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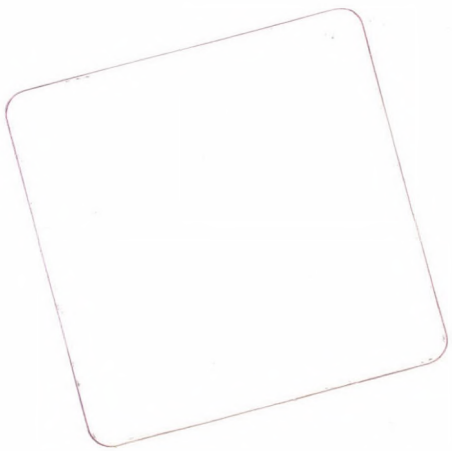

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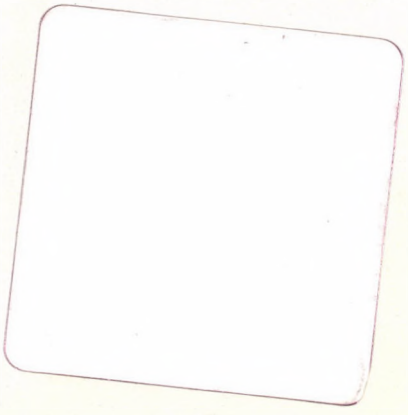
AUGUST 24–27, 1997

BUDAPEST, HUNGARY

LECTURES OF THE PLENARY SESSIONS



MAGYAR
TUDOMÁNYOS AKADÉMIA
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OPENING SPEECH TO THE FIRST REGIONAL INTERASMA CONGRESS OF THE CENTRAL AND EASTERN EUROPEAN COUNTRIES

(Received July 14, 1997)
(Accepted October 16, 1997)

Ladies and Gentlemen!

Welcome to Hungary, welcome to Budapest, welcome to the first INTERASMA Meeting of our region.

As you all know well, INTERASMA is an international organization having some peculiarities. One of them is that its main activity is to organize mostly local, regional meetings, i.e. congresses, conferences etc. The reason is obvious, each region has its own problems, characteristics, singular difficulties or given specialities. The significant dilemmas can be outlined, defined well only by the regional experts and also the solution could be found by the local professionals.

Unfortunately enough, till today there was no such a regional meeting organized and held in the Eastern and Central part of Europe. That was the reason why the International Board of INTERASMA appointed Professor Kazimierz MAY and myself to prepare, organize and perform the First INTERASMA Congress of this region in Budapest, this year. All this happened at the Jerusalem INTERASMA Congress in 1993. We were both really eager to fulfil this requirement, had created the hard-working Organizing Committe, asked of help from the pharmaceutical industry as well as of the Semmelweis Medical University and last but not least the two medical societies, namely the Hungarian Society of Pulmonology and the Hungarian Society of Allergology and Clinical Immunology. We feel obliged to express our gratitude to all of the above mentioned organizations and principally to the co-workers, organizers, members of the bodies, especially to Drs László ENDRE and Kristóf NÉKÁM and COOPCONGRESS, which is our scientific meetings organizing agent.

As co-chairmen of this Congress we would call your attention to the unique and outstanding occasion, which occurs for the first time in our life, namely

– the possibility to continue to organize a long series of forthcoming Congresses, in frames of INTERASMA, in other words to decide on the location, time and the responsible persons for the next regional meetings,

– the formation of a loose but active regional organization inside INTERASMA, having the task to keep together and to organize the efforts of the national societies, in the field of asthma research and diagnostic and therapeutic activities of our region,

– the opportunity to get to know each other, the representatives of several countries and to build up good relations with the pharmaceutical companies,

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– finally having the chance to visit our capital Budapest and our country Hungary, where you all can feel constantly our hospitality and friendship.

We have to realize that our future and fortune are in our own hands, we will have what we create, organize and perform.

Our cooperation will happen to be so fruitful and successful as we prepare and develop it. The profit of our collaboration depends on us.

We wish all of you a very pleasant stay and useful participation in the Congress, and declare the First Regional INTERASMA Congress of the Central and Eastern European Countries to be open.

Prof Paul Magyar Prof Paul A. Szemere
co-chairmen of the Congress

Acta Microbiologica et Immunologica Hungarica, 45 (1), pp. 3–13 (1998)

THE GENETICS OF ATOPY

SERGIO BONINI and ANNA RUFFILLI

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(Received July 14, 1997)
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Since the original introduction by Coca and Coombs in 1923, the concept of atopy has always been linked to the relevance of an inherited predisposition in causing immediate-type hypersensitivity reactions to common allergens. However, the mechanisms underlying this predisposition have long been vague and controversial. Only recently, the better knowledge of the basic pathophysiological abnormalities of atopy as well as the availability of molecular biology techniques for gene mapping, have renewed the interest of several research groups for the genetics of atopy and have produced substantial progress in its understanding.

We have recently reviewed the most recent data on susceptibility genes for allergy and asthma [1]. In this parallel presentation, we shall summarize the main information from this review, which however is growing and modifying every day for new important contributions.

From an historical point of view, the studies of genetics of human diseases in general and of atopy in particular can be divided in three different phases, characterized by different methodological approaches and ultimate goals.

The first phase is represented by formal genetics, aimed at defining for diseases with familial aggregation the relative role of genetic and environmental factors through population, family and twin studies. Formal genetic studies do not provide information on the genes causing the disease but measure their general effect by defining the heritability of the disease (excessive risk for relatives of affected subjects). They can also suggest the best model of inheritance for the disease or specific traits of it fitting with experimental data (monogenic, polygenic, dominant, codominant, recessive).

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A second phase is represented by HLA and immunogenetic studies, stimulated by the advances in basic immunology on the role of the major histocompatibility complex in immune recognition and response. Interesting results in this area refer to the association between specific HLA haplotypes and specific antibody response to purified antigens. On the other hand, results about associations between HLA antigens and disease showed a relevant clinical and pathogenetic role in a few diseases only, while very weak or conflicting data are reported for multifactorial and heterogeneous diseases such as atopic disordes.

Recent progress in molecular biology and genetics has led to a third phase aimed at direct mapping of genes on chromosomes through a series of complex methodologies which will be summarized below. Mapping strategies involve complex methodologies and a specific background. A close collaboration between geneticists and clinicians is needed to face genetics of atopy, in order to avoid that geneticists, from one side, while looking at sophisticated models of mapping and analysis lose contact with clinical phenotypes, as well as that clinicians, from the other side, come to simplistic extrapolations from evidence which, although sound, is confined to specific experimental models.

Mapping strategies

Two different approaches are currently used for mapping genes on human chromosomes: the random genomic search and the candidate gene approach.

Random genomic search aims at screening the entire genome through numerous markers for different chromosomes and loci in order to identify regions linked to the disease or to specific traits of it.

Candidate gene approach aims at screening selected chromosomal regions, where it has already been possible to locate genes that might influence the disease or specific traits of it. Of course, this approach requires that pathogenetic mechanisms for the disease are known and that relative genes possibly influencing them have already been identified.

With reference to allergic and hypersensitivity reactions and disease in general, an example of random genomic search is represented by the study of Cookson et al. [2] reporting the linkage between chromosome 11q13 markers and atopy, while an example of the candidate gene approach is the study of Marsh et al. [3] looking for a linkage between markers at chromosome 5q 31–35 (where the gene cluster for several candidate genes for allergy are located) and the occurrence of high serum IgE.

Usually, however, these approaches are complementary or sequential rather than alternative. In fact, when a linked region has been identified through a random search, the next step is to search a potential candidate gene in the region. For example, in the study of Cookson et al. [2] above reported, the linkage of atopy with 11q13 markers was followed by the working hypothesis – tested by the candidate gene approach – that the FcER1-B gene, located in that region, might have been the major candidate gene responsible for the linkage [4].

Both random genomic search and candidate gene approach aim at detecting a linkage between chromosomal markers and disease (or traits). This linkage can be detected by two different methodologies: gametic recombination mapping (or linkage analysis) and linkage disequilibrium mapping (or allelic association analysis).

In gametic (or meiotic) recombination mapping – or linkage analysis – linkage is defined by meiotic co-segregation (coinheritance) of alleles at a markers locus with the disease (or trait) in family studies.

In linkage disequilibrium mapping – or allelic association analysis – linkage is defined by a statistical association between a specific allele at a marker locus and the disease (or trait) in case – control population studies.

The underlying concept, the study design and the resolution power are different with the two methodologies. Also the statistical parametric and non-parametric tests used are different and quite complex [1]. These differences can possibly account for the contrasting results observed in some circumstances. For example, a marker allele associated with an increased risk in the population may not segregate with the diseases phenotype if the disease expression requires other genetics and environmental factors or because the sample is too small. The choice of the one rather than the other methodology should be therefore dictated by the polimorphism and density of markers available, the characteristics of the population and the magnitude of the study group.

It is obvious that, as the chances of recombination between two loci are greater in the population that in a family, linkage disequilibrium mapping has a higher resolution power than gametic recombination mapping. Whereas the latter defines regions which are approximately within 1–2 Mb of a marker, linkage disequilibrium mapping can define regions within up to 50 Kb of a marker. In general, the sample size necessary to provide evidence of linkage disequilibrium between two loci is much smaller than that necessary for gametic mapping over the same interval. In gene mapping, gametic recombination is normally used to identify regions of interest and linkage disequilibrium to confirm the involvement of the candidate loci and/or to reduce the candidate region to a size accessible to cloning and sequencing [1]. In fact, when a candidate region has been identified, it is important to narrow it down to approximately 1 cM (corresponding approximately to 1 Mb), an area containing 30–50 genes, using additional markers (high resolution mapping), to allow for cloning and sequencing.

The next step is usually the identification of allelic variants at candidate loci and the comparison of the variant frequency between patients and controls.

Finally, the formal proof that a mutated variant is causing the disease is reached by defining the functional or regulatory abnormality of the encoded polypeptide and its relationship to the disease phenotype.

Susceptibility genes of atopy

Candidate susceptibility genes for allergy and asthma were recently object of extensive reviews [1, 5, 6] which might be consulted for more extensive information and references. We summarize here the most significant data on regions which at present are

considered to be most relevant candidates for locating susceptibility genes of allergy (Table I).

Chromosome 5q 31-33. This region contains a cluster of genes encoding several cytokines (form the centromere: IL-13, IL-4, IL-5, IL-3, IL-9 and IL-2 B chain) [7], the beta 2 adrenergic receptor (B2ADR) [8], some important transcription factors such as the interferon regulator factor 1 (IRF1) the early growth factor response 1 (EGR1), as well as other factors which are important for inflammatory cell growth, differentiation, survival and functions, such as the granulocyte macrophage colony stimulating factor 2 (CSF2), the receptor for the colony stimulating factor 1 (CSF1) and the macrophage marker CD14.

Table I

Candidate regions for allergy susceptibility genes

Chromosome/region	Candidate locus, markers, allele	Methodology used	Linked trait
Chr. 5q 31–33	Several	LA, AA, LA	Total IgE, BHR
Chr. 6p 21.3	HLA	LA	Specific IgE
Chr. 11q13	FcERI-beta	AA, LA	Total serum IgE
	L181/L183		
	G237/D11S197		Specific IgE
	D11S527/168		
	D11S534/853		Atopy, Asthma, BHR
Chr. 14	TCR alfa, gamma	LA	Total and specific IgE
Chr. 13	D13S153	LA	Atopy
Chr. 16	D16S289	LA	Atopy, asthma
Chr. 4	D4S426	LA	Total IgE, Atopy

LA = Linkage analysis
AA= Association Analysis
See Ruffilli and Bonini, 1997 for references.

In particular, the cytokine gene cluster can be considered one of the most relevant genetic region in regulating the Th2 profile of allergic subjects [9]. In fact, IL-4 and IL-13 are the major signals for up-regulating IgE-isotypic switch and production, IL-5 influences eosinophil growth, differentiation and function and survival, IL-3 primes and enhances mast cell and basophil releasability.

Several studies have shown significant linkage between 5q31–33 and total serum IgE (or bronchial hyperreactivity in asthma) both through linkage analysis in family studies and through allelic association analysis in population studies.

Chromosome 6p21.3. This regions contains loci for the highly polymorphic HLA system. HLA class II products are membrane proteins involved in the presentation of peptides to CD4+T helper cells. In allergic subjects an association with HLA alleles has

been reported for IgE and IgG response to several allergens [10]. The contribution of HLA class II genes to the risk of developing allergy is more significant in patients who are monosensitized and have low serum total IgE levels [11]. Two additional potential candidate genes which map within the HLA region are the NTF alfa and beta genes. A large British and Australian study indicates that a TNF-beta promoter polymorphism, known to be associated with the upregulation of the TNF-beta gene expression, is significantly increased in subjects with asthma regardless of atopy [12].

Chromosome 11q13. The chromosome 11q13 region received a certain amount of attention when, in a random gnomic search, the Oxford group of Cookson et al. [2] reported the linkage of the region marker D11S97 to atopy, in a study of seven large families. In subsequent reports the linkage of atopy with D11S97 and other centromeric 11q13 markers was confirmed and the postulated susceptibility allele referred to the maternal chromosome as the linkage was detected in sibling sharing maternal but not in those sharing paternal alleles. The gene encoding the beta chain of the high affinity IgE receptor 1 (FcERIB) [4] was then suggested as candidate gene for explaining the association found. In fact, the FcERI has a central role in the initiation of the allergic response since its crosslinking on the mast cell surface leads to cell activation and to the synthesis and release of mediators of anaphylaxis and IL-4 [13]. The beta chain is neither essential for ligand binding nor for signal transmission, but contains an antigen receptor activation motif (ARAM), which binds an Src family tyrosine kinase, regulating the phosphorylation of several substrates and functions as a signal amplifier [14].

Although the definition of atopy used by Cookson and coworkers in early studies can be questioned and several studies were not able to confirm the association, C11q13 still represents an interesting region for genetics of atopy.

Another potential candidate gene in this region is also the CC10 gene which has recently been mapped at chromosome 11q13. Its product is the Clara cell, a 10 kDa protein which has antiinflammatory and immune-modulating properties (as it inhibits phospholipase A, the rate limiting enzyme in the synthesis of prostaglandin and leukotriene mediators) and may be important in modulating inflammation within the airways [15].

Chromosome 14q12. The T-cell receptor complex (TCR/CD23) is composed of multiple subunits and delivers one of the two signals essential for T cell mediated induction of B cell isotype switch to IgE [16]. The subunits include TCR alfa-beta or, less frequently, gamma – delta chains which confer to the complex property of specific recognition of class II molecule-peptide complexes on the membrane of antigen presenting cells.

TCR allelic polymorphisms have been identified and there is evidence suggesting that, perhaps in synergy with HLA class II alleles, they contribute to genetic susceptibility to certain autoimmune diseases. Moffat et al. [17] investigated whether TCR alleles contribute susceptibility to allergy and found a TCR alfa locus marker to be associated with specific IgE response to purified allergens.

Other chromosomal regions. Recently, genetics of allergy and asthma has attracted the interest of several research groups, this increasing the number of candidate regions deriving from random genomic searches. For example, Daniels et al. [18] in a random

genomic search for identification of new linked regions called attention on *chromosome 13, chromosome 16* and *chromosome 4* (no potential candidate genes were indicated).

On the other hand, the redundance of the allergic inflammatory network and the available information on genes encoding for several cytokines, inflammatory mediators or receptors involved in allergic inflammation represent am almost illimited reservoir for new candidate gene approaches. Special attention for instance was given to chromosome 16p12 containing the IL-4 receptor gene, chromosome 12q22–24 (INF gamma), chromosome 4q31 (IL-15), chromosome 6q16–22 (IFN-gamma R). The list of potential candidate genes can also be extended to transcription factors and genes regulating cytokine gene expression, genes of CC chemokines, adhesion molecules, etc., so that it is conceivable that Table I will prove obsolete already at the time of printing, as requiring an on-line update.

The heterogeneity of atopic phenotypes

Clinical phenotypes of atopic diseases are very far from the resolution power optimal for genetic analysis, i.e. the biunivocal relationship between a defined phenotype and a specific gene. The reason for such inadequacy mainly refers to the multiple of pathophysiological abnormalities underlying clinical symptoms and the complex organization of pathophysiological processes, where single molecular species transmit multiple signals and undergo modulation through redundant influences.

For didactic purposes we suggested [19] distinguishing four major pathophysiological abnormalities in allergic respiratory diseases, which can be possibly related to different clinical phenotypes. These are: 1. enhanced allergen specific IgE response; 2. enhanced polyclonal IgE (and IgG4) synthesis; 3. upregulation of inflammatory cells (increased number/releasability of mast cells, basophils and eosinophils, mainly); 4. tissue (skin, lung, nose and eye) hyperreactivity. These pathophysiological abnormalities possibly undergo different genetic and environmental control (Fig. 1).

1. Enhancement of allergen specific IgE response. IgE antibody response to common allergens, as defined by positive response to skin tests, is usually considered to be one of the major markers of atopy. The reported prevalence of skin test reactivity to common allergens in the general population (including non symptomatic subjects) is usually high. For example, in a large study on New Zealand children, Burrow et al. [20] reported that 46% of subjects had skin test reactivity to one or more allergenic extracts. In addition, subjects without allergen specific IgE may have IgG antibody to allergens [10] or be potentially able of developing such a response upon administration of allergen by injection [21].

The cellular and molecular mechanisms controlling the immune recognition of antigens and antibody synthesis have been largely clarified: a crucial event is the formation of a ternary complex between T cell peptide-specific receptors (TCR) and molecular complexes between peptides and the major histocompatibility complex (MHC,

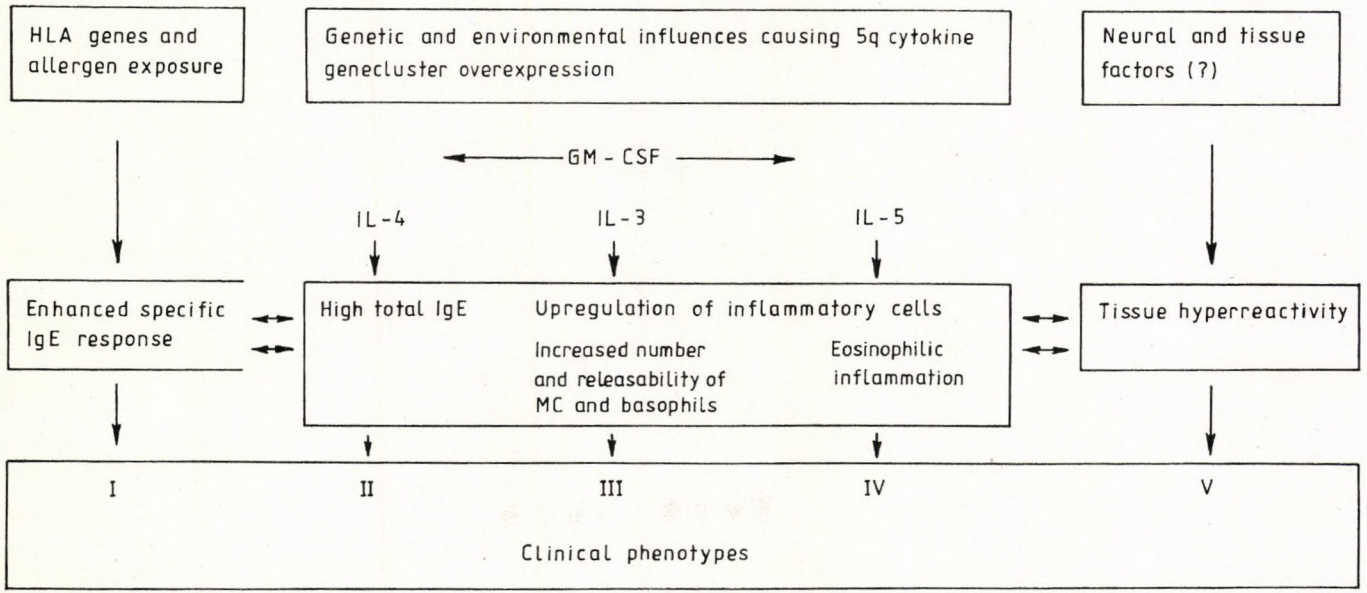

Fig. 1. Clinical phenotypes of allergic diseases distinguished on the basis of the prevalent pathophysiological mechanism causing hypersensitivity and symptoms (from Ruffilli and Bonini, 1997)

HLA in man) class II molecules. This event (cognate recognition) is at the basis of the reported and well documented association between specific HLA class II haplotypes and enhanced responsiveness to certain allergens [10, 22].

The power of a study to detect linkage/association of specific IgE with HLA class II genes may depend on the selection of the study population. For example, the selection of families through probands with clinical symptoms might increase the population prevalence of atopics with high total IgE in the study group, favouring the overlapping between the effects of genes controlling total and specific IgE. In addition to genetic factors, environmental conditions and, in particular, the level of allergen exposure play a significant role in the modulation of allergen specific IgE level in atopics [23]. Genetic and environmental confounding factors may explain why, in several studies, including studies on atopic twins, a significant genetic effect of HLA genes on specific IgE response has not been observed [24]. These considerations suggest that linkage/association of HLA class II genes to specific antibody response may be stronger in study population selected regardless to symptoms, with a well-controlled allergen experience.

2. Enhanced polyclonal total IgE synthesis. It is well known that a comparatively high level of total serum IgE (and IgG4) is usually associated with atopy and increased levels of IgE (and IgG4) in secretions are observed in various forms of allergic diseases. However, high serum IgE levels are also present in some non atopic subjects, for example in a proportion of intrinsic asthmatics [25]. Moreover, in many healthy subjects, in smokers [26] and in subjects with non-allergic diseases (such as some infections, proliferative and autoimmune diseases) high serum total IgE serum levels are not accompanied by the presence of IgE antibodies to common allergens.

Total serum IgE can be considered a composite parameter, consisting of an allergen specific and a polyclonal induced fraction. In allergic subjects, there is evidence that the enhancement of Ig constant region (CH) gene switching to the expression of IgE and IgG4 isotype reflects an abnormal expression of IL-4 and IL-13, encoded at chromosome 5q31–5q33 leading to the predominance of a Th2-type cytokine profile.

In allergic subjects, the interaction between HLA class II genes controlling the response to specific allergens and genes influencing polyclonal IgE overproduction, cannot easily be dissected. For example the sensitization to multiple allergens which usually accompanies high total IgE levels, probably does not reflect the presence of many HLA predisposing alleles but is possibly the result of polyclonal activation of allergen specific B cells.

Very little is known about total IgG4 levels, a parameter which could be interesting as an additional marker of 5q cytokine gene cluster overexpression.

3. Upregulation of inflammatory cells. Studies of late-phase reactions following allergen challenge, as well as bioptic and mediator studies of allergic organ tissues, clearly indicate that asthma and allergic diseases are characterized by different degrees of eosinophilic inflammation [27]. Mast cells, activated eosinophils and CD4 positive cells with a Th2 functional phenotype are increased in number, altered in distribution between the subepithelium and the epithelium and show increased releasability [28]. The genetic control of eosinophilia, suggested by experimental animal models, has been poorly

investigated in man up to now. Genetic influences on basophil releasability in vitro have been reported in atopic twins [29]. The localization of genes encoding several molecules involved in allergic inflammation, such as C-C chemokines or adhesion molecules, has also been reported, thus suggesting new candidate regions for the study of genetics of allergy and asthma.

Of the abnormalities of Fig. 1 the upregulation of inflammatory cells is certainly the most heterogeneous with respect to genetic and pathological mechanisms underlying. A major common factor is probably the overexpression of the 5q cytokine gene cluster, leading to a generalized Th-2 cytokine profile (IL-5 driven eosinophil differentiation, activation, recruitment and survival and IL-3 driven mast cell and basophil differentiation).

4. Tissue hyperreactivity. Studies of target organ challenge with non-specific stimuli (such as histamine) suggest that an increased responsiveness, independent from that induced by allergic inflammation, can represent a distinct basic abnormality.

The mechanisms leading to this basal hyperreactivity are still largely unexplored. Neural influences might certainly be important in this area. Nerve-mast cells interactions, neuropeptides as well as a complex neuro-endocrine immune network represent new relevant targets for studies of genetic and environmental modulation of the end-organ hyperreactivity. In asthma, for instance, genetic control of bronchial hyperreactivity, suggested on the basis of experimental data, is strongly supported by the gaussian distribution of this variable in the general population.

Although close relationships exist among the pathophysiological abnormalities reported in Fig. 1 (for example total and specific IgE response influence each other, high sensitization increases severity of inflammation and enhances tissue reactivity, etc.), their relative predominance can identify at least five different clinical phenotypes along the spectrum of allergic diseases (Fig. 1). The appreciation of the spectrum of phenotypic abnormalities may help the selection of patients for a genetic study: For instance, we suggest that it may be useful to subdivide allergic subjects into phenotypes having distinct pathological and clinical features, whereas their aggregation would probably introduce confounding factors and decrease the resolution power of genetic analysis. Finally, we would like to suggest that in allergic patients different responses to treatments with a well-defined major pharmacological activity could represent an additional criterion of classification which may help to dissect the heterogeneity of phenotypes into more punctual metabolic abnormalities.

Concluding remarks

Atopy and atopic diseases are polyfactorial conditions in which several genes and environmental factors are able to influence each variable involved in their complex pathogenesis. Accordingly, genetic studies of atopy should not deal with atopy or individual atopic disease – which represent extremely heterogeneous phenotypes –, but

should dissect and focus on the individual variables causing the final clinical picture. In this paper we have suggested that four major abnormalities are involved in causing atopic diseases, i.e. an abnormal aptitude in allergen recognition and specific IgE response, an enhanced Th2 cytokine profile with polyclonal IgE activation and inflammatory cell up-regulation and an exaggerated organ hyperreactivity. These abnormalities, which are likely to be polyfactorial too, are probably better studied in a general population or in well-defined phenotypes than in a mixed atopic population sample, where individual abnormalities can influence each others.

The list of genes of atopy is expected to continuously increase. However, since atopy is a complex disease, genes of atopy – even if completely recognized – will only represent susceptibility genes also present in a healthy population and which require other genes or environmental factors to cause the disease. Their identification and cloning will anyway make possible new strategies of prevention and pharmacological control of diseases with very high prevalence as well as individual and social costs.

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PATHOMORPHOLOGY OF THE AIRWAYS IN ASTHMA

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Asthma is a chronic inflammatory disorder of the airways with a characteristic infiltrate of eosinophils, lymphocytes, mast cells, and monocytes in the bronchial epithelium and peribronchial tissue. Bronchial-, bronchoalveolar lavage and bronchoscopic biopsies have proved to be safe and useful techniques for studying the inflammatory processes in the airways, they have contributed to our basic knowledge concerning the pathomechanism of asthma. The data derived from the clinical and experimental studies have made possible to perform an international consensus on the diagnosis and treatment of asthma.

Asthma is a chronic inflammatory disorder of the airways characterised by the infiltration of eosinophils, lymphocytes and mast cells in the bronchial epithelium and peribronchial tissue.

Former data derived from autopsy studies have suggested that the main pathologic changes are edema of the mucous membrane, hypertrophic bronchial glands, occlusion of the airway by secretion, thickening of the basement membrane, smooth muscle cell hypertrophy/hyperplasia and eosinophilia. These data generally represent the end stage of the disease and it is difficult to relate them to the early morphological changes of asthma. Evidence from autopsy data also suggested that the pathologic changes occur in both the large and small airways as well as in the alveolar area.

The application of bronchoalveolar lavage and bronchial biopsy has led to substantial advances in our understanding of the pathomorphology and pathogenesis of asthma. Despite the early fear of risks of bronchospasm, laryngospasm and hypoxaemia, nowadays, both techniques – under careful clinical monitoring – are widely used in asthma research [1, 2].

Epithelial cell damage is a common characteristic of subjects who die of asthma. This damage has been attributed to several factors, including tissue trauma, edema, increase in number of eosinophils, release of major basic protein, and reactive oxygen derivatives [3–5].

In asthma pathology a universal finding is a thick basement membrane, however, this phenomenon has not any correlation with the severity of the disease.

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Infiltration of inflammatory cells in the lamina propria of the bronchi seems to persist in asthmatics despite regular treatment with inhaled steroids. In addition, it appears that among the clinical indices of asthma severity, the degree of airway hyperresponsiveness to metacholine is most closely related to the number of leukocytes in bronchial biopsy from atopic subjects who are receiving inhaled steroids.

Ultrastructural features of the airways of asthmatic subjects demonstrated goblet cell hyperplasia, mucus plugging, increased collagen deposition beneath the epithelial basement membrane, large number of submucosal eosinophils and an extensive denudation of epithelium [6].

Several studies using different bronchoscopic techniques demonstrated an influx of inflammatory cells consisting of eosinophils, neutrophils, monocytes and T lymphocytes into the airways after antigen challenge. Natural antigen exposure, leading to clinical exacerbation of asthma, may induce an inflammatory response involving T cells, eosinophils and mast cells. The changes were more prominent in the T cell population resulting in a significant increase of CD4+ cells in the submucosa and in the increase of the IL-2R on CD3+ cells in BAL. The number of metachromatic cells (basophil- and mast cells) decreased significantly during the season, which can be interpreted as a sign of degranulation [5, 7–11].

The presence of large numbers of lymphocytes in asthmatic airways has been described, however their importance has been ignored. There is evidence of alterations in lymphocytes' number and subsets in peripheral blood and BAL fluid in asthmatics. These studies confirm the observations that lymphocytes and lymphokines are involved in the pathogenesis of asthma.

In the lamina propria of asthmatic airways exact role of the monocytes is not yet clearly defined, but it is known that they have the capacity to synthesize and release leukotrienes, prostaglandins and a variety of other cytokines.

While many different cell types contribute to airway inflammation, increasing evidence suggest that the eosinophils may be particularly important. Mediators and proteins released from eosinophils are able to contract airway smooth muscle, damage the bronchial epithelium, and may induce airway hyperresponsiveness. The increased number of eosinophils in asthmatic airways reflect an increase in eosinophil recruitment and/or survival. However, the activation of eosinophils may be of greater importance than their number in the airways. The granulocyte-macrophage colony stimulating factor (GM-CSF) may be involved in this process. Woolley et al. [12] found that mild asthmatics show an increases of eosinophils, eosinophil cationic protein and GM-CSF compared to nonasthmatics. Relationship was evident between the number and function of eosinophils and GM-CSF, furthermore these markers of airway inflammation were related to airway function.

Martin et al. [13] demonstrated that there was a significant increase in bronchoalveolar lavage fluid inflammatory cells in asthmatics, whose lung function was worse during sleep. Neutrophils and eosinophils increased while lymphocytes and epithelial sloughing appeared to be more prominent in these subjects early morning. These circadian alterations in BAL total cells, neutrophils, and eosinophils were not apparent in the peripheral leukocyte count [5, 11, 14, 15].

These findings were later strengthened, and it was also demonstrated that the greatest circadian morphological changes could be observed also in the alveolar tissue. Van Vyvre et al. [16], however, demonstrated that the total and differential cell counts of bronchial and alveolar samples differ in asthmatic and in normal subjects. In both groups, bronchial samples contained significantly more neutrophils and epithelial cells than alveolar samples. In asthmatic subjects an increased percentage of eosinophils were found in the bronchial sample. The only significant difference between asthmatic and nonasthmatic subjects was the increased number of eosinophils in both the bronchial and alveolar samples of asthmatics.

The data derived from the clinical and experimental morphological studies have made possible to perform an international consensus on the diagnosis and treatment of asthma.

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T CELLS AND EOSINOPHILS IN ASTHMA

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The bronchial inflammation characterising asthma represents a specialised form of cell-mediated immune reactions, in which products of activated $CD4^+$ T cells orchestrate the accumulation and activation of granulocytes, particularly eosinophils. Through their toxic granule proteins, membrane derived lipid mediators and proinflammatory cytokines, eosinophils are suggested to be responsible for the changes in airway submucosal tissue resulting in altered airway responsiveness. T cell activation is followed by the synthesis and release of cytokines of which IL-3, IL-5 and GM-CSF are particularly important in the site-specific accumulation, prolonged survival and activation of eosinophils. This review focuses on the interaction of these two cell types with particular interest in the cytokines which may be responsible for the development of eosinophilic airway inflammation.

Eosinophilic airway inflammation

One of the fundamental problems in investigating the pathogenesis of asthma has been to explain why eosinophils preferentially accumulate in the inflamed mucosa. Allergen inhalation results in a marked increase in eosinophils in BAL fluid at the time of the late reaction and there is a close association between eosinophil counts in airway eosinophilia and hyperresponsiveness in asthmatic patients.

Eosinophil maturation in the bone marrow

Eosinophils are non-dividing, granule containing cells that arise principally in the bone marrow. Like other leukocytes, they differentiate from myeloid precursor cells in the bone marrow under the control of cytokine growth factors of which interleukin-3 (IL-3), GM-CSF and IL-5 are active [1]. IL-5 is produced principally by T lymphocytes [2]. The

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genes encoding IL-3, GM-CSF, IL-4 and IL-5 are all located on the long arm of chromosome 5 [3]. In mice a cloned subset of $CD4^+$ T-lymphocytes termed Th2 lymphocytes are characterised by their ability to secrete IL-4 and IL-5. This is in contrast to Th1 cells which secrete IL-2 and IFN-γ [4]. Eosinophilia may therefore result from the activation of Th2 type lymphocytes responding to allergen. IL-3 and GM-CSF enhance the differentiation of a number of leukocyte precursors whereas IL-5 appears to affect only eosinophil differentiation. In colony forming assays eosinophil and basophil colonies can appear together suggesting a common precursor cell [5]. Neutrophil and monocyte differentiation appear to be distinct from eosinopoesis. Eosinophils can also be cultured from human umbilical cord blood. After 28 days in culture with IL-5 and IL-3 mononuclear cells in cord blood differentiate into eosinophils (up to 95% pure) which appear to share a number of effector functions with peripheral blood eosinophils [6]. They can also be induced to differentiate from adult peripheral blood. Precursors are increased in the blood of atopics after allergen challenge [7, 8]. The morphological changes of eosinophils as they differentiate have been well described, in particular focusing on the development of eosinophil granules [9]. Uncommitted stem cells express CD34, a cell surface receptor of unknown function and a marker of myeloid progenitors. As they proliferate these cells become $CD34^-$ at which stage they are still mononuclear. They then develop non-granular pink staining cytoplasm with immature indented nuclei by May-Grunwald/Giemsa staining and so progress more mature, bilobed, granulated cells which stain with eosinophilic dyes [10].

Adhesion, chemotaxis and prolonged survival in site specific eosinophil accumulation

In adhesion to endothelium eosinophils express the $\beta 2$ integrins LFA-1 and Mac-1 as well as the $\beta 1$ integrin VLA-4 (very late activation antigen-4). They could utilise therefore both endothelial intercellular adhesion molecule (ICAM)-1 (a receptor for LFA-1 and Mac-1), as well as VCAM (vascular cell adhesion molecule)-1 (which recognises VLA4), to mediate the integrin/immunoglobulin receptor-dependent transmigration step involved in leukocyte migration into tissue [11]. While both eosinophils and neutrophils express the $\beta 2$ integrins LFA-1(CD18/CD11a), MAC-1(CD18/CD11b) and E-selectin ligand, only eosinophils seem to show increased adhesion to endothelial cells in response to IL-3 and IL-5 treatment [12]. IL-4 selectively upregulates VCAM-1 expression on endothelial cells and eosinophil adhesion to HUVEC suggesting that VLA-4/VCAM-1 may be important in eosinophil recruitment into the airways in allergic asthma [13].

Highly potent eosinophil chemoattractants, such as PAF and C5a, are nonspecific in the sense that they also attract neutrophils. However, IL-5 can specifically prime eosinophils for enhanced locomotor responses to PAF, LTB_4 and IL-8 [14]. Further, a family of potent eosinophil specific chemokines has recently been characterized, the most prominent members of which are Eotaxin, MCP-3, MCP-4 and RANTES.

Finally, a prolonged survival in the tissue may also account for accumulation of eosinophils in the asthmatic airways. Eosinophils can have their survival markedly

enhanced by a number of cytokines including IL-3, IL-5 and GM-CSF. This profile of cytokine release is associated with activation of the Th2 subset of T cells. It was also demonstrated that interaction of eosinophils with extracellular matrix protein enhanced their survival and secretory potential [15].

Activation and release of preformed and newly-synthetised eosinophil products

Activated eosinophils were shown to be able to cause chronic damage of respiratory epithelium by releasing toxic granule products, such as major basic protein (MBP), eosinophil cationic protein (ECP), eosinophil-derived neurotoxin (EDN) and eosinophil peroxidase (EPO) [16]. Eosinophils also release membrane derived inflammatory mediators including LTC_4, PAF, 15-hydroxyeicosatetraenoic acid (15-HETE), oxygen-derived free radicals and proinflammatory cytokines. Via the peroxidase-hydrogen peroxidase-halide system eosinophils were shown to increase vascular permeability [17]. MBP can induce smooth muscle hyperresponsiveness based on their highly basic charge [18, 19] and may block inhibitory M2 muscarinic receptors on cholinergic airway nerves (Gleich et al. 1993). Studies in primates and in rats have shown that administration of human MBP produced airway constriction and hyperresponsiveness [20, 21]. Significant correlations were also shown between MBP^+ cells and airway hyperresponsiveness in human [22]. Eosinophils have the capacity to elaborate certain cytokines including transforming growth factor (TGF)-α, TGF-β, GM-CSF, IL-1, IL-3, IL-5, IL-6 and IL-8 [23]. These may affect eosinophils themselves by an autocrine fashion contributing to the maintenance of inflammation. These extensive pro-inflammatory properties of eosinophils, along with their abundance in the asthmatic bronchial mucosa strengthen the current view that eosinophils are prime effector cells in the asthmatic inflammation.

Role of T-cells in eosinophilic airway inflammation in asthma

The mechanism by which T cells exert their effects on airway responsiveness is poorly understood. Interaction between activated T cells and eosinophils although presence of the latter in the airways is not always accompanied by heightened airway responsiveness [24], has been seen as an essential element in the development of allergic airway hyperresponsiveness (AHR) [25].

This view is supported by the fact that activation of T cells correlates with disease severity [26] and the extent of eosinophilia [27]. In addition, mRNA for IL-5 was detected in both bronchial biopsies [28] and peripheral blood [29, 30] of symptomatic asthmatic patients. Further, T cells from clinically corticosteroid-responsive individuals were sensitive to the inhibitory effects of steroids in vitro, while T cells from patients with corticosteroid resistant asthma were refractory to such inhibition [31, 32]. Interestingly, response of T cells from both steroid sensitive and steroid resistant patients were similar to other immunosuppressants such as cyclosporine A and rapamycin [32]. These data

suggest that T-lymphocyte inhibition may be one mechanism of action of glucocorticoids in asthma, and further, that alternative drugs which inhibit T cell function may be effective for asthma therapy. This was confirmed in a clinical trial, where cyclosporin-A therapy of steroid-dependent asthmatic patients significantly improved their morning peak expiratory flow rate and FEV_1 as well as the frequency of exacerbations [33].

Regulation of eosinophil production and function by T cells

Along with other polymorphonuclear leukocytes, eosinophils originate from bone marrow precursor cells under the influence of growth- or colony-stimulating factors. Because the eosinophilia that normally accompanies helminthic infections is suppressed in athymic mice [34] and in normal rats or mice that have been depleted of their T cells [35, 36], it has been concluded that eosinopoiesis is modulated by T-cell factors. In fact, several of these T-cell derived cytokines have been identified and include GM-CSF, IL-3, and IL-5 [37]. Both GM-CSF and IL-3 are multilineage hematopoietic regulators in that in addition to stimulating eosinophil growth, they also promote the differentiation of neutrophil, macrophage and mixed colonies. The selectivity of eosinophil production in asthma and other allergic conditions as well as in parasitic, helminthic infections suggested the possibility that a lineage-specific growth factor may control development of eosinophilia. The murine T-cell cytokine, eosinophil differentiation factor (EDF, IL-5), was shown to selectively stimulate the proliferation, differentiation, survival and function of eosinophils [38, 39]. Using cross-species hybridisation with a murine IL-5 complementary DNA (cDNA) probe, Campbell and colleagues (1987) [40] cloned and characterised the human gene for IL-5. The human and murine proteins are highly homologous (70% identity) and the predicted size of both proteins is 115 amino acids. Recombinant human IL-5 is lineage specific for eosinophils in both liquid bone marrow cultures and the bone marrow colony assay. Not only is recombinant human IL-5 a selective hematopoietin for eosinophil growth, it also is a potent and selective stimulator of human eosinophil function. In a dose-dependent fashion, recombinant human IL-5 induces shape changes, membrane ruffling and granule polarisation in eosinophils. These morphologic changes are accompanied by enhanced cellular cytotoxicity, phagocytosis and superoxide generation. In contrast to eosinophils, neutrophils are not activated by this cytokine [39].

Th1 and Th2-type cytokines

There has been a considerable interest in the classification of T cells according to their profiles of cytokine secretion. Landmark studies by Mosmann and Coffmann in the mid-to-late 1980s established the division of a number of murine $CD4^+$ T cell clones into distinct subsets designated Th1 and Th2 cells, that mediate their effector functions through elaborating distinct groups of cytokines [4, 41]. Mouse T cells can be subdivided on the basis of selective secretion of IL-2, lymphotoxin, and IFN-γ by Th1 cells and IL-4,

IL-5, IL-9, IL-10 and IL-13 by Th2 cells. Both subsets produce additional cytokines, such as IL-3 and GM-CSF. Th1 cells are adept at macrophage activation and immunoglobulin secretion for isotypes (IgG2a and IgG3) that mediate antibody-dependent cellular cytotoxicity and complement activation. These cells have been shown in models of infectious diseases to activate appropriate host defense against intracellular microbes including viruses, bacteria, yeast and protozoa. In some of these models of infectious diseases Th2 cells have proven to be deleterious mainly through their ability to produce IL-4, IL-10 and IL-13 to downregulate macrophage activation, even in the presence of IFN-γ [42]. Apart from downregulating Th1 cell activity, cytokines produced by Th2 cells promote maturation of mast cells and eosinophils and stimulate B cell growth, differentiation and isotype switching to IgE and IgG1 [4]. The mechanism underlying initial polarisation of the Th responses remains unclear, however, it may involve the nature and dose of antigen as well as its processing and presentation [43]. In addition, the nature of T-cell responses in vivo may depend on factors present in the local microenvironment during the period of primary antigen contact, such as the cytokines produced by nonspecific inflammatory cells [44]. Data derived from limiting dilution analysis [45] and T cell receptor transgenic mice [46, 47] demonstrated that naive $CD4^+$ T cells have the capacity to differentiate into either Th1 or Th2 cells and that this process is markedly influenced by the cytokine milieu during initial priming by specific antigen. These subsets arise from a common naive precursor which secretes high levels of IL-2 but little IL-4 or IFN-γ through a process of antigen-driven extrathymic maturation [48]. Although many immune responses probably retain this mixed phenotype (Th0), it is possible that distinct forms of primitive, nonspecific inflammatory responses which initially arose to counter infection have determined the polarisation of adaptive cell mediated immune responses [43]. Organisms that induce Th1 responses such as *Listeria*, *Toxoplasma* and the tissue form of *Leishmania* contain antigens that trigger IL-12 release from macrophages [49–51]. IL-12 in turn directly primes $CD4^+$ cells towards a Th1 phenotype. In a murine model of schistosomiasis an egg-derived oligosaccharide was shown to induce IL-10 production from B cells [52] which in turn may promote Th2 development of naive $CD4^+$ T cells [46]. The mechanism by which common environmental allergens in atopic patients prevalently stimulate Th2 responses is less clear. Since Th cells seem to be unable to differentiate into IL-4-producing cells in the absence of IL-4, early IL-4 production by another cell type must be involved. Possible candidates include mast cells and basophils which have recently been shown to release stored IL-4 in response to Fcε receptor triggering [53–55]. In both atopic and non-atopic human subjects, Th1 and Th2 type T-cell clones can be distinguished in vitro [56], furthermore, there is increasing evidence that these cell types may also be involved in human inflammatory processes in vivo.

T cells in the BAL fluid of mild, atopic asthmatics showed elevated expression of IL-4 and IL-5 mRNA as compared with non-atopic controls, reflecting a Th2-like pattern [58]. However, it would appear that IL-4 and IL-5 expression need not always be co-ordinately regulated in vivo: T cells purified from the peripheral blood of non-atopic asthmatics spontaneously secreted elevated quantities of IL-5, but not IL-4, as compared with normal controls, whereas those from atopic patients secreted elevated quantities of

both IL-4 and IL-5 [27]. It is possible, therefore, that increased IL-4 synthesis is associated with the atopic state and is not a prerequisite for the development of asthma.

Besides its effects on Th2-cell development and IgE synthesis IL-4 may contribute to the selective eosinophil accumulation in sites of allergic inflammation. In an in vitro study, Schleimer et al. (1992) found an upregulation of VCAM-1 expression by IL-4 in a time and dose-dependent manner which paralleled eosinophil adhesion. Anti-VCAM-1 or anti-VLA-4 inhibited adhesion of eosinophils and basophils to IL-4 stimulated endothelial cells [13]. In in vivo studies, injection of a Th2 cell line into mouse footpad induced a local tissue inflammation which was IL-4 dependent [59], while in the lung schistosoma-induced eosinophilia was abrogated by anti-IL-4 antibody treatment [60]. sIL-4R treatment of OA-sensitised mice inhibited immediate hypersensitivity reaction, IgE and IgG1 production and airway hyperresponsiveness [61]. Finally, it was shown that mice homozygous for a mutation that inactivates the IL-4 gene had remarkably reduced eosinophil influx (in addition to IgE non-responsiveness) and were unable to develop airway hyperresponsiveness following sensitisation and exposure to OA [62, 63]. A key cytokine in selective eosinophil (as opposed to neutrophil) recruitment [64] and activation [39] is IL-5. It can promote terminal differentiation of the committed eosinophil precursor [1] and enhance its survival and chemotaxis [38]. It was shown that an antibody against IL-5 prevented antigen-induced eosinophilia and bronchial hyperreactivity in guinea pigs [65, 66] and mice and finally, that IL-5 deficiency abolished airway eosinophil inflammation and hyperresponsiveness in mice [67].

Skin model

Early and late allergic reactions have been observed in the lung but also in the nose and skin. The influx of inflammatory cells that characterises the late phase allergic reaction (LAR) is reminiscent of a chronic allergic inflammation. Much of our understanding of the immunopathogenesis of allergic changes comes from dermatologic studies. A small number of resident $CD3^+/CD4^+$ T-lymphocytes are found in the skin [68, 69]. A study on cell traffic and activation in allergen induced human late-phase reactions in the skin revealed a lymphocytic infiltration that was almost exclusively $CD4^+$ [69]. As determined by positive staining for IL-2R, some of these T cells were activated. Additional evidence for T-cell activation was provided by the observation that endothelial cells in the allergen challenged biopsy specimens demonstrated increased HLA-DR expression, a presumed result of T-cell secreted IFN-γ. The presence of activated T cells at these sites was accompanied by the presence of activated eosinophils [69]. Using the technique of in situ hybridisation, Kay et al. (1991) determined the cytokine profile of the infiltrating $CD4^+$ T cells seen in the late phase skin response to allergen challenge [70]. At the allergen injected sites, most biopsy specimens had positive hybridisation signals for IL-3, IL-4, IL-5 and GM-CSF, a group of cytokines whose genes are clustered in the region of the long arm of human chromosome 5. In addition, at the allergen injected sites there were significant correlations between IL-5 mRNA-positive cells and IL-3,IL-4 and GM-CSF mRNA-positive cells. Further, Tsicopoulos et al. (1992) [71] demonstrated a

Th2-like pattern in allergen-induced late-phase cutaneous reactions relatively early (within 1–6 hours) and a Th1-like pattern of cytokine mRNA expression in cutaneous tuberculin reactions at 48–96 hours after allergen and tuberculin intradermal injections.

Animal models

These observations do not necessarily mean that $CD4^+$ T cells and their products actually cause the allergic reaction. However, they do suggest that T-cell recruitment sets the scene for the intense inflammation and possibly that they are responsible for the persistent airway narrowing that accompanies repeated allergen exposure. We have described a series of studies investigating the role of T cells in the pathogenesis of allergic airway hyperresponsiveness (AHR) using a rat model [72]. In these experiments, AHR was associated with ovalbumin specific IgE production, significant increases in eosinophil, neutrophil and lymphocyte counts and an elevation of $CD25^+$ cells in the bronchoalveolar lavage fluid [73] and in the airway mucosal tissue in rats actively sensitized to and challenged with ovalbumin [74]. There were significant correlations between the numbers of $CD25^+$ cells, eosinophils and the extent of AHR suggesting a causal relationship. In addition, lung tissue from rats sensitised and exposed to ovalbumin expressed mRNA for IL-4 and IL-5 with a reduction in IFN-γ [75]. These studies demonstrated that along with AHR, increased serum IgE and airway eosinophilia, the BN-rat model shares cardinal features with human atopic asthma including T cell activation and an involvement of Th2-type cytokines. In addition, using T cell transfer, it was demonstrated that $CD4^+$ T cells but not $CD8^+$ T cells, when injected intravenously, are capable of directly inducing allergic airway hyperresponsiveness following single challenge of the recipient animals [76].

These results strengthen an independent role of $CD4^+$ T cells in allergic AHR and form an important step in the elucidation of asthma pathogenesis.

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NEURO-IMMUNO REGULATORY MECHANISMS OF ASTHMA

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The normal regulation of bronchomotor tone is mainly achieved by parasympathetic and non adrenergic non cholinergic (NANC) impulses. These systems are responsible for maintaining the adjustment of bronchomotor tone by release of their neurogenic transmitters (Acytylcholine, VIP, PHM) to guarantee homeostasis. In case of disease (inflammation) this homeostasis is imbalanced and as a consequence of the disease other factors like the liberation of inflammatory mediators, interleukins, other neural transmitters like SP, NKA, NKY, and CGRP are involved. Many neuropeptides have been identified in peptidergic fibres in the airways [1–3]. Calcitonin gene-related peptide and the tachykinins substance P (SP) and neurokinin A have been localized to airways in animals and man [3–6] and have been shown to have potent bronchoconstrictor actions in human airways [5–8].

While the precise physiological and pathophysiological role of neuropeptides in the airways remains uncertain, the potent effects of these peptides on various aspects of airway function suggest that they may be involved in controlling airway tone, mucus secretion and plasma extravasation. Neural and neuropeptide control mechanisms might be affected by inflammation [9]. Therefore, it is possible that defective function of neuropeptide containing nerves might be involved in nasal and bronchial hyperreactivity [7].

Release of SP from sensory nerves can be induced by several stimuli including capsaicin in animals [10], antidromic vagal stimulation or physiological stimuli like bradykinin, histamine and nicotine [11].

In addition, baseline concentrations of SP were higher in nasal and bronchoalveolar lavage fluids obtained from patients with grass pollen asthma compared to healthy non-atopic subjects [12, 13]. Furthermore a significant release of SP to nasal and bronchial antigen challenge was shown [14, 15] (Fig. 1).

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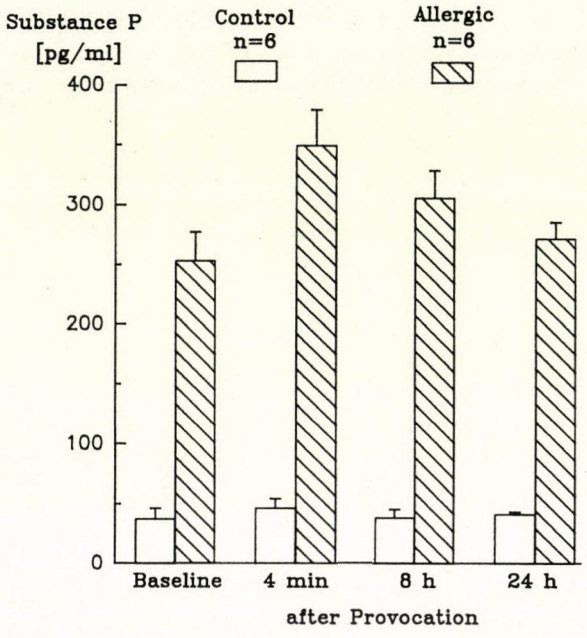


Fig. 1. SP-Concentration in BAL fluids before and after intrasegmental allergen provocation in nonallergic controls (n=6) and grass pollen allergic patients (n=6), $p<0.05$.
From Nieber et al., J Allergy Clin Immunol, 1992.

The liberation of neurotransmitters can activate the immune system and inflammatory cells liberating interleukins and inflammatory mediators like bradykinin, PAF, and arachidonic acid metabolites like LTB4, LTC4, D4, E4 resulting in an augmentation of inflammation. The peripheral target cells (epithelium, smooth muscles, blood vessels and secretory glands) do respond directly to peptide transmitters or via the liberation of inflammatory mediators and interleukins.

The property of substance P of mast cell degranulation have attracted attention [16–19]. Human skin mast cells respond to neuropeptide stimulation with a rapid release of histamine and minimal generation of prostaglandin D_2 and leukotriene C_4 [20, 21]. This ability of skin mast cells to release mediators in response to neuropeptide stimulation is evidence in favour of a neuro-immune interaction within the human skin. Unlike mast cells of the skin, mast cells of the lung, adenoids, tonsils and intestine, whose primary role is thought to be IgE-mediated hostdefence, do not respond to neuropeptide stimulation. While mast cells of different tissue release histamine in a concentration dependent manner following IgE-dependent or calcium ionophore A 23187 stimulation [21], the neuropeptide SP induces histamine release only from skin mast cells but not from mast cells derived from lung, adenoids, tonsils or intestinal mucosa and intestinal muscle [22].

Sensory fibers are extensively branched and densely spread to and innervate the walls of arterioles, venous vessels and gland acini and moreover extend to the epithelium.

The local release of neuropeptides, known as axon reflex and a defense mechanism, has been demonstrated in rodent nasal mucosa [17, 18]. It occurs when epithelial or mucosal injury or mast cell degranulation leads to the release of factors such as histamine or bradykinin, which can depolarize sensory nerves [16, 23, 24]. The observations of epithelial cell secretion [25], vasodilation and plasma influx in the skin and respiratory tract [26, 27] are somewhat contradictory in humans. As regards the topical application of substance P on the nasal mucosa, Wolf et al. [28] and Devillier et al. [29] demonstrated an increase in nasal airway resistance, whereas others were unable to confirm this [30]. These reactions can partially blocked by atropine and oxytropium bromide and augmented by serotonin [29–32].

Our results of a recent study indicate that exogenous SP induces nasal obstruction in a dose-dependent manner in both normal volunteers and patients with allergic rhinitis [33] (Fig. 2, upper panel). In addition, with the highest dose of locally applied SP systemic effects like flush, rise in heart rate and fall in blood pressure were observed in some cases in both groups. Since the symptoms were mostly nasal blockage it could be that SP's primary effect was vasodilation. It has been shown that SP could augment cholinergic activity [11]. It is conceivable therefore that SP triggers the release of acetylcholine with a subsequent induction of nasal congestion and a rise in the symptom score. Likewise, SP-induced increase in nasal congestion could also be mediated through a direct neurokinin receptor activation.

Unlike human skin mast cells, human nasal mast cells do not respond to SP stimulation with a release of inflammatory mediators, especially histamine in vivo [20, 21]. There is also no involvement of plasma-derived mediators. Kinins and TAME-esterase activity – the substrates for kinins, high and low mol. weight kininogen as well as 70% of TAME-esterase activity, plasma kallikrein-a_2 macroglobuline, are plasma derived at least during the early allergic reaction [34, 35] – do not show any increase (Fig. 2, lower panel).

In the skin SP elicit release of preformed and newly generated mediators from mast cells at concentrations of 0.1–30 μM [36]. Nerve growth factor enhances the release of histamine from rodent peritoneal mast cells activated by a variety of secretagogues [37]. In addition, neuropeptides can be generated by lymphocytes (B-endorphin) and eosinophils (SP and VIP) [38, 39] indistinguishable from the corresponding neuroendocrine peptides.

On the other hand it was shown in an organ culture model of nasal mucosa that the histamine content in the culture bath was significantly elevated after SP stimulation compared to controls. Histomorphometry showed a decreased density of mast cells and an increased percentage of degranulated mast cells [40]. The results also support the hypothesis of a close interaction between C-sensory nerves and mast cells.

The failure of topically applied SP to the nasal mucosa in vivo to evoke an increase in mast cell and plasma-derived mediators in vivo could be explained by a rapid local enzymatic degradation of SP in the secretion. SP can be degraded by several peptidases, including neutral endopeptidase (NEP), kininase II, serine proteases and aminopeptidases. A key role in this process is obviously the degrading enzyme NEP. This enzyme is found

on cell membranes very close to the neurokinin receptor, obviously to regulate the homeostasis. Studies on airway submucosal gland secretions in animals demonstrate that NEP inhibitors potentiate the secretagogue effects of tachykinins [41]. NEP inhibitors also potentiate the effects of tachykinins on airway smooth muscle [42], and cough [27] in animal studies, indicating an involvement in neurogenic inflammation. In fact, active enkephalinase has been detected in human plasma [43], lung [44] and in human nasal secretions [45].

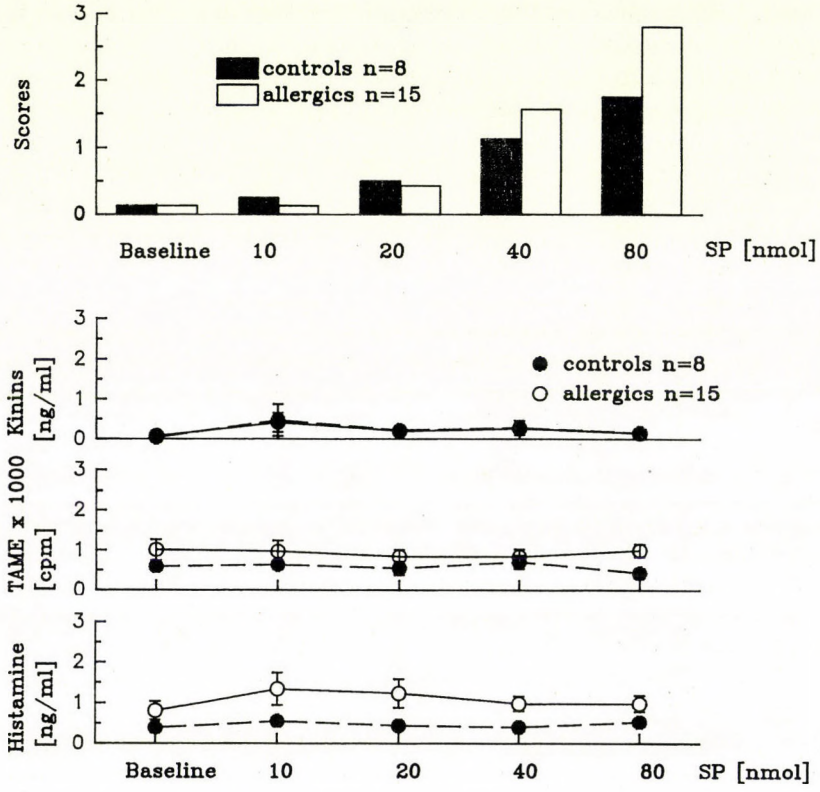


Fig. 2. Combined figure showing the symptom scores (mainly congestion) and the mediator concentrations (histamine, TAME-esterase activity and kinins) after applying SP to the nasal mucosa. The upper panel shows increasing symptom scores in a dose-dependent manner (baseline vs. 80 nmol SP challenge scores $p<0.001$ for controls and allergics), the lower panels show the results for kinins, TAME-esterase activity and histamine: there is no significant change in the levels of the various markers in the nasal lavage fluid. Results are shown as mean and standard deviation.

From Baumgarten et al., Peptides, 1996.

Nevertheless, these data together with the fact that SP is located in sensory nerve endings in the nasal mucosa of humans as well as in experimental animals [18, 46], and the dose dependent increase in SP levels in nasal lavage fluid following antigen challenge of allergic patients [14] suggests that neuropeptides could act in concert with inflammatory mediators and/or potentiate the IgE-dependent mediator release.

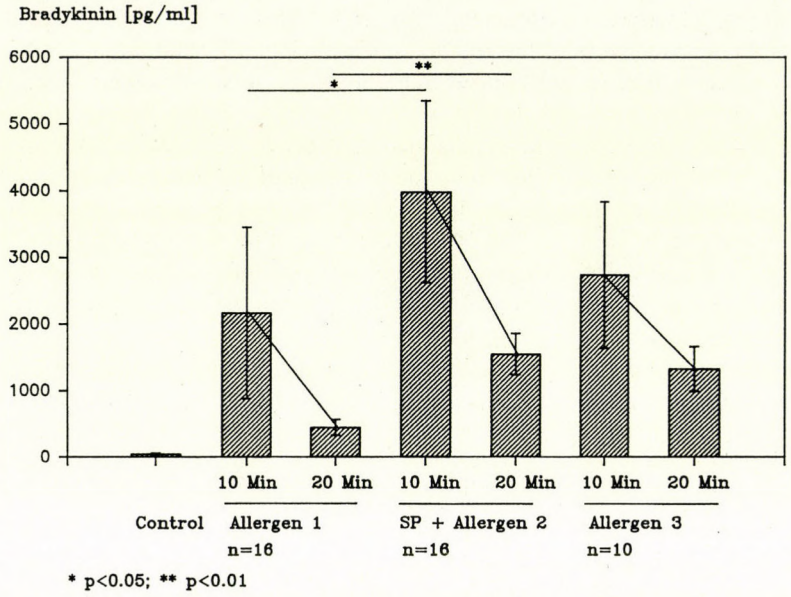
As also demonstrated in this study, pretreatment of the nasal mucosa with SP leads to an augmentation of the IgE-mediated mediator release, whereas sequential nasal challenges without pretreatment of SP do not [33] (Fig. 3). Although we could not demonstrate a neuropeptide-induced mediator secretion in the nose, we could provide direct evidence for this substance P-mast cell interaction by the significant increase of histamine concentration in the lavage [33].

In part, this could arise from a direct effect of SP, probably as a result of hyperemia and microvascular leakage with a more rapid plasma influx following the IgE-dependent mast cell mediator release or caused by a change in the breakdown of SP. NEP, bound to the membranes of multiple cells in the airways that contain tachykinin receptors, limits the effects of tachykinins by cleaving and inactivating them [42, 47]. Decreased NEP activity occurs after exposure to various stimuli such as TDI [48], experimental influenza infection [49] or inhalation of cigarette smoke [50] and was responsible for the exaggerated response to SP. It seems acceptable that NEP activity could also be decreased during the acute allergic reaction leading to an exaggerated neurogenic inflammatory response. Since NEP also cleaves kinins [51], a liberator for SP [52], its effect is even more important. In addition, kinins are released following antigen challenge [34, 53]; they could be competitors of SP and other tachykinins for the degradation potential of NEP, thus limiting the ability of NEP to act on SP, potentiating the SP effects of neurogenic inflammation. Further studies showing that antigen-induced responses inhibit neutral endopeptidase activity have to be done in order to support this speculation.

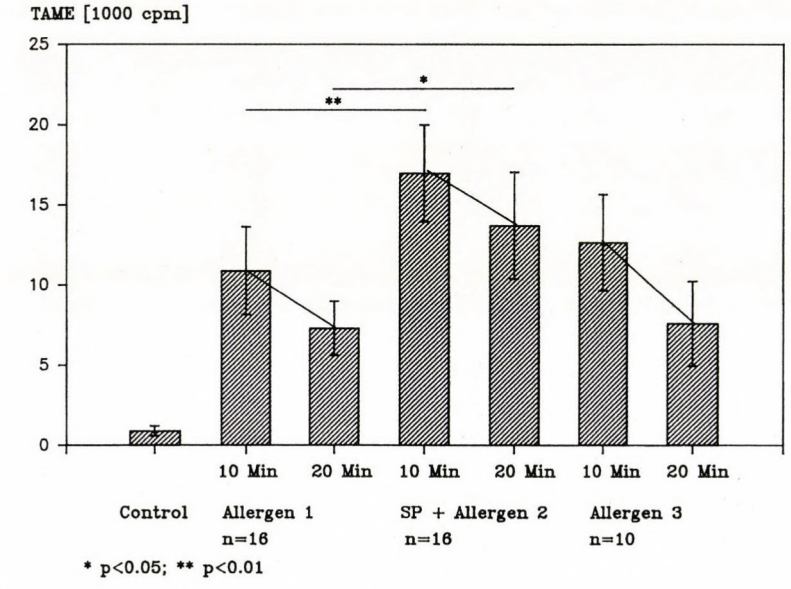
The relationship between mediator release following antigen challenge and the augmentation by SP and probably vice versa might induce a circulus vitiosus in long-term natural allergen exposure.

The increase in airway responsiveness is associated with airway inflammation as evidenced by an increase of inflammatory mediators such as cyclooxygenase and lipoxygenase products. In human nasal mucosa derived from patients undergoing surgical therapy for chronic nasal obstruction, cultured with a specially designed organ culture model and stimulated with Substance P, Calcitonin Gene-Related Peptide and Vasoactive Intestinal Peptide for 1 h we could demonstrate eicosanoid formation in the supernatant [54]. Experiments revealed neuropeptide-mediated changes in cyclooxygenase and lipoxygenase product formation with significant increases in $PGF_{2\alpha}$, TXB_2 and elevated levels of LTB_4 and $LTC_4/D_4/E_4$ after stimulation with SP. Moreover we found an increase in the concentration of $PGF_{2\alpha}$, TXB_2, LTB_4, and $LTC_4/D_4/E_4$ of CGRP-exposed and a decreased content of these mediators in the supernatant of VIP-stimulated mucosal samples.

It was shown in experimental setups that the inhibition of NEP did augment the response to neurokinins considerably and that the NEP content in the tissue was reduced by e.g. infectious stimuli and also allergen exposition. There is also a distinct correlation


Bradykinin [pg/ml]
6000
5000
4000
3000
2000
1000
0
**
*
10 Min
20 Min
10 Min
20 Min
10 Min
20 Min
Control
Allergen 1
n=16
SP + Allergen 2
n=16
Allergen 3
n=10
* p<0.05; ** p<0.01

3a


TAME [1000 cpm]
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SP + Allergen 2
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Allergen 3
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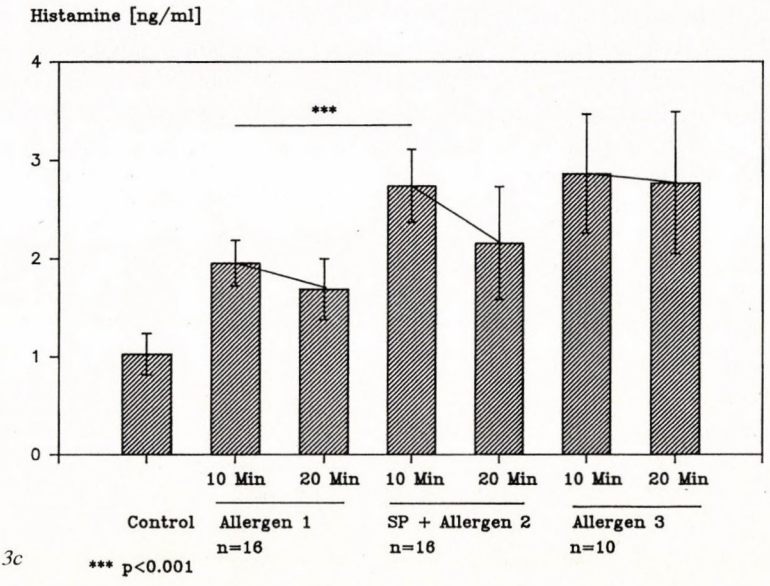
* p<0.05; ** p<0.01

3b



3c

Fig. 3. Enhancement of IgE-dependent mediator release by SP: a sequence of three successive nasal allergen challenges each followed by a lavage 10 and 20 minutes later and an additional substance P challenge 5 minutes before the second antigen challenge results in significantly higher mediator concentrations (bradykinin, TAME-esterase activity and histamine) in the 10 minute and partly in the 20 minute lavages compared to the first allergen challenge. The differences between the lavage levels of the first and third challenge procedures are still present, but not significant.
From Baumgarten et al., Peptides, 1996.

between the grade of bronchial hyperreactivity and the NEP content in the serum of asthmatic patients. The lower the serum concentration the higher the grade of hyperreactivity. It is still unclear if the inflammation per se does reduce NEP synthesis or NEP activity or augments the liberation of neuropeptides. It also has to be discussed, if the regulation of NEP synthesis and NEP activity might be an underlying cause of the inflammatory diseases of the airways.

In the above-mentioned studies we have shown that the baseline concentrations of SP were significantly higher in nasal and bronchoalveolar lavage fluids of allergic asthmatics compared with normal subjects [12, 13] and a significant release of SP to local antigen challenge could be demonstrated [14]. The fact that bradykinin is present in rhinitis of different aetiology [55] and a number of pulmonary inflammatory diseases [15], and induces a sore throat and pain following nasal challenge [56, 57] and cough and retrosternal discomfort following bronchial inhalation [58] suggests that sensory nerve stimulation must be involved. Earlier studies have shown that bradykinin stimulates sensory afferents in dogs [59] supporting this hypothesis. Our present results clearly show that bradykinin induces in vivo a dose dependent plasma leakage into the nasal cavity without effecting mast cells, but stimulates nerve endings resulting in the release of the

neuropeptide SP in atopic subjects (Fig. 4). The lack of effect of bradykinin on mast cells is consistent with previous reports of in vivo studies [56, 57, 60]. In addition, there was a clear relationship between the generation of SP and the onset of clinical symptoms observed following bradykinin challenge. Furthermore, the generation of SP following nasal challenge with bradykinin in non-allergic individuals suggests that the observed finding was specific to bradykinin.

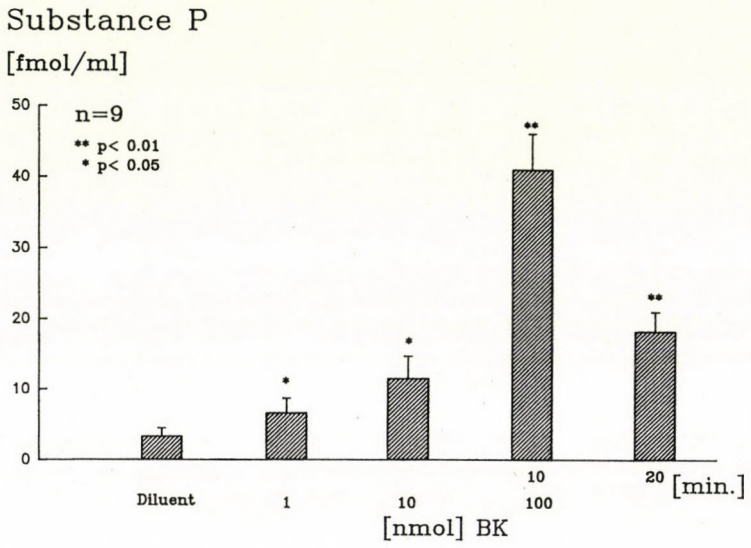


Fig. 4. Nasal challenge of allergic individuals with increasing dosages of bradykinin liberated SP in a dose-dependent response
From Baumgarten et al., Clin Exp Allergy, 1997.

The release of SP was highly correlated with that of TAME-esterase activity. The concentration of histamine, representing a mast cell or basophil-derived mediator, was not influenced by locally applied bradykinin. Therefore, the increase in TAME-esterase activity represents plasma derived proteases, mainly plasma kallikrein, since it was shown that TAME-esterase activity following antigen challenge represents 70% plasma kallikrein, 25% mast cell tryptase and 5% tissue kallikrein [34].

SP and bradykinin are released during the allergic reaction in man [13, 14, 35, 53] and as shown in the present study SP is also released following bradykinin challenge. Although we do not know, whether mast cell mediators trigger SP release or not, one explanation of SP release in allergics might be via IgE dependent mast cell degranulation leading to increased vascular permeability and plasma influx with consecutive activation of the kallikrein-kinin-system and bradykinin generation. Bradykinin then induces the demonstrated SP release. On the other hand, bradykinin does not act via mast cells and we

have demonstrated the generation of SP following bradykinin challenge also in non-allergic controls. This means that there is possibly a different mechanism by which SP is released. Since bradykinin is released in almost all inflammatory nasal reactions in humans [15, 35, 53], our data suggest a direct action of the unspecific inflammatory mediator bradykinin on the release of SP as a representative of neurokinins in neurogenic inflammation. These data could explain, at least in part, the bradykinin-evoked clinical symptoms in the nose. Bradykinin is a potent bronchoconstrictor too, but it has few direct constrictor effects on human bronchi in vitro [61]. Therefore, the released SP could cause the bradykinin-induced bronchoconstriction in human airways directly via neurokinin receptors [62] or indirectly through exaggeration of cholinergic reflex mechanisms, since Fuller et al. have demonstrated that the bradykinin-induced bronchoconstrictor responses were partly inhibited by anticholinergic agents in asthmatic patients [61].

In summary: Substance P (SP) and β-endorphin baseline concentrations are significantly higher in bronchoalveolar lavage fluids and the former also in nasal lavage fluids of allergic patients compared to normal controls. After local allergen provocation there was a rise of both neuropeptides in bronchoalveolar lavage fluids of allergic subjects immediately after provocation. Exogenous SP [10–80 nmol/ml) induced a dose dependent increase in nasal symptoms in asymptomatic allergics with rhinitis and controls but did not release any mediators. However, comparing the antigen-evoked release of mediators into nasal secretions with that of a SP-pretreated allergen challenge, a significant enhancement of relevant inflammatory mediators was found 10 and 20 min after allergen challenge.

These results suggest a direct neurokinin receptor activation independently of mast cell activation, and during the allergic reaction there is a SP-mast cell interaction that enhances significantly the inflammatory mediator response of the IgE mediated reaction. Furthermore, SP is also released following challenge with the unspecific inflammatory mediator bradykinin.

The liberation of neurotransmitters itself can activate the immune system and inflammatory cells, liberating interleukins and inflammatory mediators like bradykinin, PAF, and arachidonic acid metabolites like LTB4, LTC4, D4, E4 resulting in an augmentation of inflammation. The peripheral target cells (epithelium, smooth muscles, blood vessels and secretory glands) do respond directly to peptide transmitters or via the liberation of inflammatory mediators and interleukins.

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VIRAL INFECTIONS AND ASTHMA

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It has been known for years that patients presenting with acute severe asthma attacks, usually give a history suggestive of a respiratory tract infection. Unfortunately, direct proof is usually lacking and the history is, therefore, ambiguous.

During the last 30 years particular attention has been paid to the significance of viral infections as a trigger of bronchial asthma attacks [1–11]. Many epidemiological studies have been done since 1960s [1–11]. Their results indicate two main relations between viral respiratory infections and asthma: (i) positive correlation between viral infections and the subsequent development of asthma; (ii) temporal connection of viral infections with asthma exacerbations.

Up to 56% of the infants who experienced bronchiolitis during the first year of their life subsequently developed asthma as documented in both clinical manifestations and lung function tests [1–5]. The functional test abnormalities persisted several years after the resolution of the infection. In addition, in most studies, a family history of atopy was associated with higher probability of asthma manifestations later in life. These data indicate the existence of an association between acute viral bronchiolitis in infancy and airway obstruction and hyperresponsiveness later in life [1–5]. The fact that many of these changes can persist 10 years and longer following viral infection indicates a possibility that these infections may be of significance in the pathogenesis of asthma [4].

Clinical, serological and virological evidence of viral respiratory infection in children with acute episodes of wheezing allowed to establish the temporal relationship between asthma and these infections [8–12]. The studies show 24% to 54% incidence of acute exacerbations of asthma linked to concurrent viral infection. Although a causal relationship between viral respiratory infections and acute exacerbations of asthma has not yet been defined, the temporal relationship, is quite evident.

In contrast to the strong association of viral respiratory infections and acute exacerbations of asthma in children, the same relationship in adults is apparent only in 11% to 21% (depending on the study) [10, 13, 14].

The reason for the decreased association between viral respiratory infections and acute exacerbations of asthma in adults is not entirely understood. It may reflect the

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chronicity of asthma in adults that would make individual exacerbations more difficult to fully delineate.

All epidemiologic studies have shown that several factors are important in determining an individual's pulmonary response to viral infection [15–17]. The most important risk factors for wheezing with viral lower respiratory infection are presented below:

1. genetic
   - male gender
   - atopy (especially house dust mite allergy)
2. developmental
   - age less than 2 years
   - underlying pathologic conditions
3. environmental
   - strain of infecting virus
   - concurrent presence of cigarette smoke

The participation of these individual factors in the development of overall bronchial response to viral infection has yet to be determined.

Etiology. The spectrum of viruses responsible for acute airway infections changes with age. The most frequently isolated viruses in association with younger children are respiratory syncytial viruses (RSV), parainfluenza viruses (PIV), and coronaviruses, whereas influenza A is more predominant in older children and adolescents [8–10, 16, 20]. The rhinovirus (RV) is isolated in children of all ages.

Recent studies showed that wheezing children below 2 years of age were most frequently infected with RSV while older children and adults most often had RV infection [16, 19, 20]. This may reflect the overall prevalence of specific viral infections: rhinoviruses account for 50–70% of upper respiratory infections, coronaviruses for 15–20% and influenza, PIV, RSV and adenoviruses for the remaining infections.

Mechanisms. Viral, like any other infection, induces many protective responses in the airway of the normal host [21]. Invading agents provoke a broad spectrum of inflammatory reactions including: activation of alveolar macrophages, activation of complement, production of antibodies of multiple immunoglobulin classes, chemotactic mediator release from mast cells and basophils, recruitment and stimulation of lymphocytes, recruitment of additional inflammatory cells.

Although all these mechanisms are designed to remove the infectious agent, in some situations the inflammatory process can develop in an uncontrolled way influencing the airway function. It is still not known why some "predisposed" patients develop chronic inflammation and symptoms. In these subjects viral infections produce hyperreactivity which may cause irreversible damage.

Many studies have been carried out in order to elicit the mechanisms of viral invasion which can be responsible for airway dysfunction. Although many possible mechanisms have been described it is important to keep in mind that any single reaction is unlikely to be responsible for each of the observed changes. The effects of viral infection on airway function are multifactorial and interrelated. However, the final "common

pathway" of each individual interaction is the ability to trigger and/or promote airway inflammation.

The main events of virus–host interaction important to the development of asthma are listed below:

– loss of the protective function of the epithelium
– endothelium dysfunction
– effects on inflammatory cells
– effects on the autonomic nervous system.

Loss of the protective function of the epithelium. Viruses may cause denudation of the bronchial epithelium that: facilitates access of noxious agents (specific-allergens and nonspecific-irritants) from the airway lumen to smooth muscle and submucosa; exposes irritant or cough receptors which may respond to minimal stimulation; deprives the airways of epithelial-derived relaxant factors; stimulates epithelial cells for expression of proinflammatory cytokines, and adhesion molecules thus promoting the inflammatory process [22, 23].

The airway epithelial cells are important regulators of host defense. These cells not only provide a passive protective barrier, but also produce substances which can influence overall bronchial function. Neutral endopeptidase, an enzyme derived from bronchial epithelium cells, is known to take part in the metabolism of tachykinins such as substance P [24]. Other enzymes, such as cyclooxygenase with potentially important roles in modulating airway function, are also found in the bronchial epithelium [25].

Human cultured epithelial cells have also been shown to express adhesion molecules in response to some inflammatory cytokines and viruses promoting in this way adhesion of both neutrophils and eosinophils with consequent cell infiltration and inflammation [26].

Endothelium dysfunction. Increased endothelial permeability with subsequent exudation of plasma fluids and proteins (including immunoglobulins and complement) into the extravascular space are well-known contributors to the viral infection induced inflammatory processes.

Effects on the autonomic nervous system. The human lungs receive impulses from the sympathetic and parasympathetic branches of the autonomic nervous system. Parasympathetic fibers acting via muscarinic receptors (M2) exert the primary excitatory control on bronchial smooth muscle, resulting in bronchoconstriction, while fibers conducting sympathetic signals via adrenergic receptors (β2) exert the opposite effect. Viruses appear to change the existing equilibrium favoring the parasympathetic control, thus promoting the development of bronchoconstriction.

As previously mentioned inflammation and desquamation of bronchial epithelium caused by viruses increase the susceptibility of nerve endings to various stimuli. These processes may increase the sensitivity of sensory airway receptors thus enhancing reflex bronchospasm. It has been shown that airway hyperreactivity seen after viral infection can be prevented or significantly diminished by pretreatment with atropine [27]. This suggests that parasympathetic control is at least partially responsible for viral-induced bronchial susceptibility.

Busse et al. showed that granulocytes from asthmatic patients with upper respiratory infections have a decreased response to β2 mimetics [20]. Similar decrease in adrenergic response has been demonstrated on incubating granulocytes with influenza virus or rhinovirus in vitro [29]. It thus appears that impairment of β2-adrenergic receptor function, which in turn gives rise to bronchial smooth muscle hyperreactivity, may be due to viral infection.

Effects on inflammatory cells

Effects on mast cells and basophils. Mediator release from mast cells and other inflammatory cells is crucial to the development of bronchospasm and inflammation in asthma. Mast cells and basophils are most easily triggered through IgE mediated reactions. IgE antibodies to RSV and PIV have been demonstrated in children with bronchiolitis [30]. However, there is no direct evidence that these antibodies are functional in vivo and contribute to the inflammatory process in affected subjects.

Viruses may activate mast cells and basophils by other mechanisms. RSV and PIV can activate human basophils directly without the participation of antibodies or other serum constituents [31]. In addition, RSV has been shown to activate the complement and the complement-derived anaphylatoxins have a potential for triggering mast cells and basophils [32]. It has been suggested that during a respiratory infection immune complexes of virus and antibody may become deposited in the airways and activate complement [28]. Influenza or RSV induced production of histamine-releasing factors by mononuclear leukocytes in vitro has also been demonstrated [33]. Thus, there are many possible ways that respiratory viruses might activate mast cells and basophils.

It has been shown that basophils, but not monocytes, exerted enhanced chemotactic activity to C5 after incubation with PIV [34]. Furthermore basophil chemotaxis was also enhanced when interferon was substituted for virus, again suggesting a role for interferon in the inflammatory response.

Histamine release from pulmonary mast cells and basophils which is responsible for airway hyperresponsiveness, may be triggered either directly or by the generation of cellular products such as interferon due to viral infection.

Effects on lymphocytes. Viruses induce lymphocytes to produce interferon which was shown to enhance histamine release from basophils and mast cells exposed to a specific antigen [35]. Basophil recruitment induced by interferon also may contribute to the inflammation of viral respiratory infection. Respiratory viruses also may induce cell-mediated immune responses. A positive correlation between the degree of cell mediated immunity and subsequent episodes of wheezing has been reported [36].

Other studies suggested that a defect in suppressor cell number and function in patients with RSV bronchiolitis might cause an exaggerated lymphoproliferative response to RSV antigen as well as overproduction of antiRSV-IgE. It is not known whether such a defect may be the result of RSV infection, or not [37].

Effects on neutrophils. It is likely that neutrophils also contribute to airway inflammation and, therefore, bronchial hyperreactivity in viral respiratory infection. Influenza A enhances superoxide generation by neutrophils in vitro [38]. It is suggested

that in this way viral infection can enhance the already triggered inflammation and airway injury.

Effects on eosinophils. Recently, investigators began to evaluate the effect of viral respiratory tract infections on eosinophil functions. The eosinophils were primed by RSV to release increased quantities of LTC4 and superoxide anion on exposure to some nonspecific activators [39]. Significantly higher levels of ECP were found in children with RSV bronchiolitis than in children with RSV upper respiratory infections or non-RSV infections [40]. These data suggest that eosinophil degranulation during RSV infection may play an important role in the subsequent development of airway obstruction. Furthermore, the degree of degranulation may dictate the clinical severity of disease.

Table I

Main effects of viral infection on inflammatory cells

Mast cells and basophils	Eosinophils	Neutrophils	Lymphocytes
Activation: direct via virus specific IgE by complement products by histamine releasing factors	Enhanced migration and adhesion	Enhanced migration and adhesion Activation	Induction of interferon Induction of cell mediated immunity Alterations of T cell subsets

Table II

Main immunological mechanisms of virus induced asthma

Antibody production	Inflammatory cytokine production	Adhesion molecule expression
virus specific IgE	IL-1β TNF-α INF α, β IL-6 IL-8	ICAM-1

Molecular basis of virus-induced changes in inflammatory response. It has been demonstrated that ICAM-1 is the major receptor for human rhinovirus [41, 42]. Adhesion molecules are surface proteins found on antigen presenting cells, vascular endothelium

and airway epithelium. Increased endothelial expression of ICAM-1 is responsible for leukocyte recruitment and activation. As a result of interaction with ICAM-1 rhinovirus can potentially interfere with leukocyte migration to the site of inflammation and with the initial steps of antigen presentation. On the other hand, binding of rhinovirus to ICAM-1 can enhance the inflammatory response via induction of interleukin 1 (IL-1) secretion from antigen-presenting cells and in turn, stimulation of antigen-specific T cell proliferation. In summary, it seems to be obvious that rhinovirus can affect several distinct arms of the cell-mediated immune response.

Epidemiological data undoubtedly provide evidence for the relation between viral infections and asthma. Many experimental studies shed light on the possible mechanisms involved in this interaction. Tables I and II present the main effects of viral infections on inflammatory cells and immunological processes, respectively. The final question for the future seems to address the possibility of preventing both viral infections and if they occur treatment directed on minimizing possibility of developing asthma.

It is well known that hygiene in everyday life reduces the prevalence of most viral infections although it does not eliminate the risk of infection. Effective anti-viral vaccination seems to be the specific prophylactic measure of the future.

Treatment of viral infections of the airways in selected patients should include early application of antiinflammatory drugs, thus abolishing self-perpetuating events that lead to chronic inflammation and asthma.

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CHARACTERIZATION OF AEROALLERGENS

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Aeroallergens originate from a diverse range of animals, insects, moulds, and plants. Usually they consist of a mixture of different – allergenic and non-allergic – components. This heterogeneity was for a long time expected. But only after the introduction of CIE and CRIE by Henning Löwenstein in 1978 [1] this could be confirmed and analyzed.

By means of the CIE (crossed immunoelectrophoresis) the allergenic extract is first separated by an electrophoresis. After crossing the electrodes for 90° the different – antigenic – components move into an agarose gel containing antisera against the material and can be identified as "rockets". In the CRIE (crossed radioimmuno-electrophoresis) additional information is gained by incorporating the preparation with IgE-antibody containing sera. The rockets binding IgE – this means the allergenic components – can be identified by ^{125}I-anti-IgE and autoradiography This procedure confirmed the enormous heterogeneity of typical allergenic extracts such as from pollen, moulds, flour etc. It could be shown that they contain between 27 and 52 antigens and 3–5 allergens.

The disadvantage of these procedures was the necessity to produce at first an antiserum against the material to be analyzed. Therefore they have been replaced by separation techniques using physico-chemical peculiarities (blotting techniques). The most commonly used methods are the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the isoelectrofocusing (IEF). SDS-PAGE separates proteins on the basis of their molecular size. If standard marker proteins are run in parallel, it is possible to obtain a fairly accurate estimate of the molecular weight. The molecular weight of glycoproteins tend to be overestimated, and the glycosylation also reduces resolution. The separated proteins are blotted onto nitrocellulose membranes. Allergens are identified by incubating the blots with sera from allergic patients (western blot technique). IEF separates proteins according to their isoelectric point (pI) in a pH gradient stabilized with appropriate mixtures of amphoteric molecules. The blotting procedure is performed analogous to SDS-PAGE. However, interpretation can often prove difficult, because of charge heterogeneity attributable to isoforms, various glycosylation and deamidation. Finally, the combination of SDS-PAGE and IEF can be

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used (2D-immunoblot). It is especially appropriate to identify isoallergens. Beside the information about physico-chemical characteristics of allergens the fraction identified can be cut and used for sequencing procedures.

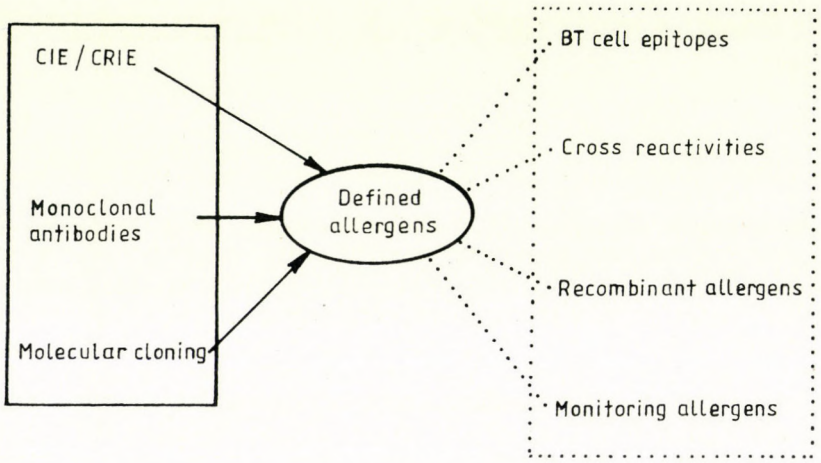

Fig. 1. Analysis of allergens

Another progress has been made by the introduction of monoclonal antibodies and genetic procedures. By means of monoclonal antibodies it became possible i, to purify allergenic molecules, e.g. for further analysis, ii, to establish quantitative and qualitative assays and iii, to identify functional sequences e.g. IgE binding epitopes.

Molecular cloning of allergens leads to their encoding genes which are easily analyzed. It starts from mRNA, which has to be prepared from the allergen source. This mRNA is then used as template to synthesize the cDNA. After preparation of the cDNA it is made ready for ligation into a suitable vector e.g. the λZAP II vector, a bacteriophage cloning vector. This ligated vector is packed with bacteriophages. After amplification of the cDNA immunoscreening is performed by transfection of *E. coli.* The expressed fusion proteins are blotted onto NC-membranes. The identification of relevant structures can be performed by means of IgE antibodies or by means of monoclonal antibodies. The prepared double stranded plasmid DNA is sequenced in both direction. By this the corresponding sequence of the amino acids can be determined. Another and more recent technique is to utilize knowledge of the amino acid sequence derived from the allergen to design primers which, in a PCR amplification, will specifically generate a DNA fragment carrying the gene of interest [2].

By means of all this techniques by the end of 1996 more then 200 individual allergens have been identified and analyzed [6]. They are designated by the nomenclature of an IUIS/WHO committee on standardization of allergens [3, 4]:

– by the first 3 letters of the genus, the first letter of the species, an Arabic numeral e.g. Der p 1 for Dermatophagoides pteronyssinus allergen 1,

– the same numeral is assigned to structurally homologous allergens from different species e.g. Phl p 5 and Lol p 5,

– if necessary, an additional letter is added to the genus or species abbreviation to avoid ambiguity e.g. Ves(pula) v(ulgaris) 5 and Ves(pula) vi(dua) 5, respectively.

– When necessary, isoallergens and variants are characterized by addition of four numbers, the first 2 identifying the isoallergens and the second 2 the variant, e.g. the 4 isoallergens of Amb a 1 are designated Amb a 1.01, Amb a 1.02 etc. and the variants of Amb a 1.01 as Amb a 1.0101, Amb a 1.0102 etc.

This progress will be exemplified for 2 especially important groups of aeroallergens – pollen from grasses and house dust mites.

Table I

Group of allergens in grasses (subfamily Pooideae)

Group	MW	pI	Glycosylation	Examples, activity	Rate of sensitization
1	26–36	5.1–9.1	+	Phl p1, Lol p 1	80– >95%
2	11	4.4–5.0	–	Dac g 2, Lol p 2	42%
3	11	8.3	–	Lol p3	45%
4	50–60	9–10	+	Lol p 4	74%
5	25–38	4.8–10	–	Dac g 5, Lol p 5	80– >95%
				ribonucleases	
10	12			Lol p 10, Poa p 10	
				cytochrome c	
11	15	5.6	+	Lol p 11	45%
Profilin	14			actin polymerization	15–30%

The grasses are a particularly large family of related allergenic sources. Already very early the problem arose, how many different types of pollen should be combined to get an extract of high efficacy. We started to answer this question by prick tests using extracts from 35 genera of grass pollens. The result was the PPL (Phleum, Poa, Lolium) mixture which could identify 98.95 % of patients with typical pollinosis [5]. But there are other combinations with similar results. In further experiments we tried to establish monoclonals for the most important allergenic fractions. We could prove the extensive cross-reactivity between the homologous groups in different species. On the other hand, we were able to identify the most important 5 groups in any genus of grass pollen,

however with extreme differences in quantity [6]. This is the reason why the use of the extract of an individual pollen genus is often insufficient.

In the meantime 8 groups are well defined – 3 of them being major allergens by definition. Group 1 usually consists of glycoproteins of between 26 and 36 kD. Lol p 1 e.g. is comprised of 240 amino acids residues. Four amino-acid substitutions have been identified in 2 isoforms that have been cloned. The pl is between 5.1 and 9.1. Similar is the situation for Phl p 1. There are several isotypes. The N-terminal amino-acid sequences show an up to 90% identity with Lol p 1. Group 2 and 3 are of minor importance. They are non-glycosylated polypeptides with a high homology. Group 4 allergens are cationic glycoproteins with a pl between 9 and 10 and molecular weights in the range of 50–60 kD. They are major allergens, more than 80% of patients with pollinosis possess IgE-antibodies against this group. The MW of the group 5 ranges from 25 to 35 kD. Obviously Poa p 9 also belongs to this group. There is a homology of more than 80 % between Phl p 5, Lol p 5 and Poa p 9, respectively. Phl p 5 consists of at least 2 isoforms (32 and 38 kD; pl 4.8–5.1 and 5.2–7.5. There is an extensive homology between the N-terminal amino acids of all group 5 allergens. Both the recombinant and the natural forms of Phl p 5 have been shown to have ribonuclease activity. This seems to hold true for other group 5 allergens, too. Beside this they seem to contain specific sequences which could be important for species recognition. It is also suggested that this allergen may play a role in host-pathogen interactions. Group 10 allergens are cytochrom C active. Profilin can be identified in all kinds of pollen. It is however a rather weak allergen and possible cross-reactivities are not relevant in clinical practice.

Table II

Allergens from house dust mites

D. pter.	MW	pl	Glycosylation	Activity	Sensitization index
p1 (f1)	25	5.7	+	cysteine protease	> 90%
p2 (f2)	14	6.6	–	?	> 90%
p3 (f3)	25	4–8	–	trypsin	51– > 90%
p4	56	5–7	?	amylase	25–46%
p5	13	?	–	?	> 55%
p6 (f6)	30	3.8–4.4	?	chromotrypsin	39%
p7 (f7)	22 (11.5–25)	4.7	+	?	53%
p8	25.5	6.3	?	glutathion transferase?	?
p9	28	4.8–10.5	?	serine protease	> 90%
Dpt 4	244	5	+	VDH-lipoprotein	?
(f10)	33		–	tropomyosin	81%

The existence of isoallergens has been exemplified especially in pollen allergens (from grasses as well as from trees; 5). Isoallergens are allergenic molecules that appear to be identical with respect to molecular size, structure and function. But when analyzed more closely they can be shown to differ with respect to their amino acid sequence up to 25%. That can be identified by 2-dimensional blotting or southern blot of DNA isolated from the plant. There are data which could speak in favour of individual isoallergens from a special plant [7]. At present it is, however, still open, whether this is clinically relevant.

The house dust mite *D. pteronyssinus* is a source of numerous allergens, an extract has been shown to contain as many as 32 different IgE-binding fractions. The major allergens from *D. pteronyssinus* and farinae have been analyzed biochemically and immunologically in detail [8]. Several of them possess enzymatic activities compatible with their role in digestive processes. Der p 1 and Der f 1 have been cloned and sequenced and found to represent proteins with 222 and 223 amino acids with the same MW of 25 kD. The homology of 81% is rather high, nevertheless there are immunologically distinct sequences. Both molecules are cysteine proteases.

Der p 2 and Der f 2 contain 129 amino acids (MW 14 kD) with 88% sequence homology. The conformations seem to be very similar as they contain 6 cysteine residues at identical positions and have the same intramolecular disulphide binding pattern. This is the cause for a high immunological cross-reactivity. By means of special monoclonal antibodies against Der 2 the total infestation of rooms can be determined. There is a strong similarity with mice lysozyme.

Der p 3 and Der f 3 are trypsin-like serine proteases derived from the digestive tract of the mites. There are N-terminal homologies between both allergens, however also with trypsins from other sources.

From *D. pteronyssinus* further allergens have been identified. Der p 4 is a 60 kD amylase. The allergenic activity depends on the intramolecular disulphide bounds. Analysis of cDNA clones led to the characterization of Der p 5, a 113 amino acid 13 kD molecule. The biological function is still unknown. Der p 6 is chymotryptic active with a MW of 30 kD. Der p 7 was first identified from an IgE-binding clone. Screening of extracts from *D. pteronyssinus* suggests that the respective structures can be found between 11.5 and 29 kD. Finally, it should be mentioned that both in *D. pteronyssinus* and cockroaches a glutathion-S-transferase with IgE-binding activity could be identified. In Dermatophagoides the MW is 25.5 kD and the pl 6.3. Major allergens are Der p 1, 2, 3 and 9 (a serine protease). Der f 10 should be mentioned as a 33 kD tropomyosin. Its cross-reactivity with tropomyosin from Crustaceae seems to be clinically relevant in selected cases.

Starting from these expanding information the old question "What makes an allergen an allergen?" can tried to be answered – at least partially

– allergens usually have MW between 5 and 70 kD,
– allergens usually are soluble in saline,
– allergens have a certain stability,
– allergens carry at least 2 (possibly) different IgE binding structures.

Table III

Aeroallergens with enzymatic activities

1. Proteases
   - 1.1. Serine proteases
     - Subtilisin
     - Trypsin: Der p 3, Der f 3; enzyme preparations
     - Chymotrypsin: Der p 6, Der f 6
     - Collagenase: Der p 9?
   - 1.2. Cysteine proteases
     - Der p 1, Der f 1, Eur m 1; Papain; Bromelin
   - 1.3. Aspartic proteases
     - Bla g 2; Candida; Pepsin
2. Carbohydrases
   - 2.1. Amylases: Der p 4; Asp o 2; flour
   - 2.2. Lysozyme: Der p 2? Der f 2?, latex (heveamine)
   - 2.3. Pectinases: Cedar (Cry j 1); moulds
3. Ribonucleases: Grass pollen (group 5), Aspergillus
4. Non-hydrolytic enzymes
   - 4.1. Enolases: moulds
   - 4.2. Alcohol dehydrogenase: Candida (Can a 1)
   - 4.3. Lipoxygenase: soy
   - 4.4. Glutathione transferase: Bla g 5; Der p 8
   - 4.5. Acyl CoA oxidase: wheat flour

Enzymatic activity is no prerequisite. Nevertheless about 20% of the allergens defined are enzymatic active (Table III) [9]. They could act as an inborn adjuvant promoting the transfer through the mucosa. It is, however not necessary. Most allergens have no demonstrable enzymatic activity, a few of them – rather active allergens – even act as enzyme inhibitors.

On the base of this expanding information it became moreover possible (Fig. 1):
- to identify IgE-binding resp. T-cell binding epitopes,
- to identify and understand cross-reactivities,
- to develop techniques for monitoring allergens,
- to produce recombinant allergens.

B- and T-cell epitopes. Based on the identification of the structure resp. the production and/or separation of highly purified allergenic molecules it became possible to identify immunologically relevant epitopes. At first so-called B-cell epitopes gained more interest. They have been identified e.g. by blocking effects of monoclonal antibodies. Soon it became obvious that B-cell and T-cell epitopes are not identical. IgE-binding epitopes very often consist of conformational structures. T-cell epitopes comprise sequences of amino acids. The latter ones gained more and more interest as they could

open the door to a scientifically-based immunotherapy. In the meantime T cell epitopes have been identified on Amb a 1 , Fel d 1 , Der p 1 etc. – usually for a limited number of patients. In own investigations we analyzed the T-cell epitopes on Phl p 5 as model allergen using T-cell clones from 15 patients with pollinosis – to get representative information. By using overlapping dodekapeptids – spanning the whole molecule – we could show that most T-cell epitopes are concentrated in 3 "hot spots", an important prerequisite for the peptide immunotherapy [8].

Cross-reactivities. These new data expanded our knowledge about cross-reactivities; this means binding of different molecules to IgE antibodies of the same specificity. This occurs when different proteins are more or less homologous and contain identical or similar epitopes. Frequently, this can be explained on the basis of taxonomic relationships (e.g. within the grasses, as exemplified; however also between Oleaceae, Compositae, mites and cockroaches). But there are sometimes surprising cross-reactivities induced by conserved structures. Well-known is profilin, an important factor for the polymerization of actin. It can be found in all eukaryotic cells – up to human ones. Usually it is, however, a rather weak allergen and cross-reactivities are more immunologic than clinically relevant phenomena "Pathogenesis related antigens" on the other hand are the basis for the well-known cross-reactivities between Bet v 1 and fruits. α-Livetin is the cause for the bird-egg-syndrome and albumins can induce a certain degree of cross-reactivity between mammals. A third type responsible for cross-reactivities between nonrelated sources are glycoproteins due to their carbohydrate side chains. Their clinical relevance is low, if there is any at all.

Recombinant allergens. Molecular cloning offers the possibility to synthesize well characterized allergens of high purity – at least their polypeptide chain. To get the recombinant protein, one has to know the nucleotide sequence of the gene coding for the allergen of interest. A standard procedure is PCR to catch and amplify the gene. After cloning and expression in a suitable vector/host system, a large amount of the allergenic protein with high purity can be extracted. However, by special hosts (yeasts, cells of mammalian origin) the glycosylation is also possible. Recombinant allergens could improve specific immunotherapy – at least for certain sensitizations.

Monitoring allergens. Monitoring allergenic exposure becomes more and more important as changes of the indoor exposure obviously contribute to the world-wide increase of bronchial asthma. The development of quantitative ELISA tests is an important tool for objective measurement of exposure [10]. In principle room air should analyzed. The concentrations of the relevant allergens are however beyond the sensitivity of the tests available at present. Therefore the content of the vacuum cleaner after vacuuming a defined surface area is analyzed and determined usually as µg/g house dust. For house dust mites and cockroaches this is adequate, as they are suspended in ambient air only temporarily. Mite allergens are inhaled above all during the night in the bed. To determine its surface concentration is therefore adequate. For cat allergen there should be a closer correlation between the content in the vacuum cleaner and in the room air. It could be shown that there are critical thresholds for indoor allergens with high Odds ratios beyond these levels up to 20 – in atopics much higher than in nonatopics. Surprisingly the levels leading to sensitization are much higher than those inducing allergic symptoms [11]. Recently a "dust-screen" has been develop ed. By a specially prepared strip the

concentrations of the most important indoor-allergens can be determined semiquantitatively. Such possibilities are not only important for diagnosis but also for monitoring the efficacy of measures to reduce exposure.

The increasing information about chemistry and immunology of allergens therefore not only satisfy a scientific interest. It opens new possibilities for the clinical practice – from the diagnosis to the therapy.

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ENVIRONMENTAL POLLUTION AND ASTHMA

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Introduction

Episodes of severe air pollution, particularly by acid aerosols and sulphur dioxide (SO_2) resulting from excessive burning of coal, frequently resulted in substantial morbidity and mortality from respiratory disease during the first few decades of this century [1–3]. Although this form of air pollution has gradually decreased since the late 1950s due to the introduction of "Clean Air Acts" and a radical change in domestic heating methods [4], the levels of SO_2 still exceed internationally agreed safety guidelines in many heavily industrialised areas of Central and Northern Europe, and to a lesser extent Western Europe. In recent years there has been a progressive increase in a new form of air pollution resulting from increased use of liquid petroleum and gas in the transport and manufacturing industries and domestic settings, and characterised by high concentrations of atmospheric hydrocarbons, oxides of nitrogen (NO_x), ozone (O_3), and respirable particulate matter (PM_{10}) [5–7].

Prevalence of allergic diseases

Several studies have shown that allergic diseases such as asthma, rhinitis, and eczema have become more common over the last 50 years. Burr and colleagues [8] have conducted two surveys 15 years apart in 12 year-old school children in South Wales (UK), and demonstrated that the number of children suffering from asthma, eczema and hay fever increased significantly from 6% to 12%, 5% to 16% and 9% to 15%, respectively, during this period. Similarly, three separate surveys conducted by Russell and colleagues [9, 10], in schoolchildren in Aberdeen (UK) over a thirty year period

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between 1964–1994, indicated that the prevalence of respiratory symptoms and atopy was increased, as demonstrated by increased diagnosis of asthma (from 4.1% in 1964 to 10.2% in 1989 to 19.5% in 1994), hay fever (from 3.2% in 1964 to 11.9% in 1989 to 12.9% in 1994) and eczema (from 5.3% in 1964 to 12% in 1989 to 17.7% in 1994).

Association between environmental factors and allergic airway disease

Although epidemiological studies, particularly from the developed countries, suggest that the increase in the prevalence of allergic disease such as asthma may be associated with air pollution resulting from increased use of liquid petroleum fuel [5–7], the findings from these studies have been difficult to interpret due to confounding effects of cigarette smoke, exposure to allergens, meteorological conditions and socioeconomic factors [11]. Additionally, these studies have investigated the effects of only the major pollutants individually, without taking into account the potential additive and/or synergistic effects of combinations of pollutants which are more relevant.

Studies from Japan have demonstrated that the incidence of rhino-conjunctivitis in residents living alongside old cedar tree-lined main roads with heavy traffic all day long was much higher than that in residents living in the cedar forest but with less traffic, despite the cedar pollen counts being similar in both areas [12]. These studies suggest that the disparity in the incidence of rhino-conjunctivitis in the different areas may be a result of vehicle exhaust pollution, which was the predominating factor in areas with high incidence. Early studies from Germany, particularly by von Mutius and colleagues, have demonstrated that allergic conditions such as hay fever were more common in West German cities than in East German cities, where chronic bronchitis was more prevalent [13]. Recent studies from Germany have even demonstrated that whilst the prevalence of atopic diseases has not altered significantly in Hamburg (West Germany), there has been a significant increase in the prevalence of these diseases in Erfurt (East Germany), suggesting that there is already a converging tendency of self-reported asthma and asthma symptoms [14]. Overall, the differences in the respiratory conditions have largely disappeared following unification of East and West Germany and it has been suggested that this is likely to be a result of both the change in air pollution patterns and living habits in the former East Germany [15].

More recent studies suggest that indoor environment is likely to be an important factor in the increased incidence of allergic disease, since as much as 90% of the time is spent indoors [16]. Von Mutius and colleagues have investigated the association between skin test reactivity and methods of heating and cooking in the homes of over 5,000 schoolchildren in West Germany and demonstrated that the prevalence of atopy and hay fever was significantly higher in children living in homes with gas ovens, oil-furnaces and central heating, compared to children living in homes where coal or wood was used for heating or cooking, indicating the possible deleterious effects of gas and oil-derived air pollutants [17]. Similarly, Luczynska and colleagues have investigated skin sensitivity to a panel of indoor and outdoor allergens in 10–11 years-old school children living in Tower Hamlets and Eltham, two areas of London segregated on the basis of their socio-

economic status and traffic pollution and demonstrated that the prevalence of skin sensitivity to house dust mite, grass pollen and cockroach allergen was higher in Tower Hamlets, the more deprived and polluted of the two areas [18].

Studies by Wallis and colleagues have suggested that certain meteorological factors may also play a role in the pathogenesis of allergic airways diseases such as asthma [19, 20]. These authors have investigated the clinical and immunological characteristics of 640 patients attending Accident and Emergency Departments of several hospitals for treatment of acute asthma attacks or other airways disease following a thunderstorm on 24 June, 1994 in London and demonstrated that 44% (283 individuals), who were not known to be asthmatic experienced asthma attacks. Additionally, twelve out of the 15 patients in whom serum specific IgE was measured demonstrated a very high RAST score (4 to 6) against grass pollen mix. Knox and colleagues have suggested that epidemics of asthma following thunderstorms are likely to be due to release into the atmosphere of pollen allergens which are associated with respirable starch granules (0.5–2.5 μm in diameter), following osmotic rupture of the pollen grains in the rainfall [21].

Association between environmental pollution and respiratory illness

i) Epidemiological studies: Several studies have demonstrated that there is a clear association between episodes of air pollution and impaired lung function, cough and infections of the lower respiratory tract. Emergency room visits for asthma in Finland have been shown to be significantly associated with NO_2 and particulate levels, although only NO_2 was an independent association after controlling for temperature [22]. Usetti and colleagues [23] examined an asthma outbreak in Barcelona in 1983, and found a clear correlation between oxides of nitrogen concentration and hospital admission for asthma. More recently, Jarvis and colleagues have studied a randomly selected population of approximately 1200 young adults living in three areas of East Anglia (UK) by an extended questionnaire, blood sampling for analysis of total and specific IgE, and lung function tests to see whether or not there were any associations between the use of domestic gas appliances and increased risk of respiratory symptoms and sensitisation to common environmental allergens [24]. These authors demonstrated that women were at a greater risk, than men, of developing asthma-like symptoms. In women who mainly used gas for cooking, the odds ratios (OR) for wheeze (OR = 2.07), waking with shortness of breath (OR = 2.32) and asthma attacks (OR = 2.60) were increased. Additionally, airway obstruction was increased and lung function was reduced in these individuals. The risk of symptoms, however, was increased more in women who were atopic than non-atopic, although this was not found to be statistically significant. Similarly, studies by Weeks and colleagues have demonstrated that the levels of night time NO_2 were increased in households with smokers and/or gas fires and that exposure of asthmatic children in such households to the increased concentrations of NO_2 correlated significantly with a decrease in the mean morning peak flow of these children, the next day [25].

Increased levels of O_3 have also been shown to adversely affect lung function in children [26, 27] and are associated with increased hospital admissions [28, 29]. Romieu

and colleagues recently studied the relationship between the number of daily emergency visits for asthma and air pollutant levels at one major paediatric hospital in Mexico City. They found that the level of atmospheric ozone exposure was significantly associated with the number of emergency visits for asthma. After adjustment for potential confounding factors, the multivariate regression model predicted that an increase of 50 ppb in the 1-hour maximum ozone level would lead to a 43% increase in the number of emergency visits for asthma on the following day. Exposure to high ozone levels (> 110 ppb) for 2 consecutive days increased the number of asthma-related emergency visits by 68 percent [29]. A study of 499 school children from schools in areas of high (100–120 ppb) or low ambient (≥ 60 ppb) O_3 levels in Austria also demonstrated that bronchial hyperresponsiveness occurred more frequently in children from areas of high O_3 levels [30].

In a recent prospective observational study lasting over a period of one year, Buchdahl and colleagues reported that day to day variations in daily average concentrations of O_3, in particular, and SO_2 were significantly associated with the incidence of acute wheezy episodes [31]. Significant associations between mean levels of winter smoke and SO_2 during hospital admissions for asthma and respiratory disease, lagged by two days, have also been demonstrated by Walters and colleagues in Birmingham, UK [32]. More recently, a study of school children at a summer camp in the Austrian Alps has demonstrated that increased mean levels of ambient acidic particles was associated with transient decreases in lung function in these children [33].

Studies on respirable particulate matter have demonstrated that increased levels of PM_{10} are also associated with worsening peak flow, inhaler usage and respiratory symptoms in asthmatic children with a lag effect of 24 hours [34]. In adults with severe asthma, peak expiratory flow rate (PEFR) worsened in association with PM_{10}, with a lag effect of 4 days [35]. In Seattle, USA, PM_{10} levels were found to be associated with emergency room visits for asthma the following day [36].

Most recently, concern has emerged regarding the health effects of environmental exposure to ultrafine particles (diameter < 0.1 μm), which are always present in the urban atmosphere in very large numbers (0.5 to $>3\times10^5$ particles/cm^3). These particles are freshly generated by combustion processes, tailpipe emissions and gas to particle conversions and can be highly reactive [37]. Peters and colleagues have investigated the effect of ultrafine particles on symptoms and peak flow of asthmatics in Germany and demonstrated that an increase of a five-day-mean of 10^4 ultra-fine particles per cm^3 was associated with a significant decrease in PEFR of 4.41 l/min in adults and a smaller decrease of 3.24 l/min in children [38].

ii) Laboratory exposure studies: Laboratory based studies have shown inconsistent effects of NO_2 exposure on lung function and non-specific bronchial responsiveness. In one study, NO_2 at low concentration (400 ppb) did not affect lung function in normals but caused bronchoconstriction and increased bronchial responsiveness in asthmatics [39]. However, lung function and bronchial response to subsequent SO_2 inhalation were not consistently affected by exposure to 250–300 ppb NO_2 in asthmatics [40, 41].

In contrast, numerous studies have agreed in finding significant effects of O_3 in increasing bronchial responsiveness and causing lung function impairment [5]. Although the effects of O_3 are enhanced at ambient concentrations in healthy exercising subjects,

asthmatics do not appear to be more sensitive to O_3 than "healthy" subjects. Studies of O_3 inhalation have also demonstrated that there are concentration- and exposure time-related changes in symptoms and lung function, including an increase in airway resistance, a reduction in lung volumes and an increase in airway responsiveness to bronchoconstrictor agents [5]. A meta-analysis by Hazucha [42], of all studies for which measurements of forced expiratory volume in 1 sec (FEV1) and forced vital capacity (FVC) were available for individuals undergoing a 2 hour exposure to different concentrations and exercising at different intensities has demonstrated that doubling the O_3 concentration has a greater effect on lung function than doubling ventilation.

Trenga and colleagues have recently investigated the effect of the nutritional status and susceptibility of subjects with asthma to toxicant damage caused by O_3 and reported that a daily intake of combined vitamin E (400 IU) and vitamin C (500 mg) attenuated O_3-induced decrease in FEV1 of these individuals. These studies suggest that supplementation of the diet with vitamins E and C may help asthmatics to cope with episodes of increased air pollution [43].

Studies of SO_2 inhalation have demonstrated that this gas also leads to bronchoconstriction in both normal healthy and asthmatic subjects and that deep breathing and intermittent exercise may potentiate this effect [6]. Although the response to SO_2 inhalation is variable in individuals, concentrations of SO_2 that have little or no effect on normal healthy subjects can produce marked symptomatic bronchoconstriction in patients with asthma.

Some studies have suggested that air pollutants may be more harmful in combination with each other rather than individually. Jörres and colleagues [40] have investigated the effect of a 30 minute exposure of asthmatic subjects to atmospheres of either air, 250 ppb NO_2 or 500 ppb SO_2, on isocapnic hyperventilation to 750 ppb SO_2 and demonstrated that preexposure to NO_2 enhanced the airways responsiveness to hyperventilation of SO_2. Similarly, Koenig and colleagues [44] have investigated the effect of a 45 minute preexposure to 120 ppb ozone on changes in pulmonary function of exercising adolescent asthmatic subjects exposed subsequently for 15 minutes to 120 ppb SO_2 and demonstrated that ozone potentiated the effect of SO_2 in these subjects.

The influence of air pollution on the airway response to inhaled allergen

Exposure chamber studies of asthmatic individuals exposed to O_3, NO_2 and a combination of NO_2 and SO_2 have indicated that these agents may increase the airway responsiveness of asthmatics to inhaled allergen. Jörres and colleagues [45] studied the effect of exposure to O_3 on the bronchial responsiveness to inhaled allergen in allergic asthmatic and rhinitic individuals and demonstrated that prior exposure for 3 hours to 250 ppb O_3 led to an increase in the bronchial responsiveness to allergen in these subjects. In asthmatics exposure to O_3 significantly decreased both the methacholine $PC_{20}FEV_1$ and allergen $PD_{20}FEV_1$. In rhinitics, whereas the allergen-induced change in FEV1 after exposure to filtered air was not significantly different from zero, the change after O_3 exposure was significantly different compared with exposure to air. Similarly, Tunnicliffe

and colleagues [46] have investigated the effect of exposure for 1 hour to 100 ppb or 400 ppb NO_2 in mild asthmatics and demonstrated that 400 ppb NO_2 significantly increased the airway response to inhaled house dust mite (HDM) allergen, during both the immediate and late phase. We have investigated the effect of exposure for 6 hours to either air, 400 ppb NO_2, 200 ppb SO_2 or a combination of the two pollutants on the airway response of non-exercising mild asthmatic patient volunteers to inhaled *Dermatophagoides pteronyssinus* allergen and have found that exposure to the combination of the two pollutants, but not to the individual gases, caused a significant reduction in the mean allergen $PD_{20}FEV_1$, when compared to exposure to air, without any significant effects on lung function [47]. We have also shown that the enhanced airway response to inhaled allergen in asthmatic individuals, resulting from exposure to the combination of 400 ppb NO_2 + 200 ppb SO_2 persists over a period of 24–48 hours and is maximal by 24 hours after exposure [48].

Putative mechanisms underlying pollution-induced increase in airway reactivity

Although there is some evidence to suggest a link between an increase in the prevalence of allergic airway disease and an increase in air pollution, there is little doubt that exposure to pollutants enhances the airway response to inhaled allergens. However, the mechanisms underlying these effects are not fully understood.

It has been suggested that air pollutants may promote sensitization and subsequent development of allergic disease by modulating the allergenicity of airborne allergens. Behrendt and colleagues have demonstrated that pollen collected from roadsides with heavy traffic and other areas with high levels of air pollution are covered with large numbers of airborne particulates ($\leq$ 5μm in size), and that incubation of pollen for 2–5 hours in aqueous solutions prepared from these particulates led to morphological alterations in pollen and extravasation of allergens with altered antigenicity [49]. More recently, Knox and colleagues have investigated the interactions between respirable diesel exhaust particles (DEP; 30–60 nm diameter) and purified rye-grass pollen allergens Lol p1 and Lol p5 and demonstrated that Lol p1 binds strongly to the DEP. These authors suggest that DEP may act as an environmental asthma trigger, particularly during episodes of air pollution in the grass pollen season [50].

Several studies have suggested that pollution-induced airway epithelial damage and impaired mucociliary clearance may allow easier penetration and access of inhaled allergens to cells of the immune system. Studies investigating the pathophysiological effects resulting from inhalation of O_3 have demonstrated that this agent leads to epithelial damage and an increased inflammatory response in the upper and lower airways, as indicated by leakage of lactate dehydrogenase, albumin and total protein, and increase in neutrophils, eosinophils, mononuclear cells, fibronectin, α-1-antitrypsin, interleukins-6 and 8, GM-CSF and prostaglandin E2, in nasal lavage (NAL), proximal airway lavage (PAL) and bronchoalveolar lavage (BAL) [51–53]. Basha and colleagues have demonstrated that although there were no significant differences in spirometric values and symptom scores between asthmatics and healthy volunteers, after exposure for

6 hours to 200 ppb O_3, there were significant increases in IL-6, IL-8 and PMN numbers in BAL fluid obtained 18 hours postexposure in asthmatics only [54]. These studies suggest that O_3 may preferentially increase the production of cytokines and inflammatory cells in asthmatics, possibly leading to an acute exacerbation at a later stage.

Peden and colleagues demonstrated that prior exposure of perennially allergic asthmatics to 400 ppb O_3 significantly increased the allergen-induced release of eosinophil cationic protein (ECP) in nasal lavage of these individuals, without significantly affecting the number of eosinophils, 4 hours after allergen challenge [55]. These results suggest that exposure to O_3 may "prime" the eosinophils to subsequent activation by inhaled allergen. Similarly, we have exposed seasonal allergic rhinitics for 6 hours to either 400 ppb NO_2 or air ± allergen challenge, following 30 minutes after exposure, and have evaluated the changes in nasal airway resistance (NAR) and presence of inflammatory mediators in NAL [56]. These studies have demonstrated that whilst exposure to NO_2 alone did not significantly alter the NAR or increase the concentration of ECP, tryptase or myeloperoxidase in NAL, exposure to NO_2 prior to allergen challenge significantly increased the concentration of ECP, but not tryptase or myeloperoxidase in NAL, compared with exposure to air, suggesting that acute exposure to NO_2 may also "prime" the eosinophils for subsequent activation by allergen.

Putative role of airway epithelial cells in the development of pollution-induced airway disease

There is increasing evidence that airway epithelial cells may play a pivotal role in the pathogenesis of allergic airways disease, since they can express and synthesise a variety of inflammatory cytokines and adhesion molecules including IL-1ß, IL-6, IL-8, IL-16, granulocyte-monocyte colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNFα), regulated on activation, normal T cell expressed and secreted (RANTES) and intercellular adhesion molecule-1 (ICAM-1), which influence the activity of eosinophils and lymphocytes, which play important roles in the allergic reaction [57–61]. RANTES and IL-8 and GM-CSF in combination are chemoattractant for eosinophils and GM-CSF plays an important part in delaying apoptosis of eosinophils. Studies from our laboratory have recently shown that exposure of human bronchial epithelial cells to 400–800 ppb NO_2, in vitro, leads to increased epithelial permeability and damage of these cells, decreased ciliary activity, and release of pro-inflammatory mediators, including LTC_4, GM-CSF, TNF-α and IL-8 [62, 63]. Similarly, our studies have demonstrated that exposure of these cells to ambient concentrations of O_3 (10–50 ppb) well below the WHO safety guidelines, induced significant release of IL-8, GM-CSF, TNF-α and soluble ICAM-1, and that release of GM-CSF, TNF-α and soluble ICAM-1 was blocked by treatment of the cells with 10^{-5} M nedocromil sodium [64]. Investigations of the effects of glutathione, a naturally occurring intracellular anti-oxidant, have demonstrated that this compound attenuates the O_3-induced release of IL-8 from human bronchial epithelial cells in a dose dependent manner. Exposure of these cells for 6 hours to 10–500 ppb O_3 led to

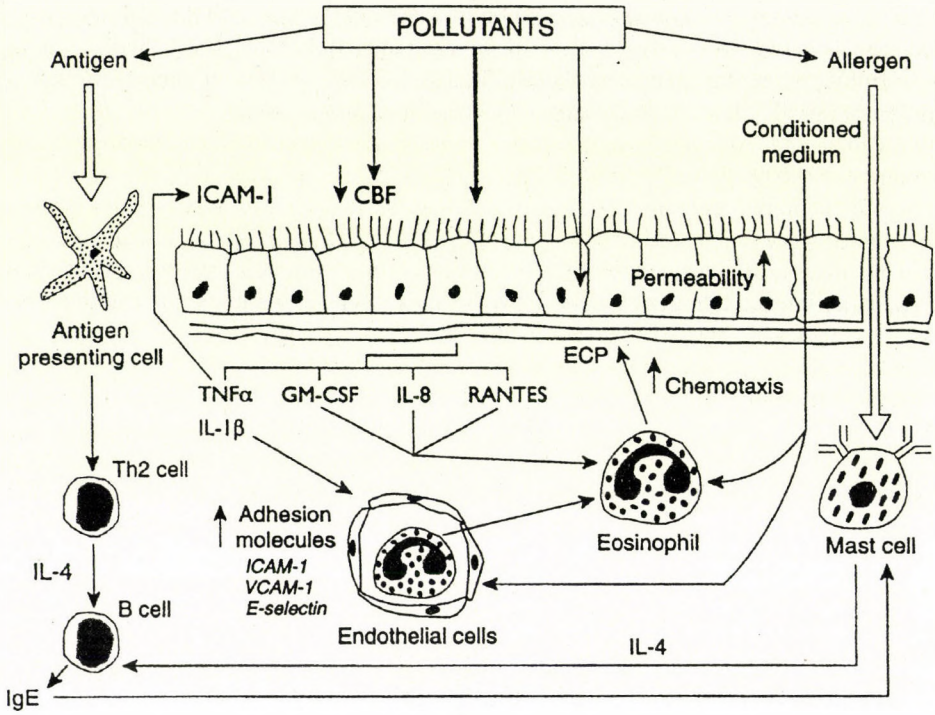

Fig. 1. Schematic view of the role of airway epithelium in the development of allergic airway diseases. (IL = interleukin, TNF = tumour-necrosis factor, GM-CSF = granulocyte-macrophage colony-stimulating factor, ICAM = intercellular adhesion molecule, CBF = ciliary beat frequency, ECP = eosinophil cationic protein, RANTES = regulated on activation, normal T cell-expressed and secreted molecule)

significant epithelial cell damage at concentrations above 100 ppb O_3 [64]. We have also investigated the effect of diesel exhaust particulates (mean aerodynamic diameter 0.4 μm) on cultured human bronchial epithelial cells and demonstrated that 10–100 μg/ml of this pollutant significantly attenuated the ciliary beat frequency of these cells and increased the release of IL-8, in vitro [65].

Most recently we have investigated the effect of cigarette smoke on human bronchial epithelial cell permeability in the absence or presence of house dust mite allergen *Dermatophagoides pteronyssinus* (Der p), in vitro. We found that exposure of these cells for 20 minutes to cigarette smoke did not significantly alter either the electrical resistance or movement of ^{14}C-BSA across the epithelial cell layer, when compared with exposure to air. In contrast, incubation of these cells with Der p led to a significant decrease in the electrical resistance over a period of 6 hours and an increase in the movement of ^{14}C-BSA over a period of 24 hours. Furthermore, exposure of the cells for 20 min to cigarette smoke significantly enhanced these effects. Movement of Der p itself

was also increased by exposure to cigarette smoke suggesting that although short-term exposure to cigarette smoke alone may not increase the non-specific epithelial permeability, it could render the epithelium more susceptible to adverse effects of allergens such as Der p. Preliminary studies from our laboratory also suggest that exposure of these cells to cigarette smoke significantly increases the release of IL-8, a potent neutrophil chemoattractant, from these cells.

Despite increasing evidence for the role of airway epithelial cells in the generation of pro-inflammatory cytokines, there is comparatively little information on the biological relevance of air pollutant-induced release of cytokines in airway inflammation. We have recently investigated the effect of conditioned medium from bronchial epithelial cell cultures exposed to 50 ppb O_3 for 6 hours and demonstrated that O_3 significantly increased eosinophil adherence to human endothelial cells, when compared to conditioned medium from cells exposed for 6 hours to air, suggesting that pollution-induced inflammation of the airways may be a consequence of increased synthesis and/or release of epithelial cell derived mediators which influence the activity of inflammatory cells [66].

Conclusions

Taken together, the findings of both epidemiological and laboratory based studies provide evidence that exposure to air pollutants generated from petrol and diesel burning engines are likely to precipitate attacks of asthma and possibly contribute to the increase in prevalence of this disorder. The mechanisms by which pollutants exert their effects may be either indirect such as modulation of allergenicity of airborne allergens or direct which include i) increased epithelial damage and permeability, ii) decreased ciliary activity, iii) depletion of naturally occurring anti-oxidants, and iv) release of pro-inflammatory cytokines and cell adhesion molecules, which orchestrate the functions of inflammatory cells such as eosinophils, mast cells and lymphocytes (Fig. 1).

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RISK FACTORS OF DEATH FROM ASTHMA

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In the present days, with all availabilities of modern asthma treatment, when properly treated, no asthmatic patient should ever die from asthma. We have all the possibilities to make true Laennec's opinion that asthma promotes longevity. His opinion and other XIX-century opinions, like e.g. Osler's that asthmatics pant into old age, proved, however, later to be wrong. Now we have asthma as potentially fatal disease. Why it is so, when we have so potent tools at hand? What are the risk factors which overgrow our medical possibilities?

They are quite a few. I am not going to speak about the diagnostic problems: what is asthma and what is not asthma. In general, I am inclined to believe that the so-called "Dutch Hypotesis" is true [1]. The asthmatic phenotype dominate during whole individual's life, both during atopic early asthma, adult (non-atopic) asthma, and "spastic" bronchitis during last years of life. During all these "periods" the risk factors of death are similar, but with different impact and severity.

The first one is the severity of childhood asthma with recurrent infections. The long duration of symptoms and the lack of their total clearance during puberty lead to the permanent impairment of the respiratory function. That is manifested by the lower values of "the personal bests" FEV_1 at the age 21–23. Such asthmatics are already handicapped during their best period (third decade of life), by greater easiness of access the "symptomatic" level of FEV_1 and later "disability" level (Fig. 1).

Before the attainment of these "levels" many other risk factors interplay in the picture. The first is the underappreciation of the symptoms (and severity) of asthma. That concerns their parents during childhood and themselves during adolescence. Quite a few cases of asthma death among adolescents happen each year. Many such deaths are due to the "negation of illness" by adolescents what results in symptomatic treatment only. Regular antiinflammatory treatment is neglected, and during fatal attack the medical service is delayed. Sometimes underlying causes are psychosomatic disturbances. The existence of depression is often not recognised and the great introversion seems to be the rule [2]. Depression, which may be diagnosed even in 25% of children hospitalized for asthma (as examined in Denver), often leads to the overactivity of the parasympathetic

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nervous system. Hypervagotony in turn, promotes bronchoconstriction. So among depressive patients the risk of severe asthma is increased in two ways: by increase of reactivity and decrease of compliance.

Such sequence of events is often forgotten by the doctors.

Commonly known risk is the sudden and high degree exposition to airborne allergens or food. Probably many sudden deaths, in the second or third decade of life, may be caused by such situations [3]. Deserve for mentioning are: thunderstorm asthma (inhalation of scrobia particles coated with allergens), exposition to many inhalant allergens, allergy to peanuts or "sea fruits", and many other asthma with anaphylactic reactions.

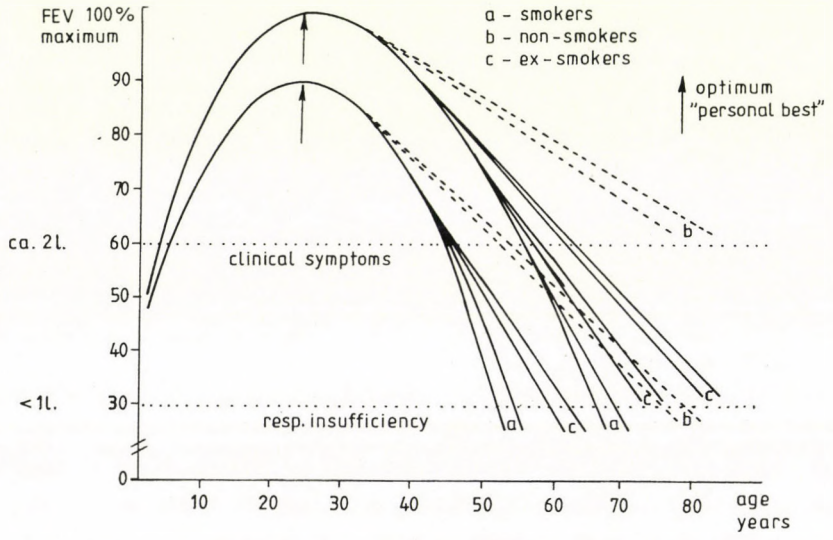

Fig. 1. The influence of smoking and "personal best" value of FEV_1 on its decline during lifespan

Among some patients chronic exposition may also be dangerous. That concerns the so-called "poor perceivers". Among asthmatics are the people who do not recognise progressive bronchial obstruction by the feeling of dyspnea or they do not react to moderate hypoxia by the increase of ventilation. Kikuchi proved that especially those asthmatics, who survived "near fatal" attack of asthma, belong to such people [4]. Using Borg's scale their results are significantly lower than asthmatics, who never experienced "near fatal" episode. So their perception of increased resistance to breathing is lower than that of other asthmatics or healthy people. Also among older asthmatics the awareness of moderate acute bronchoconstriction is much smaller than among younger asthmatics. Blunted perception no doubt, plays a significant role in many asthma deaths, especially in

non-sudden fatal events. Parallelly with perceiveness there is another physiological phenomenon which may create the general picture of severe asthma attack. That is chemoperception. Hutchinson and Olinsky already in 1981 proved that some asthmatics with common severe attacks of asthma and respiratory insufficiency demonstrate the abnormally low response of hypoxia [6]. Such decrease of response (no increase of ventilation) during asthma free period was also observed among other members of their families. So some inborn mechanisms must here be considered and most probably such low hypoxic drive is rather cause and not sequence of COPD [6]. The same concerns the response to hypercapnia. Some children of patients with far advanced COPD and hypercapnia demonstrated decreased response to hypoxia. The children with normocapnic COPD patients – did not [5, 6].

Ventilatory drive must always be considered in asthma patients especially among those who are prone to the loss of consciousness. Blunted perception and low hypoxic drive are dangerous, especially to older asthmatics with emphysema, remarkable impaired respiratory function and with other accompanying diseases.

Clinically a very important risk factor is in all age groups the patients' poor compliance. Roth and Caron were probably the first who over 30 years ago appreciated the importance of regular drug uptake in chronically ill patients (peptic ulcer) [7]. The former term – patient's cooperation in now substituted by compliance. This concerns all medical specialities. Oldridge's data show that about 70% of post heart-infarct patients do not follow their rehabilitation programme, regardless of its duration [8]. Compliance in asthma is of vital importance, it's lack is a serious risk factor.

Among those who died from asthma, up to 66% were unreliable in drug taking [9]. In adolescent death, poor-compliance has been found in 53% [10]. Only about half of average asthmatics are fully compliant as far as the use of inhaled medicine is concerned [11]. Even patients with very severe (potentially fatal) asthma were compliant in only 58%, less severe ill – in 81% [2]. Compliance is slightly better when usage of medicine (in tablets or in inhalation) is driven by symptoms [12]. When β-agonist was added to budesonid, the compliance (in its application) rose from 50 up to 94%. Other studies however have not confirmed these opinions [13]. Compliance in drug taking is influenced by many factors but in some patients it is unpredictable. Compliance is improved when the regime of the drugs is possibly simple e.g. no more than twice daily and minimal number of drugs. Still better, when the treatment is tailored to the patient's preference. Handwritten instruction for the patients improves compliance much better than the printed guidelines of treatment. Of course the patient-doctor relationship must be very good and side – effects of the treatment – minimal [14]. The average asthma patient is usually curious of the cause and mechanism of his or her illness and 84% of them agree to participate in the educational programme [15]. At risk are those who do not. Intensive out-patient management + educational programme results in 4-fold decrease in number of hospital admission in comparison to the routine care [16]. Also the number of admission for individual patients was 3 times lower in the first group. Obstacles for a good compliance are: (i) forgetfulness, (ii) prejudice against medicament e.g. steroidphobia, (iii) fear of side effects of drugs, (iv) too complicated scheme of treatment, (v) incomprehension of the purpose of treatment, (vi) price of drugs [17].

These patterns are most common among low socio-economic groups, among adolescents and among lonely people who are living without any family support [18]. As a rule mentally handicapped people are non-compliant. Very important – often forgotten or avoided for political reason – is the low socio-economical status of the asthmatic patients.

In high asthma mortality areas poverty is much more common than in low mortality areas. Such comparative studies were possible in the United States where the differences between various social groups are very big [19]. In the low mortality area the mortality from asthma was 0.38/100.000, in high mortality – 6.66/100.000. The high mortality area was inhabited mainly by African-Americans (66% of all inhabitants), 48% of inhabitants had no high school diploma and 42% lived in poverty. Low mortality area was inhabited in 97% by white Americans, 66% had high school diploma and in poverty lived only 7% [19]. In high mortality area the crowding at homes was 5 times higher than in low mortality area (10% of inhabitants versus 2%). It is known that poor living conditions promote early exposure to may allergens (mites, cockroaches) and increases the risk of many viral infections with all clinical consequences (allergisation, asthma attacks, bronchitis). Poverty and poor living conditions are associated very often (if not always) with harmful environmental factors such as higher SO_2, nitrogen compounds and dust exposure. These are the proven risk factors for exacerbation of asthma. Poverty is correlated with less efficient medical service provided by general practitioners. Emergency asthma hospital admission closely correlates with the degree of deprivation as indicated by Townsend Index. This index is calculated as a percentages of: regional unemployment, households with no cars, homes and households with more then one person to a room. The higher was Townsend index (greater deprivation) the more numerous were age standardised hospital admissions for asthma via Accident and Emergency route.

No such correlation was found when admissions were done by GPs what may be regarded as a proof of better medical supervision. So it is another argument, that the economic background is likely to be an important factor in the differences in the health care [20]. Hospital admission especially to ICU or the cases of "near fatal asthma" are by themselves another risk factors of the death from asthma.

Many "sudden asthma death" cases occurred among patients who not long before were treated in ICU or had many hospital admissions in their past history. Such cases have to be carefully evaluated [18].

All risk factors are growing alongside with age of asthmatics. The older they are the bigger are the risk factors. This fact makes remarkable contribution to the growing death rate from asthma in the older age groups.

Alongside with advanced age and the regular decrease of ventilatory reserves, the bronchial hyperreactivity and hypersensitivity are growing. Older asthmatics much easier react to many non-specific stimuli like cold air, viral infections and even to the meteorological factors (winds, weather fronts and temperature changes) [21]. Their autonomic regulatory mechanisms seem to be much less efficient. Partially it may be attributed to the existence of autoantibodies. Many elderly people otherwise in good conditions have circulating antibodies. They may be directed against immunocompetent cells like B lymphocytes [22] but also against some cellular receptors. If it concerns β_2

receptors, what is quite a common feature even in younger age [23] then it explains convincingly the increased bronchial reactivity among elderly.

These facts potenciate the action of all the risk factors operating throughout the whole life of the asthmatic patient. Over 50% of all asthma and asthma related (COPD, chronic bronchitis) deaths happens in the 7th decade of life [24]. Epidemiological data however, clearly show, that age related asthma death rates are steadily growing starting from the age of 55 (WHO data). The contributing factors are many accompanying diseases which moderate the whole clinical picture. Among these who died from asthma, spastic bronchitis or COPD (according to clinical diagnoses) diabetes was diagnosed in 17%, peptic or duodenal ulcer in 22%, psychotic disturbances in 17% [24]. Besides, 50% of all passed away asthmatics suffered also from coronary or ischemic heart diseases. Such factors may often be overlooked or underappreciated especially when the death happens outside the medical centers.

This concerns both the "cardiac" deaths, like the death due to gastrointestinal (non-recognised) bleeding. Chronic circulatory insufficiency may be the cause of bronchial mucosa edema and plasma exudation into asthmatic airways. Such phenomenon may not be significant enough for diagnosis of pulmonary oedema but sufficient to create marked bronchial hyperreactivity [25, 26]. Finally, among very old asthmatics chronic muscle fatigue and weakness of respiratory muscles play an important role. These factors may be the "final blow" to severely compromised chronic asthma patient.

So it may be said: all asthmatics during their whole lifespan are endangered by many factors and it's the duty of their doctors to know these factors even better than the most sophisticated modern antiasthmatic drugs.

Underassessment, underappreciation and undertreatment are the common features in majority of asthma-death [18]. This must be kept in mind of both the doctors and the patients.

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THE USE OF BACTERIAL ANTIGENS IN BRONCHIAL ASTHMA

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There is an ecological stability within the microcosm of the normal flora of healthy human beings. This stability is both quantitative and qualitative. It appears to have resulted from evolution of a balance of dynamic interactions rather than indifference among constituents of the flora. The discussion still considers interactions between *Streptococcus viridans* and *Moraxella catarrhalis* that are the primary normal inhabitants of the respiratory tract and other bacteria that appear to contribute to inflammatory pathology, such as *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae* and others [1].

The concept that naturally occurring antagonists between bacteria may be exploited to man's advantage is not quite new. In 1877, Pasteur noted inhibition of growth of the anthrax bacillus in urine contaminated with "common micro-organisms" and speculated that these contaminants might be useful in the treatment of anthrax (acc. 2).

The role of bacterial infection in exacerbation of bronchial asthma and chronic obstructive pulmonary disease (COPD) is well known and documented, however the mechanisms of this action still remain unclear. It was shown in experimental and clinical studies that Gram-positive and Gram-negative bacteria and their products are able to release many mediators and induce immune reaction in atopic and non-atopic asthma patients [2, 3].

Norn and co-workers have found immunological (IgE-dependent) and non-immunological (non-IgE dependent) mechanism of histamine release induced by bacterial lectins [3]. Recently, lectins have been also detected on the surface of human basophils and have been able to interact with carbohydrates on the bacterial wall leading to mediator release [1, 3]. Moreover, it was experimentally documented that bacterial exotoxines (α-toxin isolated from *Staphylococcus aureus,* leucocidin isolated from *Pseudomonas aeruginosa*) may stimulate neutrophils to produce and release leukotriens and other lipoxygenase derivates [4]. Similarly, inflammatory cells obtained from

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bronchoalveolar lavage stimulated with *Staphylococcus aureus* were demonstrated to release histamine and leukotrien B [3]. This proinflammatory reaction induced by released bacterial mediators suggests an important role of bacteria in respiratory tract pathology, especially in asthmatic patients.

It was documented that bacteria and their products interfere with inflammatory cells, e.g. eosinophils, neutrophils, and stimulate the release of inflammatory mediators involved in acute and late asthmatic response [2, 4]. The activation degree of inflammatory cells is responsible for the release of performed and newly generated mediators, and the expression of cytokine message as well as their release, e.g. GM CSF (granulocyte-macrophage colony stimulating factor), INF-α (interferon-α), IL-4 (interleukin-4) [4–7]. This mechanism promotes allergic late reaction.

It is well known that viral infections damage mucosa of the respiratory tract epithelium and cause transient bronchial hyperreactivity [3, 8, 9]. Viruses help bacteria to enter into epithelium and stimulate smooth muscles to bronchospasm [1, 5]. The specific bacterial antigen may amplify inflammatory response in asthmatic patients [10–12]. Bacteria, as we can see, contribute to asthma and chronic inflammatory diseases in the respiratory tract in numerous ways.

Links between the digestive tract and the respiratory system these two mucous membranes, which are highly open to the outside, have a similar immunological defence mechanism. Non-specific defence in the respiratory tract is realised by macrophages and neutrophils [12]. They are "a first line guard" which protects the respiratory system against infection. In the bronchus and intestine there are lymphoid systems called BALT (bronchus associated lymphoid tissue) and GALT (gut associated lymphoid tissue), which contain lymph follicles with T and B lymphocytes. This problem was recently presented in a different dimension and MALT (mucosal associated lymphoid tissue) was proposed as the largest mammalian lymphoid organ [13, 14]. It is formed by inductive sites (Peyer's patches and solitary follicles) and mucosal effector sites (BALT, GALT) [10, 13].

Antigens taken up by specialised epithelial cells (M cells – microfold cells) in mucosal inductive sites are presented to the B and T lymphocytes or transferred to professional antigen presenting cells in the mucosa-macrophages, B cells, dendritic cells. The antibody formation reaction is induced and T-cell mediated immunity comes to reality [15]. But it should be considered that after antigen induced activation and partial differentiation in the inductive site both B and T cells migrate rapidly to the regional lymph nodes and to the peripheral circulation [13]. They express adhesion molecules called "homing receptors", specific for "addressins" on endothelial cells in the mucosa [16].

The effect of the MALT activity is to produce sIgA antibodies, what is dependent on cytokines (IL-2, IL-4, IL-5, IL-10) originated from T cells and influenced by B cells [13]. It seems also that the MALT is a site of strict co-operation of Th1 and Th2 lymphocytes in the regulation of sIgA production, however segregation between T cell subsets in human mucosa is not clear [15, 17]. In the antibiotic era attention was focused upon isolation and purification of antibacterial substances to be used as either therapeutic or prophylactic agents. Following observations have appeared to have contributed to this renewed problem: a) antibiotics are of limited value in the prophylaxis of many bacterial

infections; b) suppression of the normal microbial flora by antibiotics may be followed by profound adverse effects in the host; c) selected implantation of a strain of bacteria with low virulence has been shown to protect infants from action of more virulent strains of *Staphylococcus aureus* [18, 19]. In this situation, many microbiologists have once again returned to attempts to understand the role of the normal microbial flora in resistance to infection.

The concepts regarding the antagonistic interactions of bacteria are very important from the practical point of view. The results of performed examinations suggest that respiratory pathogens become relatively more important in the microenvironment of the respiratory system during episodes of exacerbations caused by infection. A significant interaction between the normal flora and potential pathogens has been demonstrated in vitro, as well as in vivo, that *Streptococcus viridans* has the capacity to inhibit the colonisation and replication of *Streptococcus pneumoniae* [18] and *Staphylococcus aureus* and *Streptococcus β-hemolyticus* [19]. This concept of "bacterial interference" has led to the therapeutic use of *Streptococcus viridans* in the treatment of families with recurrent infection processes [19].

Great attention has been recently paid on "immune-stimulating" or "immune-modulating" agents, which influence specifically and non-specifically humoral and cellular immunity. In clinical studies has been documented that e.g. theophylline – the main bronchodilator in asthmatic patients therapy – may inhibit T cell activity in vitro but prolonged administration in moderate doses increases a number of T suppressor cells (CD8+) in peripheral blood [20]and decreases the number of activated eosinophils in the bronchial epithelial basement membrane [21]. Also the natural immunostimulators like lymphokines (IFN-γ, IL-2), thymic hormones, biologically active peptides, extracts of bacterial antigens and autovaccines have been examine as non-specific activators of phagocytosis and inductors of the specific antibody production [14, 22].

In our country in the last decade the preparates used containing extracts of bacterial antigens has been not too numerous, including Broncho-Vaxom (OM Laboratory, Ewopharma), IRS-19 (Solvay Pharma), Ribomunyl (Pierre Fabre Medicament) and Polyvaccinum (Biomed) and others. The clinical effectiveness of them has been evaluated in many studies [23–28]. The results of these observations indicate that an enhancement of bacterial killing and oxidative metabolism of neutrophils may be connected with stimulation by bacterial antigens, similarly like increase of INF-γ production [1, 14]. Some observations of the skin reactivity using Multitest CMI (BioMerieux) in some clinical centres [6, 23, 24] suggest good stimulating effect and correlation with clinical score. In another study, human phagocytes after stimulation with bacterial antigens expressed adhesion molecules MAC-1 ICAM-1 LFA-1 and produced TNF-α and IL-2 [22]. But in patients treated with extracts of bacterial antigens increased superoxide production in cells obtained from bronchoalveolar lavage fluid [22, 25] and in experimental studies [29].

The induction of memory response in MALT has been the subject of debate for a long time, since a large number of studies have demonstrated that antigen-specific IgA responses are short-lived and reimmunization may not always induce higher levels of IgA responses faster. It is well known that antigen induces the clonal expansion of antigen-specific B cells, resulting in the formation of germinal centres in lymphoid tissues. The

memory B cells are also thought to arise in these germinal centres but Th cells and cytokines are required for this reaction [30, 31]. The role of persistent antigen is very important for memory B-cell responses, and the results of experimental observations suggest that antigen presence may be essential for long-term maintenance of memory B and T cells [32, 34]. It is well established that dendritic cells in B-cell areas can retain native antigen, usually via antigen-antibody complexes [35, 36]. The slow release of these complexes may be important for restimulation and expansion of memory B-cell lines [32, 35, 37]. Recent studies have shown that oral [38, 39], nasal [38], bronchial [32, 27] or sublingual [40] specific immunotherapies are effective when the appropriate dose of standardised or modified extract is used.

In my opinion two mechanisms – local immunity and the normal indigenous flora – may act synergistically in maintaining a balanced microecology of the bacterial flora in the respiratory tract. I hope that in the future these techniques will enhance the local production of secretory IgA against specific bacteria and viruses. The present utilisation of antibiotics must be carefully considered as they may play an adverse role in this microecological balance of the respiratory tract. The advantages of combining immunomodulation and antibiotics are: higher efficacy, reducing side effects, and improving patient compliance [26]. After 50 years of antibiotic use, it appears that other strategies will be necessary in the present decade and in the 21st century to eradicate respiratory tract infections.

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SINUSITIS AND ASTHMA

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Association of bronchial asthma with disorders of the upper respiratory tract, including rhinitis and sinusitis has been known for many years. However, the nature of relationship between sinusitis and asthma is still debatable. Chronic sinusitis, if defined as an inflammation of the sinal mucosa is rarely a separate entity; usually it is accompanied by chronic rhinitis, and often coexists with nasal polyposis forming a group of inflammatory disorders of the upper respiratory tract which are related to each other. Thus, it seems that "rhinosinusitis" is a more appropriate term to describe inflammatory disorders of the upper airway when the comprehensive relationships between different portions of the same respiratory tract are discussed. However, for purpose of this review, with restricted space available, I will focus on discussing relation of bronchial asthma and sinusitis.

Sinusitis and asthma – coexistence or causal relationship?

Sinusitis and asthma often go together. Although it is estimated that 15–26% of normal asymptomatic individuals have abnormal X-rays, paranasal sinuses are affected in 31–75% of children and adults with bronchial asthma [1–3]. Recently Rossi et al. [4] evaluated the occurrence of abnormalities in paranasal sinus radiographs in 110 consecutive patients with severe attacks of asthma. An abnormal finding was detected on 87% of radiographs suggesting that sinal pathology is very common in patients with acute asthma. It is widely accepted that the presence of sinusitis aggravates asthma course, although well controlled studies to support such opinion are lacking. As discussed below some studies showed that experimental nasal provocations with specific or non-specific stimuli result in lower airway obstruction, however the relevance of these findings to clinical situation and to sinusitis is not clear [5]. Conversely, nasal breathing may alleviate exercise-induce asthma indicating that maintaining of nasal patency may be an important factor for patency of lower airways [6]. However, some data cast some doubt on the causal relationship between sinal pathology and asthma. Zimmerman et al. [7] detected

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radiographic abnormalities in 31% of asthmatic children compared to 0% in controls, but could not find any relationship between presence or absence of the sinal disease and severity of asthma. More convincing evidence for sino-bronchial relationship comes from observations that clinical course of asthma is improved following treatment of concurrent sinusitis [1, 8]. Furthermore, patients with asthma may be unresponsive to therapy until coexisting sinus disease is successfully treated. Surgical treatment of sinusitis has been shown to reduce the long term need for asthma medications in patients with sinusitis and asthma [9], and functional endoscopic sinus surgery has been also reported to have favourable effects on associated bronchial asthma [10, 11].

Unfortunately, majority of the above cited studies are retrospective and poorly controlled, thus the evidence for causal relationship between sinusitis and asthma is not clear-cut. Well-designed prospective studies are needed to establish a definite causal relationship between sinusitis and asthma.

Pathophysiology of sinusitis

Although acute sinusitis is an infectious disease, the pathophysiology of chronic sinusitis is more complex. Chronic sinusitis should be regarded as an inflammatory process of the sinal mucosa in which infection is only one of several etiological factors. The most important factor in the pathophysiology of chronic sinusitis is ostial patency, and the obstruction of osteomeatal complex is usually the basis for sinusitis. Other factors predisposing to chronic sinusitis can be divided into local (e.g. URT infections, allergic rhinitis, deviated nasal septum or hypertrophied adenoids), and systemic (e.g. immune deficiency, cystic fibrosis or immotile cilia syndrome). All of these factors seem to affect negatively (directly or indirectly) the physiological drainage of sinuses. As a result of an impaired drainage local infections may develop leading to persistent inflammation of the sinal mucosa.

The histology of the paranasal sinus mucosa has not been well studied, although the data available strongly support inflammatory character of the mucosa in chronic sinusitis. Demoly et al. [12] studied .sinus mucosa and sinus fluid with respect to the presence of, inflammatory cells and mediators, and found the infiltration of the sinus mucosa by activated eosinophils irrespective of the patients' allergic status. A role for other inflammatory cells like macrophages, mast cells and lymphocytes was also suggested by this study. Goldwyn et al. [13] performed retrospective analysis of histologic specimens obtained during endoscopic sinus surgery in patients with chronic sinusitis, as well as in control patients who had undergone trans-sinus procedures for non-sinus pathology. In paranasal mucosa from all patients with sinusitis they found mild to severe inflammatory cellular infiltrates as opposed to no inflammatory infiltrates in control subjects. Although the prevailing inflammatory cells were lymphocytes, eosinophils comprised a significant portion of influx cells.

Surprisingly, in contrast to common views, neutrophils are rarely found in the sinus mucosa, although it may be a prevailing population of cells in sinus lavage fluids [14–16]. Moreover, the number of neutrophils in the lavage fluid correlates with a

neutrophil chemoattractant chemokine (IL-8) and neutrophil derived mediator (MPO) suggesting that these cells may play a role in the pathomechanism of inflammation in chronic sinusitis [16]. Since allergy only in some cases is associated with chronic sinusitis it has been suggested that histopathology of sinusitis may differ in patients with and without presence of atopy. In the study of Demoly et al. [12] mast cells were present in the submucosa of patients with and without allergy, however, allergic patients differed in the redistribution of mast cells towards the epithelial lumen. We have recently obtained similar data by studying histopathology of nasal polyps by computer morphometric analysis [17]. Although in nasal polyps from both atopic and non-atopic patients with chronic rhinosinusitis an extensive infiltration with eosinophils and mast cells was found, the density of mast cells in the superficial layer of the mucosa was significantly higher in atopic patients. It is consistent with superficial distribution of mast cells in the nasal mucosa of patients with seasonal rhinitis further stressing similarity of allergic inflammatory responses in different parts of the airway mucosa [18]. Another hallmark of allergic sinusitis may be the presence of lymphocytes since 8 times more T cells was found in allergic than in nonallergic patients [12]. Consistent with a putative role of activated lymphocytes are observations of Hamilos et al. [19] who studied cytokine expression in chronic hyperplastic rhinosinusitis. They demonstrated that although polyps and sinus mucosa from both atopic and nonatopic patients expressed significantly higher tissue densities of GMC-SF and IL-3, these groups could be differentiated based on the density of other cytokines: most distinguishing cytokines in atopic patients were IL-4 and IL-5, and in non-allergic patients IFN-gamma. These data suggest that etiological heterogeneity of sinusitis may be reflected in distinct mechanisms of eosinophilia operating in different subgroups of patients with rhinosinusitis, but resulting in similar histopathology of the mucosa.

Pathophysiological relationship between sinusitis and asthma

Potential mechanisms of the association between asthma and sinusitis have not been elucidated. Asthma and sinusitis may coexist as different tissue responses to the same environmental factor (e.g. pollution, allergens) or they may have common pathophysiology. Several mechanism have been proposed by which sinusitis may induce or exacerbate bronchial asthma.

a. *Anatomical proximity of sinuses and bronchi.* It has been suggested that due to anatomical proximity post nasal drip of mucus, mediators or chemotactic factors into the lower airways may induce bronchial inflammation and an increased bronchial hyperreactivity. Bacterial and viral infections can be also transmitted from infected sinuses to the lower airways, contributing to the inflammation and exacerbating asthma. However, the experimental evidence for these mechanisms is lacking.

b. *Sino-naso-bronchial reflexes.* The most popular theory links the relationship between sinusitis and asthma to nasal or sinus reflexes triggering bronchial constriction in affected patients [20]. According to this theory stimulation of neural receptors within the upper airways would activate afferent trigeminal portion and vagal efferent portion of the

neural arc resulting in bronchial obstruction. Such explanation is supported by human studies demonstrating that intranasal challenge with allergen or histamine induce decrease in pulmonary function or increase in the lower airway resistance [21]. However, other studies were unable to detect changes in pulmonary function following positive nasal challenge with allergen [22, 23]. The weak side of sino-bronchial reflex theory is that the classical neural reflex may be responsible for bronchial smooth muscle constriction, typical of acute asthma, but not for chronic airway inflammation which is an underlying cause of persistent asthma.

c. *Asthma, sinusitis and neurogenic inflammation.* The concept of airway reflexes can be redefined if the current knowledge on the role of the non-adrenergic, non-cholinergic nerve fibers and neurogenic inflammation in the airway pathophysiology is considered. Microvascular permeability resulting in accumulation of plasma proteins in the airway submucosa and leading to mucosal oedema is an important component of asthmatic inflammation. It has been suggested that a portion of the airway inflammatory responses is neurogenic i.e. requires presence of the intact sensory innervation of the mucosa [24]. These nerve-dependent airway responses referred to as a neurogenic inflammation are considered to form an essential mechanism initiating a reparatory process in the airways. The scientific evidence for the involvement of the neurogenic inflammation in the development of inflammatory responses in the airways comes mostly from animal studies. Hungarian scientist Szolcsányi [24] was the first to demonstrate in 1976 that electrical stimulation of the vagus induces vascular permeability and edema formation in the mucosa of large airways in rodents. Subsequently, several studies investigated different aspects of neurogenic inflammation in the rodent airways supporting evidence that the reaction results from activation of non-adrenergic, non-cholinergic capsaicin sensitive, sensory fibres [25–27]. It has been documented that inflammatory reactions in the airway mucosa induced by several stimuli potentially relevant to asthma resembles neurogenic responses [28, 29]. Our studies on morphology of neurogenic inflammation in the rat airways confirmed that neurogenically induced vascular permeability occurs within postcapilary venules in the subepithelial layer of the tracheal and bronchial mucosa [30]. Dramatic changes in the airway epithelium involving degranulation of epithelial secretory cells, widening of the spaces between epithelial cells and the movement of plasma proteins through the airway mucosa into the airway lumen could be observed simultaneously. Thus, neurogenic inflammation in the rat airways, similarly to the allergic reaction, involves both vascular permeability and plasma protein exudation into the airway lumen [31].

Now it seems clear that this reaction is a result of activation of sensory nerves containing peptide neuromediators. These peptides are released from nerves endings upon antidromic stimulation of vagal nerves and are responsible for vasodilatation and vascular permeability. In fact, in addition to classical neurotransmitters, such neuropeptides as substance P, NKA, NKB, SOM, NT, CGRP, can be found near to epithelial surfaces both in the upper and lower airways and localize mainly to primarily sensory nerves [32]. On the other hand neuropeptides like VIP, galanine, somatostatin are also present in cholinergic efferent fibers. Several observations support involvement of neuropeptides in neurogenic inflammation in the rodent airways. Sensory neuropeptides are released from nerve endings by noxious stimulation. Substance P induces vasodilation and vascular

permeability in the airways when applied topically to the airways leading to formation of edema, but also activates leukocytes and contracts smooth muscle. Similar proinflammatory activities are shared to different degree by other neuropeptides. SP antagonists inhibit neurogenically induced edema in the airways, and finally, neurogenic responses in the airways can be prevented by local pretreatment with high doses of capsaicin, the hot, spicy essence of red peppers, and specific neurotoxin depleting neuropeptides.

When sensory neuropeptides are released from peripheral nerves, it is not clear whether they act directly on postcapillary venules to induce vasodilation and vascular permeability, or they act indirectly by inducing the release of mast cell vasoactive factors. A role for mast cells in neurogenic inflammation has been assumed for many years, but the exact nature of the interaction has not been defined. Our studies demonstrated that mast cells were not essential for neurogenic vascular permeability in the airways since we were able to evoke acute vascular responses by injecting substance P to mast cell deficient WWv mice, and did not find mast cell activation during pulmonary neurogenic inflammation [33, 34]. It seems that sensory neurotransmitters can act directly on postcapillary venules to cause vascular permeability and plasma extravasation and that this effect does not depend on histamine release from mast cells. Based on animal studies it has been proposed that also in human airways irritation of the mucosa as well as allergic reactions initiate local reflexes resulting in vasodilatation and increased vascular permeability, i.e. neurogenic inflammation, which may contribute to the asthmatic inflammation [35]. However, the evidence for the existence of neurogenic inflammation in human airway pathology is still circumstantial. Recently, M. A. Kaliner [36] proposed that neurogenic inflammatory responses in the lower airways may occur as a result of neurogenic reflexes originating in the upper airway mucosa, linking these two commonly occurring disorders. A sino-naso-bronchial reflex evoked by irritation of the afferent nerve endings in the sinus mucosa (e.g. by inflammatory mediators released due to either chronic infection or allergic reactions) could lead to activation not only of classic mediators (acetylcholine) but also peptides containing nerve fibers within vagus, leading to release of neuropeptides in the lower airways mucosa. As a result such reflex would lead to initiation and/or enhancement of secretory and inflammatory process in the airway mucosa and exacerbation of bronchial asthma.

d. *Continuity of the upper and lower airway mucosa as a basis for common inflammatory responses.* Both asthma and sinusitis are considered to be an inflammation of the airway mucosa. Since chronic sinusitis is usually associated with inflammation of the nasal mucosa (rhinosinusitis) it seems clear that in majority of cases we are dealing with chronic inflammation of both upper and lower airways mucosa. Thus, asthma and sinusitis may be regarded as two clinical manifestations of the inflammatory processes affecting both the upper and lower airway mucosa. Recent studies revealing striking similarities between histopathology of sinuses in chronic sinusitis and bronchial mucosa in bronchial asthma seem to support such assumption. Similarly to bronchial and nasal mucosa in asthma and rhinitis, respectively, eosinophils are increased in the sinal mucosa in patients with chronic sinusitis [12]. Newman et al. found strong relationship between allergy, peripheral blood eosinophilia and sinusitis in asthmatic patients [37]. The extensive parasinal disease (assessed by computed tomography) correlated with peripheral

blood eosinophilia, and to a lesser extent with the presence of IgE specific to common inhalant allergens. Moreover, the correlation with eosinophilia was noted in these patients, being evident even among patients without history of wheezing or allergy. They suggested that the inflammation in the sinus mucosa could be a source of an immunological factor (namely interleukin 5) giving rise to eosinophilia. Thus, allergy or infection in the sinuses could lead to release of chemotactic cytokines which could recruit inflammatory cells (e.g. eosinophils and mast cell precursors) into circulation. It is tempting to speculate that in the presence of ongoing inflammation in the lower airways, presence of activated inflammatory cells in the circulation could facilitate their recruitment into the bronchial mucosa, leading to exacerbation of asthmatic inflammation. Along these lines Harlin et al. [14] found a significant association of sinus mucosal eosinophilia and tissue Major Basic Protein with the presence of bronchial asthma in patients with chronic sinusitis. These data suggest that apparent association of asthma and sinusitis may reflect mucosal inflammation of the whole respiratory tract reacting as one organ to either specific or non-specific environmental stimuli. It is also possible that both diseases are linked to common genetic predisposition.

Asa-sensitive rhinosinusitis/asthma syndrome

A perfect example of association between disorders of the upper and lower respiratory tract is "the aspirin triad". A subpopulation of asthmatic patients reacts to ingestion of aspirin or other nonsteroidal anti-inflammatory drugs with severe asthmatic attack [38, 39]. Majority of ASA-sensitive asthmatics if challenged with aspirin, in addition to bronchospasm, demonstrate nasal responses heralded by rhinorrhea, sneezing and nasal obstruction [40, 41]. The nasal responses to ASA challenge, however, are superimposed on existing pathology of the upper respiratory tract including chronic rhinitis, sinusitis and nasal polyposis. The incidence of sinusitis by roentgenogram in ASA-sensitive asthmatics is up to 95%, and the frequency of nasal polyps may be as high as 70–90%, as compared to about 5% in general asthmatic population [41, 42]. A subgroup of ASA-sensitive patients manifests a reaction exclusively in the upper respiratory tract; they do not have asthma, but clinical picture of the nasal disease (hypertrophic rhinosinusitis) in these patients is similar to that observed in patients with ASA-triad [43]. A positive history of ASA intolerance, which is the hallmark of this syndrome, is a significant and reliable indicator of chronic intractable rhinosinusitis, persistent asthma of greater than average severity and higher than ordinary medication requirements, including dependence on steroids. Patients with ASA-triad have higher tendency to status asthmaticus and a fatal outcome of asthma occurs more often than in asthmatics without the triad.

Determination of severity of rhinosinusitis in ASA-sensitive patients

It is generally accepted that the clinical course of chronic rhinosinusitis in ASA-sensitive patient is more severe than in ASA-tolerant patients [41, 44]. There is high recurrency of nasal polyps, and frequent need for sinal endoscopic surgery in this group of patients. However, any systematic study comparing the extent and severity of the mucosal inflammation in this group of patients to ASA-tolerant patients has not been published. Using computer tomography (CT) and semiquantitative scoring system we have recently assessed the extent of the hypertrophic inflammation in the upper respiratory tract in ASA-sensitive patients and compared to 13 ASA-tolerant patients with nasal polyposis. CT scans were obtained using 5 mm sections, every 4 mm from the frontal sinus to the sphenoid sinus, and evaluated in the coronal projection [45, 46]. The evaluated area included individual sinuses, osteomeatal complex region, and nasal cavity and based on mucosa thickening scoring points were assigned for each sinus. Based on clinical history ASA-sensitive patients had longer lasting rhino-sinal disease with multiple polypectomies, and more frequently were treated with nasal corticosteroids. CT scan analysis demonstrated signs of mucosal disease including mucosal swelling, hypertrophy and polyp formation in sinuses in both groups of patients. However, the extent of the disease was significantly higher in ASA-sensitive patients, which had significantly higher total score as compared to ASA-tolerant patients. Thus, our study demonstrated for the first time in a quantitative manner that mucosal inflammation is more prominent in sinuses of ASA-sensitive patients.

Pathomechanism of mucosal inflammation in ASA-sensitive rhinosinusitis

It is not clear if the basic pathomechanism of chronic airway inflammation in ASA-sensitive patients is different from **ASA** tolerant patients with nasal polyps, rhinosinusitis and bronchial asthma [47]. Although nasal polyps in ASA-sensitive patients are mostly of eosinophilic type, they do not seem to differ from polyps removed from ASA-tolerant patients [47–49]. However, intranasal challenge with aspirin only in ASA-sensitive patients results in an influx of eosinophils into the nasal lavage fluid, and in an increase in the concentration of ECP, indicating specific activation of eosinophils in ASA sensitive patients [50]. Tissue eosinophilia is accompanied by infiltration with mast cells, and accordingly aspirin challenge results in increased release of mast cell tryptase into the nasal fluid of ASA sensitive patients. Although standard pathologic examinations showed that upper airway mucosa of ASA-sensitive patients cannot be distinguished from tissue of patients who tolerate aspirin, recent study of biopsy specimens of nasal polyps provided new data [19]. Aspirin sensitivity was strongly correlated with nonallergic polyps and with production of the nonallergic profile of cytokines including IFN gamma. It is possible that nasal and sinal inflammation in ASA-sensitive patients, as compared to ASA-tolerant patients, may have distinct pathomechanism, which is reflected in an increased severity of the disease in ASA-sensitive patients.

Diagnosis of sinusitis

Since early diagnosis of chronic sinusitis if often missed in asthmatic patients, it may be worthy for pulmonologists and allergologists to pay more attention to signs and symptoms suggesting such association. Sinusitis is defined as an inflammation of the sinuses, and can be subdivided into acute and chronic phases. From the practical standpoint it may be useful to distinguish recurrent sinusitis defined as two or more episodes of sinusitis per year. The leading symptom of acute sinusitis in adults is facial pain and/or headache. If frontal or maxillary sinuses are affected, pain may be elicited over the affected area, whereas with the ethmoid involvement the pain is localized between or behind the eyes. As a sign of bacterial infection mucopurulent nasal discharge is present accompanied by postnasal drip and fever. Symptoms of chronic sinusitis additionally include persistent nasal obstruction, sore throat, cough, hyposmia and anosmia.

Clinical diagnosis based on history and medical examination is usually confirmed by traditional anterior-posterior radiographic examination (in Waters view) showing diffuse opacification of one or both maxillary sinuses, mucosal thickening and/or fluid levels. Current technology offers new diagnostic modalities for assessment of the paranasal sinus pathology including computer tomography (CT). CT scan is the optimal diagnostic method for demonstration of sinus anatomy and pathology allowing for assessment of the extent of sinal involvement, as well as for measurement of mucosal thickening in all sinuses, including osteomeatal complex.

Management of chronic sinusitis in an asthmatic patient

Treatment of chronic sinusitis should be considered as an important component of asthma management plan in an asthmatic patient and effective management of chronic sinusitis requires close collaboration between allergist and oto-rhino-laryngologist. Current treatment modalities for sinusitis have been recently reviewed [51]. Basic medical treatment includes antibiotics, decongestants and topical steroids. Antibiotics should be administered for a longer period of time (usually 3–6 weeks) than in acute sinusitis. Topical steroids have an important function in reducing mucosal swelling and suppressing the upper airway inflammation but also prevent recurrence of nasal polyps, which often complicate sinusitis. Additionally nasal steroids have favourable effects on the lower airway diseases thus allowing for concomitant improvement of bronchial hyperreactivity [52, 53]. When medical treatment is not effective and the disease progresses, more aggressive, surgical management of paranasal sinus disease is necessary. At present functional endoscopic sinus surgery has become the standard procedure for most surgical cases of chronic sinusitis.

Conclusion

Chronic sinusitis and asthma are frequently associated, and seem to be causally related to each other. Active sinusitis may aggravate concomitant bronchial asthma and conversely successful treatment of sinusitis may lead to improvement of the lower airway disease. Allergist and pulmonologist should pay more attention to possible association of these two disorders

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THE SIGNIFICANCE OF LUNG FUNCTION TESTS IN THE DIFFERENTIAL DIAGNOSIS OF BRONCHIAL ASTHMA

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Measurement of airflow limitation and assessment of its reversibility are essential in estimating the correct diagnosis of asthma. The presence of at least 15% reversibility in FEV_1 or PEF after inhalation of a short-acting β_2-agonist favours the diagnosis, but lack of a short-term reversibility does not exclude asthma.

Advanced emphysema, upper airway stenosis and respiratory muscle weakness can, usually, be differentiated from asthma solely by their lung function characteristics.

Monitoring of parameters (e.g. PEF) reflecting daily variation of airways' calibre, measurement of bronchial responsiveness to exercise, and to certain bronchospasmogenic mediators, non-isosmolar solutions may help in the differential diagnosis of asthma in a symptom-free condition. Cutoff values, sensitivity and specificity for asthma of these tests are discussed.

The diagnostic procedure of asthma should aim at the recognition or detection of the most important characteristics of the disease.

Asthma is now defined as a chronic eosinophilic inflammatory disorder of the airways. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness and cough, particularly at night and early morning. These episodes are associated with airflow obstruction that is often reversible either spontaneously or with appropriate treatment. The inflammation also induces an associated increase in the existing bronchial hyperresponsiveness to a variety of stimuli [1].

The diagnostic procedure of asthma should be directed at the determination of the presence of (i) episodic symptoms of airflow obstruction, (ii) the reversibility of airflow obstruction and (iii) at the exclusion of alternative diagnoses.

Although patients' history, signs and symptoms are the most important determinants of the diagnosis of asthma, the measurement of airflow limitation, the predominant pathophysiological event of asthma, is essential in making the correct

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diagnosis, since a patient's respiratory complaints and symptoms do not necessarily reflect the presence and severity of airflow obstruction. The lack of airflow obstruction at the spot measurement of lung function does not exclude asthma characterized by episodic airflow limitation which also includes the presence of symptom-free conditions, mostly with normal values of lung function. Thus, lung function tests used for the differential diagnosis of asthma in a symptomatic condition are partly different from those used in a symptom-free condition.

Diagnostic value for asthma of lung function tests in patients presenting signs of airflow obstruction

Determination of airflow obstruction by simple spirometry using PEF (peak expiratory flow) or forced expiratory volume in one second and forced vital capacity ratio (FEV1/FVC) values objectivizes airway narrowing during forced expiration, but the presence of airflow obstruction alone does not confirm the diagnosis of asthma, since a number of other respiratory diseases (e.g. chronic bronchitis, emphysema, upper airway stenoses, respiratory muscle weakness, etc.) may also show airflow limitation.

By definition, asthma is characterized by at least partially reversible airflow limitation, thus, reversibility testing gives important information. Improvement of FEV_1 or PEF after inhalation of a potent β_2 agonist provides important data about the reversibility. Cutoff values of improvement of FEV_1 or PEF differentiating asthmatics from non-asthmatics with a high probability have not been estimated so far. The greater the post-bronchodilator improvement, the higher the possibility of the presence of asthma. Significant reversibility is indicated by an increase of >12% and 200 ml in FEV_1, after inhaling a short-acting bronchodilator [2]. The presence of at least 15% reversibility in PEF or FEV_1 after inhalation of a β_2 agonist favours the diagnosis of asthma [1]. It should be noted, however, that some of the patients with COPD may have an increase of 15% or more in FEV_1 after inhalation of a β_2 agonist aerosol [3]. Lack of immediate response to a β_2 agonist does not exclude asthma. A short course (2 or 3 weeks') treatment with glucocorticosteroids resulting in relieving airflow obstruction also favours the diagnosis of asthma superimposed upon chronic bronchitis and emphysema [1].

Detailed cross-sectional examination of lung function of patients with airflow limitation can differentiate advanced emphysema, the most common cause of irreversible expiratory airflow limitation of exobronchial type. Decreased FEV_1/VC in association with normal FIV_1/VC, normal or slightly increased airway resistance (Raw), markedly increased thoracic gas volume (TGV) and decreased transfer factor of the lung are characteristic features of the lung functional alteration of pulmonary emphysema.

Plateau-like flow-volume (V'-V) curves suggest the presence of upper (extra- or endothoracic) airway stenoses which may mimick asthma. Weakness of respiratory muscles may also present plateau-like flow-volume curves, predominantly during forced inspiration [4] but with normal Raw which allows a clearcut differentiation from upper airway stenoses.

Diagnostics of asthma by lung function tests in symptom-free condition

Patients with asthma do not, usually, show flow limitation measured by FEV_1 or PEF if they are in a symptom-free condition. They are suspected of having asthma based on their history. Monitoring of parameters reflecting daily variation of airways' calibre, measurement of bronchial response to exercise, and to certain bronchospasmogenic mediators, non-isosmolar solutions may help in the differential diagnosis of asthma in a symptom-free condition.

Monitoring of circadian variation of PEF. Assessment of the diurnal variation of PEF is recommended when patients with a suspicion of asthma are symptom-free or have asthma symptoms but normal spirometry [5].

Diurnal variation of PEF exceeding upper limit values of diurnal changes of PEF of healthy subjects [5, 6] are suggested to be of diagnostic value for asthma. This threshold value, however, cannot be applied for differentiation of asthmatics from patients with chronic bronchitis, because the latter group has higher diurnal variability of PEF than the healthy subjects [7].

Sensitivity of measuring diurnal variation of PEF is dependent on the frequency of daily measurements of this parameter and on the number of days of monitoring [7]. Measuring PEF on awakening and in the evening may be more practical and feasible than more frequent daily measurements, but PEF variability calculated from morning-evening PEF values underestimates the actual diurnal variation [1], and this results in a decreased sensitivity.

It must be stressed that patients need instructions and demonstrations because the measurement of PEF is dependent on effort and technique. Diurnal variation of more than one-third of the patients with asthma does not exceed diagnostic cutoff value during a ten-day monitoring [7].

In patients whose PEF diurnal variability is below or just above the cutoff value, at least one provocation test with exercise or other stimulus is required.

Exercise challenge test. It is well known that exercise may induce bronchospasm in patients with asthma. The test sensitivity in adult symptom-free asthmatics is low at cutoff values of 15 or 20% postchallenge decrease in FEV_1 at room temperature [8], but it can be increased while breathing cold and dry air [9]. Asthmatic children are more susceptible to developing bronchospasm after exercise. The sensitivity of exercise test in this group is over 70% [10]. Negative test result does not exclude the presence of asthma.

Bronchial hyperresponsiveness (BHR). BHR to a variety of stimuli is one of the characteristic features of asthma. Hyperresponsiveness means a greater bronchial response than that shown by healthy subjects to inhaled bronchoconstricting stimuli. Biometrically, a positive bronchial response (20% decrease in FEV_1) achieved at equal or less than mean -1.645 SD of PD_{20} or PC_{20} FEV_1 values of the group of healthy subjects is considered to be hyperresponsive (Fig. 1). This implies that 5% of healthy subjects is declared as hyperresponsive. If mean – 2SD is used as threshold value then roughly 2% of healthy subjects will be hyperresponsive to a particular stimulus!

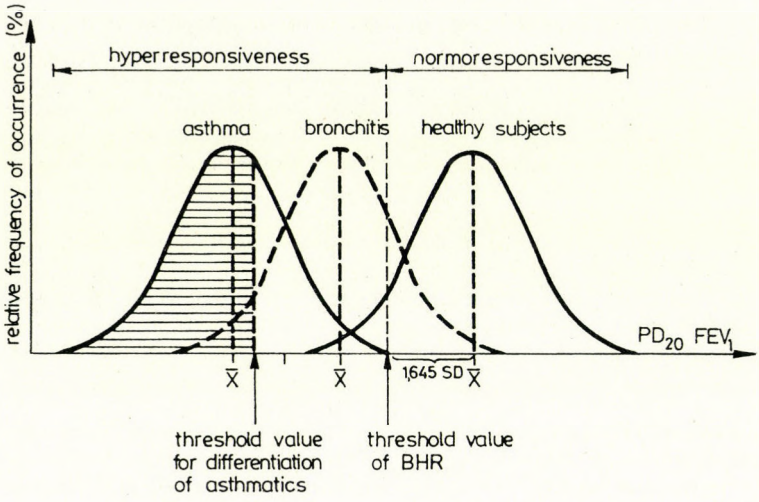

Fig. 1. Schematic representation of the estimation of normo- and hyperresponsiveness as well as threshold value for differentiation of asthmatics by using idealized distribution curves of doses required to achieve a 20% decrease in FEV_1 (PD_{20} FEV_1) in healthy subjects and in patients with chronic bronchitis and asthma

Negative test result suggests that the likelihood of the presence of asthma is fairly low. Positive test result, i.e. the presence of BHR alone, does not confirm the diagnosis of asthma because a large proportion of patients with respiratory disease other than asthma (e.g. patients with chronic bronchitis) may also show BHR [12]. Therefore, the presence of BHR is of limited differential diagnostic value for asthma [11]. There is a wide overlapping in PD_{20} FEV_1 of asthmatics and patients with chronic bronchitis [12]. Only patients having PD_{20} FEV_1 values exceeding by more than 95% of non-asthmatics can be considered as asthmatics with a great probability. PD_{20} FEV_1 values being higher than the threshold value for asthma, but less than that for BHR (Fig. 1), do not play a significant role in the differential diagnosis of asthma.

Bronchial provocation test with non-isosmolar solutions. Bronchial provocation tests (BPTs) performed by inhalation of hypo- and hyperosmolar solutions seem to be more specific for asthma than the assessment of BHR [13] to metacholine or histamine. Inhalation of ultrasonically nebulized' distilled water [14], hypertonic saline [15] and potassium chloride solution [16] provoke bronchospasm in the vast majority of patients with asthma.

The sensitivity for asthma in adults of BPTs performed with distilled water and hypertonic (10%) KCl solution is 80 and over 95%, respectively [15]. Hypertonic (10%) saline BPT proved to have roughly 90% sensitivity (unpublished data of our laboratory). Thus, in contrast to KCl test – which has not only an osmotic but also a specific K^+ ionic effect [14] – the negativity of distilled water and hypertonic NaCl tests do not exclude the presence of asthma.

About 12% of the patients with chronic bronchitis exhibit more than 20% decrease in FEV_1 after KCl BPT. The specificity of the test exceeds 95% by using a threshold value of the test positivity of 25% decrease in FEV_1. In this case, KCl challenge tests with a positive result confirm the diagnosis of asthma [17].

Challenge test with adenosine. BPT performed by inhalation of adenosine is also a useful tool for differentiating asthmatics from healthy subjects because adenosine does provoke bronchospasm in asthmatics as opposed to healthy subjects [18].

BPT with specific antigens. Due to their possibly hazardous effect, specific BPTs are not recommended to be used in the diagnostics of asthma with the exception of occupational asthma. Careful inhalational exposure to a suspected causative factor of asthma can be performed if serial peak flow records at work and away from work [19] confirm the work association.

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THE VALUE OF IN VITRO TESTS IN THE DIAGNOSIS OF BRONCHIAL ASTHMA

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There is a strong association between allergic rhinitis and asthma. A significant linkage between these diseases has recently been recognized. More than 58% of adult asthmatics have been reported to have allergic rhinitis [1]. Recent epidemiologic data suggest that both allergic disease and asthma are increasing in parallel. A study of Swedish conscripts into the military from 1971–1981 indicated that among teenage males the prevalence of allergic rhinitis increased from 4.4 to 8.4% in parallel with an increase in the prevalence in asthma from 1.9% to 2.8% [2]. Among patients with asthma, up to 78% may demonstrate nasal symptoms compared with 20% of the normal population [3].

Nineteen to 38% of patients with allergic rhinitis have concomitant asthma, whereas only 5% of the general population has asthma [1]. There is a two- to four-fold greater prevalence of allergic rhinitis or asthma among individuals who are diagnosed with either one of these diseases [4]. The relationship between allergic rhinitis and asthma is further substantiated by the temporal nature of the onset of both illnesses typically occurring concurrently, especially among children and adolescents.

Often, allergic rhinitis precedes asthma, and in fact, may be a harbinger of the development of asthma. A study of college students revealed that those students having symptoms of allergic rhinitis were three times more likely to develop asthma than students who did not have symptoms of rhinitis [5]. Furthermore, patients with allergic rhinitis who do not have clinical asthma may develop non-specific bronchial hyperreactivity during the allergy season. Up to one-third of patients with allergic rhinitis without clinical asthma will demonstrate positive bronchoconstrictive responses to methacholine, similar to responses generated in asthmatic patients [6].

Responsivity of the airways often increases among patients with allergic rhinitis who do not demonstrate clinical asthma when such rhinitis patients are exposed to pollen. A study of 27 patients with seasonal allergic rhinitis before, during and after the pollen season revealed that the incidence of bronchial hyperresponsivity increased from 11%

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prior to the pollen season to 48% during the pollen season [7]. It appears that many patients with allergic rhinitis have subclinical asthma as documented by bronchial reactivity to methacholine with responses such as a methacholine PC_{20} less than 8 mg/mL frequently found in allergic rhinitis subjects [8].

A number of studies have concluded that improving allergic rhinitis symptoms may significantly improve symptoms of allergic asthma [9]. To identify those persons at risk for the development of allergen-driven asthma, it is essential to make the definitive diagnosis of the atopic state. Studies utilizing skin-prick testing have demonstrated a frequent association between allergic rhinitis and asthma. Patients with allergic rhinitis having positive skin tests have been noted to be at risk for the subsequent development of asthma [10].

Essential to the treatment of allergic asthma is the recognition of the allergen-triggering factors for this disease. Such factors can be identified either by skin-prick testing or via in vitro tests for allergen-specific IgE. Such testing can allow for appropriate precautions to avoid allergens that may exacerbate both allergic rhinitis and asthma [11].

In vitro diagnostic tests can be utilized to assist in the diagnosis of allergic asthma. The results of such tests must be carefully interpreted in light of the clinical history. A laboratory test result does not in itself indicate that clinically significant atopy is present. All test results must be clinically interpreted and carefully correlated with the patient's history. In general, in vitro diagnostic tests to diagnose allergic disease are less sensitive than in vivo tests such as allergen skin testing. The sensitivity of in vitro diagnostic tests, however, has been rapidly improving with advances that have been made in the methodologic techniques employed. When contrasted to bronchoprovocation tests or allergen skin test, in general, in vitro tests maintain good specificity but tend to have less sensitivity than in vivo tests [12].

Appropriate selection of tests to be performed for the diagnosis of allergic asthma requires that a clinical history be obtained from the patient. Only those allergens to which one suspects a clinically-relevant allergic response should be evaluated. Screening of patients for reactivity towards a large number of potential allergens should not be undertaken as the likelihood exists that some of the test results obtained will be clinically irrelevant [13].

A variety of methodologies are currently available for conducting allergen-specific IgE testing. The traditional RAST test was based upon having an allergen coupled to a solid phase which was subsequently reacted with serum from the patient. If the serum contained IgE specifically directed to the allergen, the specific IgE would bind to the allergen with non-specific IgE washed away. The bound IgE would then be detected using an anti-IgE antibody which had been coupled to a radiolabeled tracer. Subsequent advances included replacing the radiolabeled tracer with non-radioactive moieties such as enzymes producing a fluorescence upon reaction with an appropriate substrate, or enzymes producing an ultraviolet light product upon reaction with an appropriate substrate. Such assays have been termed enzyme-linked immunosorbent assays. To further enhance the sensitivity of the tracer beyond that which can be achieved using an ultraviolet or fluorescent product, chemiluminescent assays have been developed. These assays employ anti-IgE which is coupled to a substance capable of generating a chemiluminescent reaction when appropriately reacted. Such assays have the potential for

enhanced sensitivity, with the chemiluminescent product approximating the potential of a radioisotope with respect to signal amplification, thus enhancing the assay sensitivity.

Comparative performance data contrasting different immunodiagnostic methodologies for the detection of allergen-specific IgE have been difficult to obtain. A study comparing three immunoassays for allergen-specific IgE versus skin testing for the diagnosis of an inhalant allergen documented the finding that results obtained using different methodologies for detection of allergen-specific IgE may not be strictly comparable. Although the results obtained using one method for allergen-specific IgE when done in duplicate or triplicate may show good reproducibility, less than identical results may occur when the same aliquot of serum is assayed using different methodologies for detection of allergen-specific IgE [15]. These differences are related to significant variations between manufacturers in the way allergen is chosen and coupled resulting in differences in the way allergen is expressed within the immunoassay. All allergens which are employed in in vitro tests for allergen-specific IgE must first be carefully processed. Selected protein constituents from the relevant allergen are coupled to either a solid phase matrix or coupled to a particle to be reacted within a liquid phase.

The coupling procedure itself may change the epitopes expressed on the allergen. Selection of the allergen by the manufacturer may also determine the relative reactivity of the allergen with specific IgE. A myriad of factors may influence the results of the allergen-specific IgE test when different methodologies and systems are employed for the detection of allergen-specific IgE. These differences in both methods and allergen source materials make cross-system comparisons problematic. Overall assay performance, including sensitivity and specificity when compared to allergen .skin testing, clinical history, and whole blood leukocyte histamine release was most consistent utilizing the Pharmacia CAP system, especially when assaying for a wide range of allergen-specific IgE analytes, including trees, grasses, weeds, dust mites, and animal danders [15].

Advances in the diagnosis of an inhalant allergy using allergen-specific IgE have included the utilization of liquid phase assays such as the paramagnetic particle liquid allergen chemiluminescent enzyme immunoassays developed by both Ciba-Corning (©Magic-Lite) and also by Sanofi-Diagnostics Pasteur (©ACCESS). Such assays have the potential for a significantly enhanced sensitivity as the serum is reacted with the allergen within the liquid phase, decreasing the confounding problem of allergen conformational/stoichiometric limitations noted when two-dimensional paper discs are used as the allergen binding surface. The allergen is coupled to paramagnetic particles, then separated from the serum using a magnetic field. The particles containing the allergen and allergen-bound specific IgE are then reacted in the liquid phase with anti-IgE containing a chemiluminescent-producing reactant. These assays, however, are still dependent upon utilizing appropriate allergen within the assay. In this regard, for instance, the ©Magic Lite SQ utilizing SQ standardized allergen in the High Sensitivity (HS) format has been found to have enhanced diagnostic performance in contrast to some earlier methodologies [16]. Excellent performances have also been noted utilizing the ACCESS technology, especially in individuals having a high prior probability of the presence of allergic disease [17].

Some attempts have been made to improve contemporary methodologies utilizing enhanced range scoring systems. Such attempts either lower the threshold for detection of

allergen-specific IgE through methodologic improvements or simply decrease the threshold while still using standard methodology. In the latter instances, however, specificity may be lost as sensitivity is enhanced. This is similar to the situation which occurs utilizing the modified RAST test, an assay especially popular in the United States among otolaryngologists. Such assays extend the assay range while sacrificing specificity. In certain instances these tests, however, have been found useful, especially when sensitivity is of greater importance than specificity [18].

Some newer technologies have recently developed employing older methodologies with more sophisticated computerization or mechanistics. Technologies, such as the Hycor HY-TEC system, may be especially appropriate in instances where cost-effectiveness has been mandated by medical-economic constraints [19].

Whole blood basophil histamine release testing has been demonstrated to correlate very well with allergen challenges in patients with inhalant allergy [20]. Studies contrasting the results of in vitro tests for allergen-specific IgE with the results of the whole blood leukocyte histamine release tests utilizing the glass fiber-based assay developed by Skov and Nolte have clearly demonstrated that the whole blood histamine release test correlates better with the results of allergen skin testing than do results of RAST tests [15]. This assay employs a micro-titer well format with glass fibers that adhere to the bottom of the micro-titer wells, the fibers binding histamine released by the incubation of basophils with relevant allergen or anti-IgE in their whole blood milieu.

The histamine that is bound to the glass fibers remains ionically attached. Subsequently, the histamine is condensed to *o*-phthalaldehyde with an enzyme-reaction-derived product being detected by a spectrophotometer. The assay has a sensitivity of 5 ng/mL of histamine and an assay linearity from 5 to 50 ng/mL of histamine. The assay is useful in that many samples can be rapidly analyzed utilizing allergen that has not been adversely affected by coupling to a solid phase or to particles as is the case when allergen is coupled for employment in a RAST test [21]. The ability to use a wide range of allergens that do not have to be processed, as is the case when allergens are employed in the RAST test, allows for a closer approximation to in vivo allergic events utilizing this. unique technology.

Recently, the association between asthma and allergic disease has been appreciated among those patients suffering from latex allergy. The sensitivity of the whole blood leukocyte histamine release test was found to be greater than that of either the Pharmacia CAP or the Hycor HY-TEC system in the diagnosis of latex allergy among hospital employees at Brigham and Women's Hospital in Boston [22, 23]. Among hospital employees having latex allergy, inhalant allergy, including rhinitis and/or asthma, has been noted to be present in greater than 50% of latex sensitive persons. Asthmatic responses may be generated consequent to airborne exposure to latex allergen.

It has been noted that a number of persons developing such allergic asthmatic response to latex allergen will also concurrently become sensitized to a number of foods, among which are bananas and avocados [24]. Although these employees may have tolerated such foods prior to development of latex sensitization, in vitro tests allow us to advise the latex-sensitive hospital worker of the potential risk of ingestion of foods which cross-react to latex. In several instances, it has been noted that employees who subsequently ate these foods developed clinically significant anaphylactic responses to the

food. In one such case, a dental clinic worker who had previously tolerated avocados ate an avocado-containing food after development of latex allergy and anaphylaxis developed. In another case, a nurse who had tolerated bananas very well in the past ate a banana and subsequently exercised, developing exercise-induced anaphylaxis after becoming cross sensitized to latex. In such instances, the in vitro diagnostic tests identifying the cross sensitization with the foods was predictive of the subsequent development of the anaphylactic reaction.

In general, RAST tests for latex sensitivity have identified from 5 to 10% of hospital employees as possibly being sensitized to latex. Among highly selective employees demonstrating reactivity to latex, the prevalence of positive RAST test to latex may exceed 20%, whereas among hospital employees exposed to latex, greater than 40% may demonstrate positive reaction to latex using the whole blood histamine release test. In general, more persons exposed to latex in a hospital setting are identified as being latex allergic when utilizing the histamine methodology than when using RAST testing for latex-specific IgE. Most of these persons have been exposed to latex via the airborne route, suggesting that basophil histamine release testing may be a more sensitive method of identifying airborne sensitized individuals who manifest allergic asthma.

The laboratory diagnosis of allergic asthma may be significantly assisted through utilization of in vitro diagnostic tests. The whole blood leukocyte histamine release test appears to have some diagnostic advantages over in vitro tests for allergen-specific IgE. All test results obtained in the laboratory for the diagnosis of inhalant allergy in the asthmatic individual, however, must be carefully interpreted. Clinically relevant test results are critically dependent on the presence of a significant clinical history of reaction to a given allergen. Test results should never be interpreted in the absence of an adequate clinical history. Just as is the case with methacholine challenges wherein the clinical history must be included in the interpretation of test results to determine a true positive from a false positive, so too must the clinical history be carefully weighed when interpreting the test results in the in vitro diagnostic assessment of an inhalant allergy in asthmatic individuals.

The best assay performance for in vitro tests for allergen-specific IgE in patients with inhalant allergy occurs in those individuals with the greatest prior probability of having inhalant allergy. Persons with a very strong clinical history of reaction to a given allergen will most likely demonstrate positivity for allergen-specific IgE. Patients having a more equivocal history of inhalant allergy usually have more equivocal results in such assays. The assay performance of tests for allergen-specific is therefore critically dependent on the prior probability of having inhalant allergy. This prior probability can only be established by first obtaining a clinical history. Therefore, it is essential to interpret the results for allergen-specific IgE and whole blood leukocyte histamine release in the context of the patient's clinical history of inhalant allergy-derived symptoms. It is impossible to separate the clinical diagnosis from the laboratory diagnosis of inhalant allergy in the asthmatic patient. All test result interpretations should flow naturally from the clinical history.

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OCCUPATIONAL ASTHMA

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Occupational asthma is the most common work related respiratory disorder in developed countries, and has a rising tendency. It is said that about 2% of the 10 million Americans who have asthma acquired it as a result of some chemical irritant or immunogen in their working environment, and probably accounts for about 2–6% of adult asthma in the [1]. The frequency of the disease in less developed countries is unknown but is potentially very large. In Hungary the number of registered cases of occupational asthma is far from the real value and highly underestimated, because in Hungary occupational asthma is not a compensated occupational disease.

Aetiological agents. At present, more than 200 agents have been implicated in causing occupational asthma in the workplace [2]. New initiators of occupational asthma are continuously being reported. Still, diisoisocyanates and flours remain major causes. Sometimes two different occupational sensitizing agents can be present at the same patient with occupational asthma.

Pathogenesis. The aetiological agents can be divided into two categories by their mechanism of action: immunological and non-immunological.

Immunological causes can be further divided into those that induce asthma through an IgE-dependent mechanism, by producing specific IgE antibodies to the compound or its protein-conjugate, and those that induce asthma through a non-IgE-dependent mechanism. In the latter category, specific IgE antibodies are found only in a small percentage of the patients with proven disease, even though the clinical picture is compatible with an allergic reaction.

More than one mechanism may be operative in occupational asthma. Among the mechanisms proposed, immunological mechanisms and airway inflammation play an important role.

There is evidence to confirm that T-lymphocyte activation and local accumulation in the bronchial wall of activated eosinophils occurs also in occupational asthma [3–5]. Some compounds, when inhaled in high concentrations, act as irritants and produce bronchoconstriction probably by inducing acute airway inflammation. Respiratory irritants can lead to asthma and rhinitis through the interaction with chemical irritant

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receptors in the airway, leading to release of substance P from sensory nerves and neurogenic inflammation. The best-known example of nonimmunological asthma is Reactive Airways Dysfunction Syndrome (RADS) or irritant-induced asthma [6].

The two types of occupational asthma are classified on the basis of their temporal relationship to onset. Occupational asthma with latency reflects allergic occupational asthma and is a condition characterized by a preceding latent period of workplace exposure during which allergic sensitization to a material present at the work site occurs.

The RADS is a chronic asthma-like syndrome resulting from a single acute exposure to a respiratory irritant. The disregulation of neurogenic inflammation by chemical exposures may be an important mechanism in the toxic induction of RADS [7].

Definition and diagnosis. The diagnosis of occupational asthma, defined as variable airways narrowing, causally related to exposure in the working environment to specific airborne dusts, gases, vapours or fumes, needs to be confirmed by objective means [8, 9]. Specific bronchial provocation tests can confirm the diagnosis of occupational respiratory disease and also identify the causal agent of occupational asthma. The different diagnostic methods used for occupational asthma are: history taking, immunological investigations, pulmonary function tests, measurement of peak flow during occupational activity and holidays, sequential measurements of nonspecific bronchial hyperreactivity.

The difficulty in standardizing specific provocation tests used for occupational asthma is related to the wide variety of causal agents which can be inhaled as gas, aerosols or powders. Because of the required precautions, these test must be performed by specialized personnel in hospital units.

There are also limitations to specific provocation tests. False positives require placebo test and false negatives may result from insufficient identification of the causal agent. Exposure time may be too short or concentrations too low, and may depend on how long the causal agent has been eliminated [10].

Specific provocation tests may be avoided when the clinical history reveals a typical situation due to an agent known to cause occupational asthma and repeated PEF measurements at the working site and/or immunologic sensitisation tests provide objective evidence. But in a certain number of cases, specific investigations are required to obtain the precise etiological diagnosis required to evict the allergen rapidly and avoid chronic asthma.

Host and environmental determinants. It is clear that both host and environmental factors are important determinants of occupational asthma once an individual enters a high-risk industry. Concentrations and timing of exposure are significant environmental factors. Recent findings suggest that sensitization to low molecular weight agents requires a shorter interval than sensitisation to high molecular weight compounds [1]. The effect of smoking interacts with atopy and predisposes to sensitisation to high molecular weight compounds.

Prognosis. Follow-up studies of workers suffering from occupational asthma have reported persistence of symptoms over long periods (70% of the cases of occupational asthma remains symptomatic after removal from the occupational exposure) [12]. The number of years of asthma existed at diagnosis is also higher in these subjects.

After removal from the occupational exposure, a majority of subjects (60%) show a decrease of specific bronchial responsiveness to the offending agent [13]. Once the diagnosis is established, the worker should be removed from the work-place. If the diagnosis is made in time, the patient should experience a significant improvement. The major factor in determining a poor prognosis in occupational asthma is the duration of exposure before the diagnosis is established.

Prevention and treatment. Prevention of the disorder is the best therapeutic intervention. However, prevention is a multidisciplinary enterprise needing the commitment of chemists, industrial hygienist, allergologist, in addition that of occupational health personnel.

Inhaled corticosteroid treatment induces improvement of the asthmatic condition in subjects with occupational asthma caused by high and low-molecular weight agents after withdrawal from exposure. The beneficial effect is, however, pronounced if inhaled steroids are given early after diagnosis [14].

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NUTRITIONAL TRIGGERS IN ASTHMA

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Food and food additive triggers play an important role in approximately 5–8% of all asthma cases. Exact epidemiological data are lacking, partly because the etiological link is not always obvious, the diagnosis of food hypersensitivity is often complicated and ambiguous, food triggers usually act in concert with other trigger(s), and intraspecies and intrabotanic cross-reactivities between inhalant and nutritional allergens can make the time-course of the symptomes confusing.

The participation of airway symptomes in food allergy goes up to 40%. Relevant diagnosis can only be established by the combination of procedures used for both food allergy and asthma.

In the therapy avoidance measures are of great importance besides usual asthma therapy, and probably in combination with the reduction of gut permeability.

Asthma and nutrition have a very intimate, antigen-dependent relationship under certain circumstances, as it can be proven by the representation of airway symptomes among those of adverse reactions to food on one side, and foods, additives (and nonetheless, drugs) as triggers of asthmatic reactions (non-classified per se as food allergy) on the other – obviously two sides of the same coin.

The presence of some fatty acids in the diet, which are very suitable precursors of proinflammatory lipid mediators, might be a well-known example of non-antigen-specific relations.

An antigen-specific nutritional trigger, however, is not necessarily a single essential factor in provoking bronchial hyperreactivity response or even acute asthma attack: exercise, alcohol consumption e.g. may (and indeed, usually do) act as further triggers [1, 2].

Cross-reacting (usually with inhalatory antigens) food epitopes, sometimes in volatile form are particularly suitable as causes, triggers of asthma – a phenomenon occurring often among workers in the food processing industry [3, 4].

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The first large series of investigations for the identification of nutritional asthma triggers were performed by Onorato et al. in1986, who had proven by the gold standard of the diagnosis of adverse reactions to food, DBPCFC (double-blind placebo-controlled food challenge) the relevance of certain foods in the provocation of airway-symptomes [5].

The role of aspirin and other non-steroid antiinflammatory agents as asthma triggers was discussed extensively since the late 70s [6, 7]. WHO also has taken a position against a certain group of medicines (beta-blockers) in asthma, because of their aggravating effects [8].

Asthma cannot only be triggered by food, drugs or additives like sulphites, but, as mentioned previously, by means of exercise, alcohol, cold air or cold drinks, as well [9–11].

Although several mechanisms may play an important role in food or additive-induced asthma, e.g. immune complexes (cf. Heiner Syndrome [12]), or direct activation of committed T-lymphocytes, the most important (early) symptomes and mechanisms are to our present knowledge IgE-bound [13]. IgG is less often suspected. It is by no means easy to identify any food triggering asthma via non-IgE mediated pathways, as complaints may occur sometimes more than 24 h after ingestion. This leaves ample time for symptomes caused by the spontaneous variability of the course of the disease in chronic asthmatics. Further, it may be difficult, sometimes even impossible to identify nutritional factors as asthma triggers in situations where possible cross-reactivities are unknown or not investigated in detail.

Even if only few direct evidences exist, it is also generally accepted that – similar to other forms of food allergy, normal or abnormal digestion, degradation of food components, allergic reactions in the gastrointestinal system, or some non-immunological gastrointestinal diseases may facilitate gastrointestinal absorption so much that it may play decisive role as trigger [14].

Clinical symptomes, at one end of the spectrum may include acute asthma attacks [2] and multiorgan hypersensitivity reactions (i.e. anaphylaxis) in case of idiosyncrasy, intolerance, or real food allergy if the allergen is rarely ingested and/or if the reactivity is high.

Alternatively, it may happen, if the presence of the offending food component is not obvious because of incomplete labeling. Milder upper and/or lower airway symptomes causing severe diagnostic problems usually occur when the triggering food is ingested on a more frequent basis [15].

Typical symptomes include runny nose, discharge and itching, laryngeal oedema, and bronchoconstriction, cough, wheezing etc. – the complete symptomatology of asthma bronchiale and sometimes of allergic rhinitis. According to Wüthrich, second to dermatological symptomes (45%) come airway symptomes (24%) in food allergy [16]. Less relevant data exist on food triggers of asthma, the prevalence being estimated as 4–6% for children [17] and 1–4% for adults [5].

As most important triggers, cow's milk and peanut are usually mentioned [18]. For the diagnosis of food triggered asthma, primarily the proven diagnostic methods of adverse reactions to food should be used. It should be mentioned that some widely used tests (skin prick tests, basophil histamine release, migration inhibition assay, lymphocyte

stimulation assays, leukotriene assays) are accepted mainly for the reduction of the list of possible offending foods before in vivo provocation tests are performed. DBPCFC should be done parallel to lung function tests and/or the determination of biomarkers before, during and after the provocation (ECP and/ or tryptase are suggested) [19].

Very often, however, the amount of food suitable for "blind" provocation (=encapsuled) is not enough to provoke symptomes at all, not even a slight change in bronchial hyperreactivity representing the mildest reaction to challenge [20, 21]. In this case open challenge may replace the blinded test. In vitro determinations of (IgE, IgG, IgA) antibodies to food epitopes did not prove to be of much use in the diagnosis of ARF or, similarly, food triggered asthma. In rare cases high concentrations of serum antibodies toward a specific food allergen and parallel relevant anamnestic data make in vivo testing unnecessary or even dangerous. Food additives of practical importance, causing asthma are sulphiting agents, benzoates, salicylates and tartrazin: the most important among them is probably sulfur dioxide [9]. The prevalence of sulphit triggering is less than 5%, all others show much less frequency. Some cases of MSG have also been reported [22].

Suspected is the role of food allergy in asthmatics not only in infants having had food allergy (mainly to cow 's milk) earlier in their life with gastrointestinal symptomes or gastrointestinal and skin symptomes (even though, when these latter may vanish with the time), when total IgE levels are high, typically exceeding 1000 IU/l, when anaphylaxis is present in the past history, and in some cases of poorly controlled asthma with no obvious background [5].

A drop of at least 20% in FEV_1 following food challenge – in a time course parallel to the symptomes in open challenge, or during everyday life on usual diet – is considered as minimum criteria for the possibility of food induced/triggered asthma [3, 23].

The epidemiology of food triggered asthma cannot be determined at present – mainly because of the pitfalls and difficulties in the reliable diagnosis of adverse reactions to food.

In therapy, unless the decisive role of ingestion of any food or additive is proven, there is no real value of complicated dietary measurements, taking into account the possible hidden foods and the possibility of cross reactivities, too. Excluding the group of poorly controlled asthma as well, where the restricted intake of major food allergens (milk proteins, eggs, soy, peanuts) might be of value in some cases, nutrition triggered asthma should be treated by anti-asthma treatment [24, 25]. A larger room for antiallergics ("antihistaminics") as adjuvants in the treatment of these types of asthma might be justified – taking in account that some observations support the reducing effect of these drugs on gut permeability in intestinal allergies [26].

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INHALATION DEVICES IN CHILDHOOD ASTHMA

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Inhalation therapy is currently the most appropriate way to treat asthma also in childhood. This route of administration provides the best clinical effect with the least systemic side effects. The use of inhalative therapy in childhood involves special considerations. The age of the child is a major determinant of the method which can be used. The accuracy of dosing and the technically perfect use of devices are extremely important, especially when drugs with potential systemic side effects are administered. Successful therapy needs a good partnership between the patient, their parents, the family doctor and the pulmologist.

The correct use of pressurized metered dose inhalers (pMDI-s) and breath-actuated pMDI-s can be expected only in 40–50% of children over 7 years of age [1]. When children reach school age many of them will be able to use dry-powder inhalers correctly [2].

The use of pMDI-s with large-volume, valved spacer devices (Nebuhaler, Volumatic) increases the proportion of particles likely to reach the lower airways. Local deposition in the mouth and throat is also decreased which is important when using inhaled corticosteroids. Important advantage is, especially in children, that there is no need to coordinate actuation and inhalation. But it is important to know that multiple actuation and electrostatic charging of the spacer walls reduce the delivered dose. Even a short delay between actuation of the pMDI and inhalation from the spacer reduces the proportion of small inhalable particles [3]. Many of children between 3 and 6 years can co-operate in the use of a mouthpiece. In this age group pMDI-s with large- or small volume spacers are recommended both for long-term antiinflammatory medications and short-term relievers [4].

Special considerations exist in infants and small children [5]. For inhaled therapy currently two types of system are available: nebulizers and pressurised metered dose inhalers with spacer devices.

Nebulizers have a number of disadvantages: they are expensive and not portable, require power supply, the administration time is long, only small proportion of inhaled

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drug reaches the lung. In addition several studies in wheezy babies have shown an initial bronchoconstrictor response to nebulized bronchodilators [6, 7]. It has concluded that in wheezy children under two years of age inhaled beta-2 stimulants are recommended as first line therapy using spacer rather than nebulizer [8]. Nebulized therapy is indicated in children under 2 who cannot use other inhalers and in all ages for acute severe asthma [9].

During the past decade different devices were developed for drug delivery from pressurized metered dose inhalers to infants and young children.

The coffee-cup delivery system was the first and simpliest method for delivery pressurised aerosols to babies [10], later on large volume spacers with face-mask have been used successfully in this age group. These methods have considerable disadvantages compared with an ideal device for this age-group.

Characteristics of an ideal spacer for infants and small children:

– simple
– portable
– cheep
– inhalation time is short
– acceptable for small children (soft and closely fitted face-mask)
– acceptable for parents
– favourable technical parameters: small volume holding chamber, 2 one-way valve with small dead-space, non electrostatic material of the chamber

NES-Spacer (ASTRA), babyhaler (GLAXOWELLCOME), and aerochamber (TRUDELL) fulfil many of these criteria and have been used in our department in the therapy of wheezy bronchitis and infantile asthma.

We studied the efficacy of inhaled beta-2 agonists in 172 acute wheezing episodes of 52 children under 3 years of age. The aim was to judge the clinical efficacy of these spacer-devices.

Method

Two puffs of Salbutamol (200 µg) or 1 puff of terbutaline (250 µg) were administered via NES-spacer, babyhaler or aerochamber. When there was no improvement, the same dose was repeated twice with 5-minute intervals. So the maximum dose were 600 µg salbutamol or 750 µg terbutaline. The outcome was detected with a clinical symptom-score (respiratory rate, cough, wheezing, auscultation-finding, maximum 8 points). When the symptom-score decreased with 3 or more the effect was evaluated as good. A decrease with 2 points was considered as moderate, with 1 or 0 as ineffective result The pulse-rate and oxygen-saturation was also monitored in all children but was not evaluated in the clinical symptom-score. We did not observe a tachycardia above 180/minute at any child.

Results

Good effect was demonstrated in 54% of children, moderate in 28%.The treatment was ineffective in 18% of patients (Fig. 1).

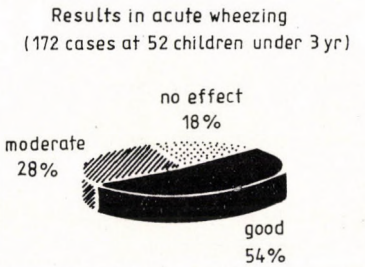

Fig. 1. Inhaled beta-2 agonists via small-spacer devices

Spacer devices were also used for maintenance therapy with inhaled corticosteroids (budesonide and fluticasone) in 62 children under the age of 3. In this study 210 children (aged 1–17 years) were investigated during long-term (1–3.5 years) inhaled corticosteroid treatment. The improvement in frequency and severity of asthmatic symptoms was scored. When compared the results in all children with results of children under 3 years of age (who used small-spacer devices for inhaled corticosteroid therapy) the same clinical improvement was found (Fig. 2).

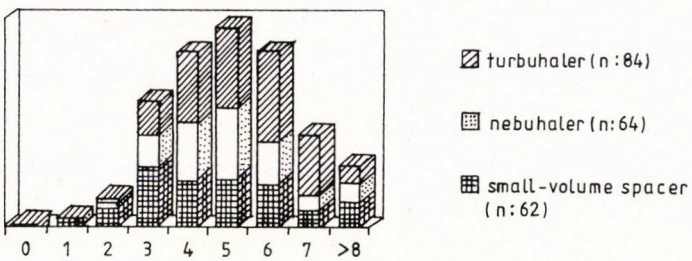

Fig. 2. Clinical improvement during inhaled corticosteroid therapy. Decrease in disease severity-score

Conclusion

Our results also demonstrated that pMDI-s via small spacer devices can be successfully used both for first line therapy of acute wheezing and for long-term antiinflammatory treatment in infantile asthma and wheezy bronchitis.

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INHALATION DEVICES IN ADULT ASTHMA

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Local treatment of the respiratory tract with inhaled remedies dates back to ancient times. Inhalation of the smoke of various roots and plants was a well-known method of folk medicine. The administration of "asthma cigarettes" (containing among others stramonium leaves and *Atropa belladonna*) for therapeutic purposes was started in the 19th century. Although the first inhalers were developed in the 1850s, the major role of inhalation treatment in the case of bronchial asthma and other obstructive respiratory disorders developed during the past 40 years. The pressurized metered dose inhaler (pMDI), introduced in 1956, was the first effective portable device suitable for aerosol administration. During the 1970s and 1980s, different new generations of inhalers were developed.

Physical properties of aerosols

Aerosols involve solid, liquid or mixed particles 0.001–100 μm diameter suspended in gases. Most aerosols contain droplets with a wide range of diameters, characterized by the mass median aerodynamic diameter (MMAD). The mass of a particle with a diameter of 0.5 μm is 8000 times less than that of a 10 μm particle. Particles with a diameter above 10 μm are entirely deposited in the oropharynx. The diameter of therapeutically usable particles is between 0.5 and 5 μm. 1–5 μm particles are deposited in the conducting airways, while particles below 1 μm in diameter reach the terminal bronchioles and alveoli. The small airways are the optimal sites for the inhalative treatment of obstructive pulmonary diseases. Particles are deposited mainly by impaction, and less so by sedimentation. Diffusion as a mechanism of deposition is relevant for particles smaller than 1 μm. The weight and hygroscopic properties of a particle also modify its deposition. Besides the properties of the aerosol, the delivery device, the inhalation technique and the airways of the patient also have a great influence on the

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amount of drug delivered. The ideal delivery method would produce a pure drug aerosol with particles only in the diameter range 2–3 μm.

The most important advantages of inhalative treatment in respiratory diseases:

Drugs are delivered to the site of action. The onset of effect is much more rapid, which is particularly important in the case of bronchodilators; this is why inhalation is the optimal route for the administration of beta-2 agonists. The applied doses are 10–20 times less than the oral or parenteral doses. The risk of systemic side-effect is minimal.

Some drugs can be administered only in this way, e.g. sodium cromoglycate and nedocromil. (In contrast, theophyllin is not effective when administered by inhalation.)

Types of inhalation devices. Aerosols produced by nebulizers are also suitable for the treatment of obstructive pulmonary disorders, but such nebulizers are not portable devices. The two main types are compressed air-driven nozzle nebulizers and ultrasonic nebulizers.

The most important indications for the use of nebulizers. Treatment of acute severe asthma with large doses of beta-2 agonists and ipratropium bromide in hospitals and in ambulances. Supervised treatment of chronic asthma in young children and selected adults at home. Administration of antibiotics in diseases such as cystic fibrosis and bronchiectasis. Inhalation of hypertonic saline to induce sputum production.

There are three main groups of currently available portable devices that generate aerosols suitable for inhalation into the lungs: Pressurized Metered Dose Inhalers (pMDIs), pMDIs with added spacer devices, and Dry Powder Inhalers (DPIs).

I. *Pressurized metered dose inhalers.* pMDIs were introduced in 1956 as the first truly portable inhaler devices. Being multi-dose inhalers (usually containing 200 doses), they deliver repeated, precisely measured doses of drugs. The canister, the actuator and the mouthpiece comprise their most important parts. The aerosol in the canister contains: drug substance, up to three different propellants, and surfactants or lubricants.

The pMDIs are closed with dosing valves, each actuation of which delivers from the dosing chamber an exactly defined amount of propellant/active drug mixture. Most drugs used for inhaled anti-asthma therapy are available in pMDI form, including beta-2 agonists, steroids, cromoglycate, nedocromil and anticholinergs. Bronchodilators administered by pMDI came into clinical practice in the 1960s, and the use of inhaled corticosteroids started in 1972. Inside the canister the vapour pressure is 300–500 kPa. The MMAD of the drug particles is less than 5 μm, but the effective aerosol size is much larger because the particles are within the unevaporated propellant droplets. Surfactants (usually sorbitan trioleate, oleic acid or lecithin) and lubricants ensure aerosolization of the drug on release and lubricate the valve mechanism of the canister. Chlorofluorocarbon (CFC) gases are generally applied as propellants in pMDIs. Most of the mentioned drug substances are not soluble in CFC gases, so pMDIs usually contain a suspension. The drug particles are usually less dense than the propellants, so in an unshaken inhaler the drug gradually separates out and floats on the propellant/surfactant mix. After actuation, the initial velocity of the droplets exceeds 30 m/s.

In order to use pMDIs correctly and to achieve maximal drug delivery to the lower airways, patients have to follow several important instructions: take the cap off the inhaler mouthpiece; shake the inhaler before use; hold the inhaler upright and make a

comfortable expiration; place the mouthpiece between the lips; actuate the inhaler while breathing in slowly and deeply; hold the breath in for 5–10 seconds; if another puff is needed, repeat these steps after about half a minute.

Disadvantages of pMDIs. Coordination problem: for effective drug deposition in the lungs, accurate coordination between actuation of the device and inhalation by the patient is essential, which causes difficulties for many patients. Despite repeated verbal and written instructions and regular checking of the technique: many patients are not able to use their inhalers correctly.

To overcome this coordination problem, breath-actuated pMDIs have been developed (e.g. the Autohaler), but they are not widely used. "Cold freon effect": some patients stop inhaling when they feel the aerosol cloud in their mouth. The propellants and surfactants may cause initial coughing and bronchoconstriction.

The CFC gases used as propellants in pMDIs damage the ozone layer of the Earth's stratosphere. In 1987 the delegates of 24 countries came to an agreement concerning the restriction of the production and use of CFCs. This agreement became known as the so-called the Montreal protocol. The revised protocol decided to ban all CFCs in developed countries by the year 2000.

Efforts are being made to develop pMDI which can be operated by non-ozone-depleting propellants. The use of the alternative hydrofluorocarbon (HFC) propellants requires changes in the structure and materials of pMDIs. Although the ozone layer is not damaged by HFCs, they are called "greenhouse gases" and may have an effect on global warming.

The delivered doses are not always the same during different actuations. If the inhaler is not shaken before use, the dose delivered will differ from the expected one. Some physicians recommend "priming", which means firing several puffs when a pMDI has not been used for several days.

The patient has no information about the quantity of drug remaining in the pMDI. High humidity may cause problems in their use.

II. *PMDIs with added spacer devices.* The use of large volume spacer devices or holding chambers such (as the Volumatic or the Nebuhaler) with pMDIs started more than 10 years ago.

The main advantages of the use of spacer devices with pMDIs. There is no coordination problem. They allow sufficient time and distance for the propellant to evaporate. A one-way valve at the mouthpiece allows the containment of the aerosol in the spacer before inhalation, decreasing the speed and size of the particles. The inspiratory flow needed to open the valves is very low (approx. 10 ml/min).

They decrease oropharyngeal deposition of the drug, which means fewer localside effects. This is of great importance when high-dose inhaled steroids are administered. (With the pMDI alone, the oropharyngeal deposition is about 80% of the dose; when a spacer device is used, the deposition in the oropharynx is only about 15%.)

They increase the proportion of inspirable particles. With a pMDI alone, only about 15% of the total dose reaches the lung. If a spacer device is applied, this increases to approximately 20% of the inhaled drug.

They are easier to use for old patients and young children and in acute asthma attacks. In acute severe asthma, large doses of bronchodilators can be administered with a pMDI and a spacer combination, producing sufficient bronchodilation. Under these circumstances, spacers may provide good alternatives to nebulizers.

Disadvantages of large spacer devices. Because of their size, they are not really portable. The delay between actuation and inhalation reduces the amount of small inspirable particles. The inhalation flow modifies the delivered dose. Two deep breaths is sufficient to inhale the contents of the 750 ml Nebuhaler. Multiple actuation of the PMDI into the spacer reduces the proportion of respirable particles within the spacer device. The mixing of two different drugs may also lead to a reduction in the delivered doses. The bronchoconstrictor effect of the propellant and surfactant may still occur.

The inside wall of a plastic spacer device has a strong electrostatic charge. The deposition of aerosol particles on this wall reduces the available dose. This effect can be reduced by several methods (wiping the inside wall with an antistatic cloth, with previous multiple actuation of the drug into the spacer, by coating the wall with benzalkonium chloride, or by constructing the spacer out of metal).

III. *Dry powder inhalers.* On the basis of aerosolization, two types of DPIs can be distinguished: inspiratory flow-driven inhalers and inspiratory flow-actuated inhalers. The first DPI (the Spinhaler) was introduced in 1969; it was suitable only for the inhalation of disodium cromoglycate. This was followed by the Rotahaler in the late 1970s. Both of them are single-dose devices.

The micronized drug substance, alone or together with additives (most frequently lactose), is filled into a plastic capsule. This capsule has to be placed into the inhaler, where the capsule is mechanically punctured and patients can inhale its contents with the suitable inspiratory flow.

Among the second generation DPIs, the Diskhaler/Rotadisk contain 4–8 individual doses in numbered blisters within a circular disc. The patient releases a dose by turning the disc; the blister has to be mechanically punctured. The device is breath-actuated, since the patient's inhaled airstream is used to disperse the drug powder into the lungs. The number of doses remaining in the device is visible through a window on the external surface.

The Turbuhaler is the first multi-dose DPI holding up 50, 100 or 200 doses. The Turbuhaler consists of a drug reservoir and a set of rotating discs which provide a metered dose of drug in specially designed conical holes. Holding the Turbuhaler vertically, the patient has to twist the grip at the bottom. This process turns the dosing disc and the holes in it are filled with a single dose of pure drug substance. When the patient makes a quick and deep inspiration through the device, the spiral in the mouthpiece creates a turbulent airflow, producing small inspirable particles from the drug.

The advantages of the Turbuhaler. It eliminates coordination problems and is much easier to use than pMDIs. There is automatic coordination between drug delivery and inhalation. Additives and vehicles are not required; it delivers pure drug into the airways.

The accuracy of dosing is maintained for all doses. If the Turbuhaler is twisted more than once before use, one dose remains in the inhalation chamber, so that the correct dose is still delivered. Even patients with acute severe asthma can produce the sufficient

inspiratory flow (see below). The counting mechanism of the Turbuhaler warns the patient when there are only 20 doses left.

The disadvantages of the Turbuhaler. The amount of drug inhaled is so small that the patient has no sensation of having inhaled anything. The patient must be informed of this. The drug content of the turbuhaler can be proved by inhaling through black silk.

The relative humidity can modify the inhaled dose. In this connection there are differences between the drugs used in Turbuhalers, namely terbutaline and budesonide. Terbutaline is a hygroscopic drug, and therefore tends to aggregate under conditions of high relative humidity, e.g. the respirable dose decreases by 75% at a relative humidity of 85%. The Bricanyl Turbuhaler contains a desiccant to keep the powder dry. It is in a separate compartment to the drug, and cannot be inhaled when the Turbuhaler is used. The cap also has a protective role. If the cap is left on, the Turbuhaler delivers the required dose for up to two years under various conditions. If the cap is left off, this period decreases to two month up to 80% relative humidity. In the case of budesonide, which is a hydrophobic drug, the relative humidity is less of a problem. As regards other DPIs, the Rotacaps used in the Rotahaler may soften at high relative humidity.

DPIs cannot be used with spacers; this may cause problem in patients inhaling large doses of glucocorticoids. The most frequent local side-effects are oropharyngeal candidiasis, throat discomfort, inhaler-induced cough and dysphonia. Candidiasis is often asymptomatic, but may be diagnosed microbiologically. Dysphonia, the most unpleasant side-effect, is probably a partial myopathy of the vocal cords. These local side-effects can be minimised with mouth rinsing and spitting out of the rinse water. Eating immediately after inhalation helps to clear the drug from the oropharynx, but may increase systemic absorption. Pedersen states that it is worthwhile to inhale budesonide immediately before brushing teeth. This can remove up to 80% of the drug deposited in the mouth.

Lung deposition from different inhalers. The efficacy and the side-effects of inhaled drugs depend on the site and extent of aerosol deposition in different parts of the body. The amount and distribution of the drug deposited within the lung determine its efficacy. Oropharyngeal and tracheal deposition is responsible for local side-effects. Systemic side-effects are the resultants of different components: the amount of the drug which is systemically absorbed, via the gastrointestinal tract, after swallowing the oropharyngeal portion and that from the lungs.

The deposition of inhaled drugs can be investigated with two different methods: gamma-scintigraphy and the non-radioactive, pharmakokinetic method.

Gamma-scintigraphy. The deposition and distribution within the lung can be measured by gamma-scintigraphy. The aerosol is prelabelled with a radioisotope (usually technetium 99) and after the inhalation of the labelled drug the emitted gamma X-rays can be measured with a gamma-camera. With this method it is possible to visualize the distribution of the drug in the various parts of the human organ. The central (large airway) and peripheral (small airway and alveoli) lung deposition can be distinguished.

Non-radioactive method. This is an indirect method, because the pulmonary deposition cannot be determined directly by measuring local concentrations of inhaled drug. If an inhaled drug is not absorbed from the gastrointestinal tract or is completely eliminated by first-pass metabolism, its systemic availability results absolutely from the lung absorption. By means of activated charcoal, the gut adsorption of several drugs,

including budesonide, terbutaline and salbutamol, can be blocked ("charcoal-block method").

While the gamma-scintigraphy technique measures the amount of drug deposited on the lung surface, the charcoal-block method measures the amount of drug absorbed via the lung.

Deposition from pMDI. Most of the inhaled drug is deposited in the oropharynx, and only 15% of each dose reaches the lower respiratory tract. In pMDIs large-volume spacer devices the large particles of the aerosol evaporate and settle down on the wall, which decreases the oropharyngeal deposition. On the other hand, the use of spacer devices increases the lung deposition, which is about 20% of the total dose.

Deposition from DPIs. The inspiratory flow determines the quality of the aerosol cloud. A high inspiratory flow increases the number of small particles. Among dry-powder device the Turbuhaler provides the greatest lung deposition, which is approximately 30%, when the peak inspiratory flow (PIF) is about 60 liter/min. In the case of 35 liter/min inspiratory flow the rate of lung deposition is about 15%, similar to the deposition achieved with pMDI. At a PIF below 30 l/min, the Turbuhaler begins to lose efficacy, because the number of respirable drug particles is approximately 25% of the number delivered when the PIF is 60 l/min. Several studies have shown that the Bricanyl Turbuhaler for example, is effective in patients with acute severe asthma. The peak inspiratory flow through the Turbuhaler is high enough to obtain suitable lung deposition. A lower nominal dose with the Turbuhaler can produce the same clinical effect as that with a higher dose from a pMDI.

In the case of inhaled steroids, half of the dose is enough when changing from a budesonide pMDI to a budesonide Turbuhaler. As concerns the regional lung distribution of inhaled drugs, their concentration in the central airways is much higher than that in the peripheral areas. Narrowing of the airways may reduce aerosol penetration into the peripheral parts, so in patients with obstruction more central deposition can be found.

The systemic availability of inhaled drugs. Systemic availability of inhaled drugs (especially steroids) results in their systemic side-effects. As mentioned above, two potential routes of absorption can be distinguished: via the gastrointestinal tract and from the lung. After pulmonary deposition, inhaled steroids are not inactivated; a part of this amount is also available systemically because of absorption. When the first-pass metabolism is high, systemically available drug mainly results from the absorption through the lung. If the first-pass metabolism is low, the portion of drug absorbed from the gut makes a greater contribution to the systemic availability. The therapeutic index is the ratio of pulmonary deposition (desired effect) to total systemic bioavailability (unwanted effect, resulting from both pulmonary and gastrointestinal absorption into the systemic circulation). The ideal index would 1.0, where systemic effects result only from pulmonary deposition of the drug. If the Turbuhaler is used, a lower dose of budesonide (reduced by 30–50%) is enough to achieve the same clinical effect as with the higher dose by MDI. The improved therapeutic index results in a reduction in the total systemic dose.

The characteristics of the ideal inhaler (from Journal of Aerosol Medicine, 1997. Vol. 10. Suppl. 1. S.27–48): portability; ease of use; resistance to rough handling; reliable delivery of drug substance throughout inhaler life; absence of excipients or additives; multiple dose delivery; a method of confirming receipt of a dose by the patient – by taste,

sight or sound; a dose counter (counting down to zero); an "alarm clock" device (to encourage compliance); efficacy independent of inhalation flow rate; minimal loss of drug within the device; high lung deposition; uniform delivery to the airways; low oropharyngeal deposition; reusable/refillable; potential to use similar inhaler for a variety of inhaled drugs; low cost.

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PREVENTIVE THERAPY OF ASTHMA STEROID AND OTHER ANTIASTHMATIC DRUGS

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The aim in preventive therapy of asthma is to reduce the severity by treating the inflammation and by minimizing its recurrence using a combination of strategies including appropriate drugs for preventing exacerbations, reducing triggers (allergen avoidance) and treating aggravating paranasal infections' factors.

Therapeutic strategies for asthma might be divided into two groups, defined broadly by their long-term effect on bronchial hyper-responsiveness. Bronchial hyper-responsiveness (BHR) is a hallmark of asthma and can be defined as the constrictor response of the airways after standardized inhalation provocation tests with either specific/allergen or non-specific (e.g. histamine, metacholine or hypertonic KCl) stimuli. BHR correlates not only with the severity of asthmatic symptoms, drug requirements, nocturnal fluctuations in lung function parameters and hospital admissions [1] but also with airway inflammation as measured by bronchial biopsy [2] and by increases in lavage mainly of eosinophils and desquamat epithelial cells.

The ability of a drug to reduce BHR should therefore indicate its potential to reduce the morbidity associated with asthma. The importance of airway inflammation in the pathogenesis of asthma has recently been emphasized [3]. It is widely accepted that, even in the mild cases of asthma, airway inflammation with involvement of eosinophils, mast cells and lymphocytes is observed consistently.

Therapeutic strategies for asthma might be divided into two groups defined broadly by their long-term effect on BHR. Treatments acting-directly or indirectly by antiinflammatory mechanisms with long-lasting effect on BHR-include both ingested and inhaled steroids, environmental control inhaled sodium cromoglycate and nedocromil sodium.

The other treatment group, namely bronchodilators-β2-agonists anticholinergics appears from present evidences to have no antiinflammatory effects and no long-lasting effects on bronchial hyperreactivity.

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Corticosteroids

The mechanism of action of different types of corticosteroids is not known in detail. The most often described mechanism is their anti-inflammatory action. The most important targets probably include leukocytes-lymphocytes, mast cells and blood vessels. Of special interest to the treatment of asthma is the permissive effect of corticosteroids on 32-receptors. Both inhaled and ingested corticosteroids, particularly the former, are potent inhibitors of allergen induced airway hyper-reactivity, either experimental or natural [4].

Corticosteroids have a significant inhibiting effect on allergen-induced late-phase reaction but no acute effect on early reaction, but a long-term treatment seems to provide a good protection. Mainly the inhaled form of corticosteroids consistently produce a marked and sustained improvement in airway hyper-responsiveness in unselected groups of asthmatics [5].

Then work reported by Laitinen showed that after regular treatment with inhaled budesonide for 3 months the airway epithelium of patients with mild asthma was restored to normality. The total number of inflammatory cell (epitheloid, lymphocytes, and eosinophils) was significantly reduced in the group treated with steroid, showing the antiinflammatory action of steroid. Such changes were not seen in the group treated with regular β2-agonist [5[. Today the inhaled corticosteroids are accepted as potent drugs for the treatment of non-specific and specific bronchial hyper-responsiveness.

Side effects. The more relevant systemic side effects concern the adrenal function, the bone metabolism and the growth pattern of children. Based on presently available data, measurable side effects of inhaled corticosteroid on hypothalamic-pituitary-adrenal axis are likely to occur with beclamethason and budesonide at high doses (>1500 in adults and >400 μg/day in children) [6].

In a long term treatment with budesonide (200–400 μg/day) no significant effects on bone mass was observed in children, similar findings were found in adults [7]. A meta-analytical study of 21 clinical trials [8], including more than 800 children, did not demonstrate a significant effect on growth exerted by budesonide even if used at high doses and long term. On the contrary, a recent study demonstrated a significant growth impairment at the dose of 400 μg/day beclomethason diproprionate [9]. Some local side effects have also been described: oropharyngeal candidiasis, dysphonia, cough/bronchospasm. The oral candidiasis seems to be dose-related and it can be avoided by using spacers and rinsing mouth after inhalation, whereas dysphonia usually requires treatment discontinuation.

Sodium cromoglycate and nedocromil sodium

In spite of their often use and a large amount of literature little is known about how these drugs work. They stabilise isolated mucosal mast cells and reduce the release of mediators; [10] the extent of this action in vivo is not known. Their action in blocking the effects of different challenges (allergens, exercise and SO_2) is proved. It is likely that they act on other inflammatory cells and on afferent nerve endings as well as on mast cells. In

vitro nedocromil sodium seems to be more effective in preventing mast cell degranulation than sodium cromoglycate, although the two drugs appear to be similar in the clinical efficacy.

Clinical effects

In a short-term experiment the above drugs prevent the development of exercise induced bronchoconstriction in many adults and most children [11]. The degree of inhibition is dose related and short lived, because the drugs are rapidly removed from the airways during exercise. Inhaling them before an allergen challenge they prevent the development of early and late reactions as well [12]. They also inhibit the effects of other "indirect" provoking stimuli such as SO_2; in this respect nedocromil sodium is more effective than sodium cromoglycate.

On a long term basis, in patients with episodic asthma seasonal exacerbations can be prevented. The effect on bronchial hyperreactivity of cromolyns is quite controversial compared to the proved efficacy of inhalation steroids.

Side effects. These drugs are amongst the safest drugs used today and have virtually no side effects. The dry powder of sodium cromoglycate can causes minor irritation in the throat and some patients complain that nedocromil sodium has an unpleasant taste.

For long-term use, they need to be given in an adequate dose initially four times a day. When the condition is improved they are used three times a day. Sodium cromoglycate is usually administered as 20 mg spincaps and nedocromil sodium 4 mg per puff from metered-dose aerosols.

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IS IT POSSIBLE TO PREVENT ASTHMA?

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Atopic diseases and among them bronchial asthma seem to be the worldwide epidemic of the end of the 20th century and this tendency may continue even in the 3rd millenium.

The main factors and periods which are important in the genesis of bronchial asthma are the following: genetic influences; intrauterine sensitization; factors in the perinatal life; influences in infancy; environmental factors in childhood; bronchial asthma and "western lifestyle"; prevention of further developing of atopic diseases.

Genetic influences

The tendency of the baby's immune system to overreact to allergens is largely under genetic control. It seems that this control of atopy and asthma is heterogenous, that is the mutation of more than one gene can induce the same or almost the same phenotype. Among others on chromosome 11q and 5q mutations were found which show a good correlation with atopic diseases, high IgE level, interleukin-4 expression (up-regulation of IgE production), interleukin-5 expression (up-regulation of eosinophil inflammation) [1, 2].

According to Cookson [3] 40–50% of the young adult population of western countries show positive prick test results against housedust mite and/or some kinds of pollen. As a consequence there are atopic individuals in about 20–25% of the marriages, so their children may have even two or more atopic genes.

It is clearly demonstrated that the risk for the development of allergic sensitization and manifest atopic disease depends on the patients' sensitization capacity which is partly determined by the atopic family history. In the countries with western lifestyle at least 10% of the general population have one or more atopic diseases. In Hungary the data of the last years show about 8 percent allergic rhinitis in the population and about 2 percent

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atopic asthma in children [4]. A child having one atopic first grade relative (parent or sibling) has 25% risk of developing an atopic disease, more than one such relative means a risk of about 50%, and two relatives with the same disease (e.g. both parents have atopic asthma) can rise the risk to 70–80%.

Intrauterine sensitization

It is known that mature circulating T cells can be detected in the fetus from the 18th week of gestation and as a consequence antigen exposure via the mother could initiate sensitizations. These may cause the ineffectivity of food allergen avoidance in the 3rd trimester of pregnancy, namely being too late. Warner [5] has shown that positive peripheral blood mononuclear cells yield proliferative responses to specific allergens if the mothers were exposed to increased allergen concentrations after the 22nd week of pregnancy. In other cases sensitization was already found from the 17th week. In individuals with a genetic atopic predisposition the maternal and thereby fetal exposure to allergens (pollen, mite, etc.) and tobacco smoke may induce materno-placental-fetal immunological interactions.

Trials in connection with the special diet of the mother during pregnancy and/or lactation periods gave ambiguous results. Elimination diets avoiding cow's milk, soya proteins, fish, nuts, hen's egg have been recommended to mothers during pregnancy and breast feeding periods in case of an increased risk for atopy in the child. There were not too many convincing results; some authors described fewer atopic symptoms in these babies and small children [6, 7]. Others observed a reduction in the weight of these pregnant women and even malnutrition is possible [8].

Factors in the perinatal life

Among the newborns we have to find the ones who are at high risk for developing bronchial asthma or other atopic diseases.

In the German Multicentre Atopy Study the authors evaluated the predictive value of various clinical and immunological parameters and the significance of environmental exposure to trigger factors and allergens in the development of atopic diseases in young children. Out of 1314 newborns 38% were selected as having high risk of getting atopic diseases (at least 2 atopic family members and/or cord blood IgE levels > 0.9 kU/l), 62% of the newborns served as controls. The follow-up lasted 2 years. Boys developed significantly higher levels of IgE than girls. There was a significant correlation between levels of cord blood IgE and parenteral smoking habits during pregnancy. The level of cord blood IgE was a good predictor of allergic sensitization at 1 and 2 years of age, but poor predictor of allergic disease symptoms at the same age. The mother's atopic history had significant association with the cord blood IgE class. The authors have the opinion that cord blood IgE levels are determined by genetic influences on one side and by intrauterine allergen and trigger (smoking) factors on the other [9].

Others found no positive association between elevated cord blood IgE levels and atopic dermatitis or obstructive bronchitis. These authors summarize their experiences and the data of recent literature: since 1988 the empirical evidence is against the predictive value of cord blood IgE [10].

According to Hansen [11] the determination of cord blood IgE concentration in combination with the atopic family history is the best way of selecting candidates for clinical trials of preventive measures. In the Danish population the positive atopic family anamnesis is positive in 16–20 percent of all newborns but if cord blood IgE with a cut-off level of 0.3 kU/l is considered in combination with atopic disease history, the high risk group consists of only 8–10% of all newborns.

Some prospective studies demonstrated that high cord blood IgE has a predictive value for future atopic diseases; the cut off point ranged from 0.3 to 0.9 kU/l in the published papers. More recent studies have found that cord blood IgE has a low predictive value for atopy [12].

Atopic family history can be recognised as a useful and inexpensive risk indicator [13].

We have to consider that most children who develop atopic diseases come from non-atopic families, on the other side a big part of the offspring of atopic families will never have atopic diseases – so the prevention measures introduced in neonatal age must be harmless and relatively inexpensive.

Influences in infancy

Experiences and animal trials showed that the first few months of life are very important from the point of view of later sensitization, atopic diseases and above all bronchial asthma. The hyperactivity to some allergens develop from the first months of life. The nature of the allergens varies widely (house dust mite, animal hair, pollen, food, etc.) and the clinical response also shows a lot of variations (skin eruptions, gastrointestinal signs, sneezing, wheezing, ocular symptoms, etc.).

Experiments with mice gave proof that in young animals small amounts of ragweed or housedust mite allergen induce "immunologic tolerance". For 2–3 weeks we can find a transitional IgE response and later a specific IgG and/or IgA answer and a low antigen specific T helper cell memory remains. These animals give no specific IgE response to these antigens later.

Other investigations demonstrate that in the first months of life almost all children give low level IgE type reactions to food allergens and somewhat later the same thing happens against inhalative allergens. This type of reaction is transitional and disappears in a large proportion of cases, but remains in a smaller cohort of children. They will probably show allergic, atopic diseases later in their lives.

In human beings this means that a lot of immunologic interactions between antagonistic T cells will result in a Th1 or Th2 cell response dominance. The result is a remaining or disappearing T cell memory against allergens; this reaction type may persist for the whole life.

The months of birth may determine the risk of a special pollen allergy. Sensitization to tree, grass and ragweed pollen was more frequent in children born during or close before a pollen season [14]. Until now no preventive measures have been advised to families at risk of atopic diseases to plan the date of birth in a period without pollen or in a season when we have not too many housedust mites.

In infants the allergic sensitization begins in general to food proteins (cow's milk and hen's egg). To avoid the early sensitization to these foods is one of the prophylactic measures generally recommended. Breast feeding is in general the best and physiologic alternative to artificial feeding. It is a very complicated task to perform an observer-blind and randomized study with a breast fed and an artificially fed group. The families with higher risk and better education will prefer breast feeding.

The cow's milk protein allergy has a low frequency in exclusively breastfed children, namely the small amount of cow's milk protein in breastmilk induces rather tolerance than allergic sensitization. In high-risk infants fed exclusively with breastmilk and/or extensively hydrolyzed formula at least in the first 4 months of life, a significant reduction of cow's milk protein allergy in the first 4 years of life has been documented. In the same time atopic dermatitis is also less frequent. Theoretically high risk infants may benefit even from maternal diet during lactation, too [15].

Lucas [16] has found in preterm babies that until the age of 18 months the breast fed ones coming from atopic families had less frequent atopic dermatitis than the artificially fed group. Only a few studies gave evidence that breast feeding helps to prevent the usually later coming atopic asthma and allergic rhinitis [17].

To recommend exclusive breast-feeding for the first 6 months of life has practically no real contraindications. It may be a general advice to the mothers of newborn babies. It is useful for those who are at risk for atopy, and it is also useful for those, in whom the risk is not expected. So breast-feeding should be recommended to all infants for the first six months of life.

Environmental indoor allergens can induce sensitization even in the first months of life. As babies spend most of their time in flats or nurseries, this type of allergens is very important. Among the children with high risk for asthma it is useful to avoid mite and pet allergens at least in the first six months of life. Early avoidance of inhalant indoor allergens (mites, cats and other pets) can result in a primary prevention.

Housedust mite is the most important allergen in the beds where the babies spend most of their time. New beds and mattresses are in many cases infested with housedust mite within 4 months of use [18].

Sensitization to pets – in Hungary cat allergens are the most important ones – is observed more frequently in children who are pet owners, but even in schools and kindergartens high level of cat and dog allergen may be found because the children bring them on their clothes and shoes [19]. We have to consider that the major allergen of cat (Fel d 1) can also be found in houses months after the removal of the cat [20].

In a trial with infants with high risk for atopy combined food elimination and indoor allergen avoidance was organized. This intervention program resulted in fewer atopic symptoms even at the age of 4 years [21].

Viral infections – RS virus, adenovirus, rhinovirus – play an important role in early sensitization of children at risk for allergy. They are very often triggers of exacerbations

of asthma. Early wheezing occurs often in children attending nurseries and kindergartens and in those who are in contact with children attending day-care institutions [22].

It seems that in this period of life respiratory tract infections can give rise to a Th1 type reaction with production of interferon-γ and interleukin 12 and this results in a suppression of Th2 type of immunreactions and acts against sensitization and atopic diseases [23]. Some recent publications indicate that infections may inhibit allergic sensitizations and symptoms. The "double edged sword" of infections as protective factor (switch of Th2 dominance to Th1 dominance) and as sensitizing and triggering factor need further observations and trials [24].

Environmental factors in childhood

Air pollution and other triggering factors have an important role in the development of atopic diseases and in the exacerbations of bronchial asthma.

The effects of air pollution on pulmonary function and asthma were evaluated in school children (6–15 years) living at least for 3 years in the Austrian alpine region. A significant association was found between increased SO_2, NO_2, O_3 level and decreased lung function. Maternal smoking and decreased $FEV_{0,75}$ showed a significant correlation, too. Increased level of O_3 and maternal smoking were associated with higher asthma prevalence. According to the authors air pollution and passive smoking are risk factors for lung function deterioration and respiratory diseases in children [25].

Many adjuvant or triggering factors (cigarette smoke, out-puff gases, ozone, SO_2, NO_2, free radicals, etc.) are known. These can facilitate the allergen specific sensitization and the development of bronchial hyperreactivity.

Tobacco smoke has a continuous negative effect on pulmonologic function and atopic diseases. It is harmful for fetus, babies and children. The reduction of tobacco smoke exposure has been found to be effective in ameliorating asthma symptoms [26].

Bronchial asthma and "western lifestyle"

New epidemiologic studies have shown that bronchial asthma and other atopic diseases are more frequent in countries where the "western lifestyle" has developed in the last few decades. Overprotected infants, few infections in the early months of life, manipulated foods with artificial components, isolated flats with high humidity, etc. together with air pollution were the most important factors causing this new "epidemy" of the high developed countries.

In studies comparing the data of 9–11 year old children in Munich (former West Germany) and in Leipzig (former East Germany) the following results were found: no significant differences in lifetime prevalences of asthma, wheezing and bronchial hyperresponsiveness; higher prevalence of bronchitis in Leipzig (30.9% vs 15.9%, $p<0.01$); higher prevalence of hay fever in Munich (8.6% vs 2.4%, $p<0.005$). The prevalence of current asthma was significantly higher in the west (5.9% vs 3.9%). The

authors are of the opinion that these differences are associated with western lifestyle and conditions including nutrition, parental smoking habits, housing and respiratory infections early in life.

The same authors have found that atopy (positive skin reaction to one or more of six aeroallergens) was twice more frequent in children of West Germany (33.5%) than in children living in former East Germany (16.7%). Rather the same differences were found in housedust mite sensitization (8.3% vs 3.4%) and bronchial hyperreactivity assessed by cold air challenge (6.4% vs 4.2%) [27, 28].

Prevention of further developing of atopic diseases

There are a few studies with the aim to prevent airway atopic diseases with the help of a long-lasting antiallergic drug intervention. Iikura [29] hoped to prevent asthma in children suffering from atopic dermatitis with the help of long-lasting ketotifen treatment.

A study with the name ETAC (Early Treatment of the Atopic Child) is running now [30]. This is an advanced clinical study to test the possibility of stopping the progression from atopic dermatitis to asthma. The probability that an infant will develop asthma is 50% if he/she has atopic dermatitis. The investigators hope that with the help of a long lasting treatment with a second generation antihistamine (cetirizine) they can prevent asthma in these children.

Another study with long lasting loratadine therapy aims to protect children and toddlers against viral respiratory infections. Knowing the role of viral infections in initiation of wheezing these investigators hope to prevent some cases of bronchial asthma [31].

Some points where we can do something against asthma

Two parents with atopic disease mean a high risk for the offspring. Taking into account the large number of atopic individuals in the population and the possibility of good medical care of these asthmatic patients no family planning has been advised to these individuals.

Elimination diet of the pregnant mother has no convincing beneficial prophylactic effect on the development of bronchial asthma.

A low concentration of inhalative allergens (mites, pollen, etc.) during pregnancy may help to avoid early sensitization of the fetus.

Tobacco smoke in the environment of the pregnant mother and her active smoking enhances atopic risk in the expected child.

The season of birth may have an influence on later sensitization: high allergenic pollen count in the first months of life, may induce sensitization. Avoiding indoor allergens (mites, pets, etc.) and triggers (smoke, gas heating and gas cooking, etc.) may

help in the prophylaxis of bronchial asthma. An environment with low inhalative allergen concentration in the first 1–2 years of life may help in this prophylaxis.

Breast feeding in the first six months of life, or feeding with hydrolyzed formula may prevent or postpone gastrointestinal and skin atopic diseases but has no convincing effect on asthma prophylaxis.

According to our knowledge, infections – first of all the viral ones – can induce allergic sensitization. RS virus, adeno- and rhinovirus infections have a proven effect on this process. Most of asthma exacerbations in infancy and small children are induced by these kinds of airway infections. Avoiding nurseries and the contact with children attending nurseries or kindergartens can have positive result.

Very recent data gave rise to the theory that children with frequent infections in infancy and young childhood – living in overcrowded flats, together with many siblings – gain some protection against atopic diseases.

Many of the components (exhaust fumes, flats and houses with technical isolation, SO_2, NO_2, ozone, inhaled particles, "sterile" circumstances in the first month of life, manipulated foods, etc.) of "western lifestyle" have an influence on developing asthmatic diseases.

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BRONCHODILATOR THERAPY IN ASTHMA

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Asthma is a world wide problem and a similar problem world wide. The Global Asthma Initiative published in 1995 highlighted the problems of under-diagnosis and under-treatment throughout the world. Guidelines for the use of drugs in relation to the management of asthma have now been available on a world wide basis via the International Consensus Document of 1992, updated by the GINA Document 1995 and now by a series of updates in different countries with UK modification, agreed in 1995 and subsequently published in 1997 and the latest US updated guidelines published in March, 1997.

There is general agreement that in the early stages of mild intermittent asthma the use of short acting Beta-agonist drugs "as required" to control symptoms is appropriate. When mild persistent symptoms occur, the need for continuous therapy and longer term management plans have to be considered. It is at this stage that anti-inflammatory medication on a regular basis is added to the intermittent use of short acting Beta-agonists. Within the most recent US updated guidelines, whilst stressing that inhaled corticosteroids are the basis of anti-inflammatory medication, support is also given to the use of Cromolyn Sodium, Nedocromil Sodium or anti-leukotrienes as alternatives.

Perhaps the most interesting advance in therapy and the adjustment that has been incorporated into the latest published guidelines is the increasing awareness that long acting inhaled Beta-agonists reduce the requirement for inhaled steroid therapy. An additional approach is, therefore, available to control those asthmatics with more severe and persistent symptoms where conventional doses of inhaled corticosteroids drugs fail to achieve adequate symptom control. There are three main pieces of evidence which have influenced thinking in this area. The first relates to the demonstration that the introduction of Salmeterol 50 mcg. twice daily achieved a more effective balance of symptom control in over half the patients in a controlled study where individuals who were inadequately controlled with standard dose of Beclomethasone dipropionate 200 mcg. twice daily were treated by, either increasing the Beclomethasone dipropionate to 500 mcg. twice daily, or by adding Salmeterol 50 mcg. twice daily to the standard regime. Subsequently, several studies have shown that by adding Salmeterol to the treatment regime of patients who

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were dependent on inhaled steroid therapy for the control of their symptoms, the level of inhaled corticosteroid could be reduced by at least 50% without any change in the peak expiratory flow rate or FEV1 measurement and with some improvement also in the level of symptom control. Furthermore, the introduction of Salmeterol in asthmatic patients whose symptoms were such as to require consideration for maintenance oral corticosteroid therapy improved in the level of symptoms particularly at night-time and reduced the requirement for additional bronchodilator medication overall. This was a further demonstration that the use of long acting inhaled Beta-agonist therapy offered an effective therapeutic option to the increased level of steroid therapy that otherwise would be required.

Concern regarding possible dangers in the regular extended treatment with long acting inhaled bronchodilators has proved unfounded. There is clear evidence that the responsiveness to these long acting drugs is not diminished with time, that tachyphylaxis does not occur and that the level of responsiveness to Salmeterol before and after six months treatment with Salmeterol 50 mcg. twice daily was unchanged. The side effect profiles for the long acting Beta-agonist preparations do not suggest any increase in complications with time. The incorporation of Salmeterol 50 mcg. twice daily into the routine treatment of patients with Chronic Obstructive Pulmonary Disease has been shown to offer a small but significant benefit in pulmonary function and, more significantly, in terms of improved symptom control.

The growing confidence regarding the safety and efficacy of long acting inhaled Beta-agonists is now rewarded by evidence that the dose of inhaled anti-inflammatory steroid preparations that is required to maintain control in asthmatic subjects can be reduced by the incorporation of long acting bronchodilator drugs within the management plan. In addition, the long-term management of Chronic Obstructive Pulmonary Disease and of severe-chronic asthma is improved with better symptom control, less need for additional medication and improvement in quality of life.

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ACUTE SEVERE ASTHMA

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Epidemiology

Many persons with asthma have a mild clinical course, reflecting chronic disease activity with occasional exacerbations rather than a more severe illness. Fortunately, in most of the asthma cases symptoms are easily controlled with a limited number of medications and education. But in a group of patients, the disease paints a much more malignant picture: developing sudden and unexpected increases in airflow obstruction resulting primarily from bronchial smooth muscle-mediated bronchospasm ("sudden asphyxic asthma") or a more slowly progressive form of airflow obstruction due to airway wall inflammation and edema [1]. This is reflected in the low rates of asthma mortality. Thus, mortality alone is a poor measure of severity.

The first challenge in describing the epidemiology of severe asthma is to define severity. One of the most fully developed models characterizing the multidimensional configuration of disease severity was developed by Stein et al. [2]. In this model, severity is defined by four components: biological severity, physiological severity, functional severity, and burden of illness. Biological severity is defined as an abstraction that cannot be directly measured but must be inferred from the other determinants of severity. Even if the biological determinants of asthma were fully understood, their measurement alone would not be sufficient to define a level of severity because of the complex interplay of biochemical determinants and environmental factors.

The biological impact of asthma on the lung is assessed by determining lung function. Therefore, the key measurement of physiological severity are pulmonary function testing and assessment of symptom scores. In epidemiologic studies of asthma severity, the forced expiratory volume in one second (FEV1) has been the most common measure of baseline function and is considered to be the most reliable measure of change in pulmonary function. It is more sensitive to airway obstruction than physical examination or symptom scoring and is linearly related to the severity of airway

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obstruction. The percent predicted FEV1 is the basis for many grading systems of asthma severity [3].

Early morning peak flow measurement provides a less precise surrogate measure of airflow obstruction than FEV1. Peak expiratory flow rate (PEFR) has twice the measurement variability of FEV1 [3] and is therefore not as good a measurement of baseline function. It is, however, very useful in establishing disease lability. Bronchial challenge testing with histamine or metacholine is widely used to establish the initial diagnosis of asthma. Similarly, it has been posed to grade asthma severity by metacholine challenge PC_{20} values [4].

The physical findings of asthma (i.e. wheezing, rhonchi, pulsus paradoxus, tachypnea, and accessory muscle use) vary over time and may be normal between episodes. Though they may correlate with the severity of a given episode, they do not correlate with the severity of the disease as a whole in a given individual. Certain individuals may perceive minimal dyspnea despite fairly severe airflow obstruction. This is particularly true if obstruction has progressed gradually over a period of days. Several population-based studies attempt to characterize the proportion of persons with severe asthma, based, in part, on symptoms' assessment. Newacheck and Taylor (1992) analyzed data of the 2.7 million American children with active asthma from the U.S. National Health Interview Survey. Based on their data, they classified asthma a severe in 10%, moderate in 32%, and mild in 58% of the children studied [5]. Marquette et al. found a death rate of 2.4 per 100 000 population in a study of patients with asthma requiring mechanical ventilation [6]. A history of near fatal asthma requiring mechanical ventilation is the greatest single predictor of subsequent asthma death. Patients with a prior history of near fatal asthma, but in clinical remission, have a blunted hypoxic ventilatory response and diminished dyspnea during inspiratory resistive loading as compared with asthmatics without prior severe exacerbations. Accordingly, diminished patient perception of dyspnea or gas exchange abnormalities likely increase the risk of future life-threatening attacks [7]. The fatality-prone asthmatic patient should be followed very carefully and should be instructed to seek medical attention promptly upon a significant worsening of symptoms.

Clinical assessment

The patient with acute severe asthma presents with dyspnea, a feeling of chest tightness, and marked and understandable anxiety. The respiratory rate is highly variable, hypoventilation and hypercapnia are signs of imminent respiratory arrest. Wheezing is often loud and its pitch is determined by the mass and elasticity of the oscillating bronchial wall, not by the diameter of the airways [8]. Widespread rhonchi on auscultation is common. In the most severe cases, the chest becomes more silent and wheezing less audible. Marked hyperinflation, prolonged expiration and inspiratory retraction arc characteristic signs of severe asthma. Tachycardia and pulsus paradoxus are also observed frequently.

The increase in work of breathing associated with asthma cannot be explained simply by widespread narrowing of the airways. In order to overcome increased resistance to flow and maintain airway patency, inspiratory muscles must keep the lung at high volumes, with marked negative pleural pressure swing and increased work of breathing [9, 10]. The need to breathe on high functional residual capacity (FRC) requires marked increases in inspiratory muscle force to overcome the elastic recoil of lungs and thorax. Thus, the primary physiologic response to asthmatic triggers is hyperinflation with increased FRC and total lung capacity (TLC). Respiratory muscles are disadvantaged in terms of their length-tension curve at these high volumes, leading to significant increases in oxygen consumption [11].

While peak expiratory flow rate (PEFR) and forced expiratory volume in 1 second (FEV1) are mainstay of severity of asthma assessment, very often it may not be possible to measure them in acute severe asthma. In general, PEFR or FEV1 less than 30 to 50% of predicted or of the patient's best (usually corresponding to a PEFR less than 120 L/min and a FEV1 less than 1 L) indicates severe exacerbation. Hypoxemia of varying degrees is invariably present. Arterial blood gases initially show low $PaCO_2$: as the asthma becomes more severe, it increases. However $PaCO_2$, by itself, is not a marker of asthma severity: some patients arrest at a relatively low $PaCO_2$, whereas others maintain respiration at a relatively high $PaCO_2$ [12]. Airway narrowing is not uniform in asthma. Bronchial obstruction causes low ventilation-perfusion ratios (V/Q), resulting in functional shunt and hypoxia. Initially, the ventilatory drive is constant or increased, leading to hypocapnia despite greater work of breathing. Later lung volume and respiratory rate increases, tidal volume and alveolar ventilation decreases due to respiratory muscle fatigue, resulting in hypercapnia [13]. A $PaCO_2$ that is in the normal range in the face of clinically apparent severe airflow obstruction should be viewed as a sign of potential deterioration with respiratory failure. Hypercapnia per se, however, although always indicative of a life-threatening exacerbation, does not predict the need for mechanical ventilation in most cases [12]. The presence of lactic acidosis in acute asthma indicates very severe airflow obstruction, unless the elevation in serum lactate can be attributed to administration of high doses of parenteral beta-adrenergic agonists. Pulmonary artery pressure always increases, probably secondary to hypoxic vasoconstriction.

Patients with findings of severe airflow obstruction (use of accessory respiratory muscles, pulsus paradoxus > 12 mmHg, diaphoresis, inability to recline, inability to speak, markedly diminished or absent breath sounds upon auscultation, hypercapnia etc.) who demonstrate a poor response to initial therapy (less than 10% increase of PEFR) or who deteriorate despite therapy should be promptly admitted to an intensive care unit [14].

Pharmacological management

Inhaled beta-agonists are the drugs of choice to treat smooth muscle-mediated bronchoconstriction in acute severe asthma (Table I). Larger and more frequent doses are needed in acute asthma since the dose-response curve and duration of activity of these

drugs are affected adversely by the degree of bronchospasm [15]. Long-acting beta-agonist, salmeterol, is not indicated in the treatment of acute asthma because of its slow onset of action, but clinical trials with formoterol are promising.

Inhalative treatments can be given continuously to severely obstructed patients until an adequate clinical response is achieved or adverse side effects limit further administration (e.g. tachycardia, arrhythmias or tremor). Prior use of inhaled beta-agonists at home should not limit their use in the emergency department unless marked adverse effects are identified [14].

Recent clinical experiences suggest that in nonintubated patients metered dose inhalers (MDIs) combined with a spacing device are just as effective as jet nebulizers [16].

Subcutaneously administered epinephrine or terbutaline sulfate carry no advantage over inhaled beta-agonists unless patients are unable to cooperate. This is not to say that an individual patient might not respond better if the parenteral route were used, but the latter should only be considered after failure of inhaled therapy. If parenteral beta-agonists are used, care must be taken to avoid hypokalaemia, lactic acidosis and cardiac arrhythmias. Known cardiac disease and age greater than 40 yr are relative contraindications to parenteral therapy. Patients older than 40 yr of age have more sinus tachycardia, premature ventricular contractions and atrial arrhythmias [17].

Table I

Treatment of acute severe asthma

Pharmacologic therapy	
Beta2-agonists	salbutamol 2.5–5 mg, terbutalin 5–8 mg (fenoterol 2–4 mg) in 2.5–4.0 ml normal saline via jet nebulizers or 4–6 puffs by MDI with spacer every 20 min × 3
Anticholinergics	ipratropium bromide 0.5 mg nebulization hourly or 4–10 puffs by MDI with spacer every 20 min × 3
Theophylline	5 mg/kg i.v. over 30 min loading dose in patients not receiving theophylline followed by 0.4 mg/kg/h i.v. maintenance dose (check serum level within 6 h of loading dose)
Corticosteroids	methylprednisolone 60–125 mg i.v. every 6 h
Supportive therapy	
Oxygen	2–5 L/min by nasal cannula
fluid and electrolyte replacement, acidosis correction, physiotherapy	
Mechanical ventilation	

Corticosteroids are essential components of therapy for acute severe asthma. They reduce the degree of recruitment and activation of inflammatory cells, potentiate the effects of beta agonists on smooth muscle relaxation, decrease beta-agonist tachyphylaxis, and decrease microvascular permeability and mucus production. Because their mechanism of action involves gene regulation and protein synthesis, the clinical effect of corticosteroids will not be immediately apparent but will take, at minimum, a number of hours to be expressed. Many physicians and patients fail to intervene early or agressively in the course of worsening asthma, therefore airway inflammation is allowed to proceed unchecked. Currently available data do support the common approach of giving 60 to 125 mg methylprednisolone intravenously every 6 h during the initial 24 h of treatment [14].

Anticholinergics produce less bronchodilatation at peak effect than beta-agonists and achieve a somewhat more variable clinical response, indicating that cholinergic mechanisms play a variable and less important role in acute asthma than in COPD.

Theophylline, as monotherapy is inferior to beta-agonists in the emergency-room treatment of asthma. In those severe asthma cases where there is poor response to treatment with beta-agonists and corticosteroids, theophylline can be added and the aim is to reach serum levels between 8 and 12 μg/ml to avoid toxicity. At the present time, the balance of evidence does not support routine use of aminophylline in the management of acute asthma treated with inhaled beta2-agonists and corticosteroids.

Fortunately, the vast majority of patients with acute severe asthma can be managed without *endotracheal intubation and mechanical ventilation.* There are enormous risks in applying mechanical ventilation to patients with acute severe asthma. The aim of artificial ventilation is to provide adequate gas exchange with minimum complications. Complications are related to the degree of positive intrathoracic pressure (e.g. pulmonary barotrauma and cardiovascular depression) and the length of time on the ventilator (e.g. sepsis, nosocomial pneumonia). To ventilate against an extreme high airway and elastic resistance of the hyperinflated lung in asthma means a very high risk of complications. Because pulmonary barotrauma is related directly to the magnitude of peak inspiratory pressure, ventilator settings should be adjusted to keep peak inspiratory pressure as low as consistent with adequate gas exchange (peak inspiratory pressure < 50 cmH_2O, tidal volumes 5–10 ml/kg). Darioli and Perrett [18] developed the concept of "controlled hypoventilation". The principle of this technique is to avoid high airway pressures and accept relatively high $PaCO_2$ levels, while maintaining normal oxygenation.

Intuitively, it would appear that applying positive end-expiratory pressure (PEEP) to overdistended lungs of asthmatics would decrease lung emptying, increase gas trapping, increase peak inspiratory pressure and predispose to barotrauma. However, PEEP has been used successfully in asthma [19].

The wide range of reported death rates in ventilated patients with acute severe asthma (0 to 38%), the lack of common approach to mechanical ventilation and pharmacological therapy preclude fair comparison of mortality rates among studies [14].

Despite advancing knowledge of the pathophysiology and treatment of asthma, asthma morbidity and mortality are on the rise. The factors responsible for this trend remain unclear. Overreliance on inhaled beta-agonists, underuse of anti-inflammatory agents, environmental pollutants, inadequate assessment of worsening airflow obstruction by patient and physician, lack of access to medical service and noncompliance with

prescribed therapy have been implicated in the recent rise in asthma mortality and morbidity [14]. All patients with asthma should be educated about their disease, physicians and patients must focus their attention on the early identification and treatment of asthma exacerbations. As Dr. Thomas Petty put it: "... the best treatment of status asthmaticus is to treat it three days before it occurs." [20].

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IMMUNOTHERAPY

THEORETICAL BASIS. TRADITIONAL AND ALTERNATIVE METHODS

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Allergen immunotherapy is defined as the repeated administration of specific allergens to patients with IgE-mediated conditions with the aim of reducing the patient's sensitivity to the allergen in question and providing protection against the inflammatory reactions associated with natural exposure to these allergens.

Immunotherapy is controversial. It is often used in some countries (USA, Southern Europe), while in Scandinavia and the UK a decreasing number of allergic patients receive immunotherapy. The tendency is probably caused by a combination of some degree of uncertainty as to the clinical efficacy, the risk of side effects, and the introduction of potent prophylactic, antiinflammatory treatment of allergic diseases.

Despite the varied immunological changes occurring after allergen immunotherapy, the precise mechanism or mechanisms responsible for clinical effectiveness have not been determined. It is still an open question as to whether immunotherapy can alter the natural history of the disease. It is claimed that it is efficacious for years after the injections are stopped and that it can prevent the development of new allergies, the progression of rhinitis to asthma and the development of irreversible lung damage in chronic asthma. But, at present these claims are based on theoretical thinking supported by thin experimental evidence [1].

As such fundamental questions remain unanswered, it is difficult to give precise indications for the use of immunotherapy.

According to the recommendations of the British Society of Allergy and Clinical Immunology: "Immunotherapy is recommended only for pollen rhino-conjunctivitis uncontrolled by drugs and only in patients without asthma" [2]. On the other hand the European Academy of Allergy and Clinical Immunology recommends the immunotherapy early in the disease process in order to prevent the further development of severe disease and declares: "The indication for immunotherapy should be considered to

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be equivalent to that of prophylactic drug treatment, and immunotherapy – for allergic rhinitis and asthma – should be started on the same disease severity. Patients who need daily pharmacotherapy should be offered supplementary immunotherapy" [3].

Mechanisms of immunotherapy

Immunological changes associated with allergen immunotherapy include an increase in IgG (blocking) antibodies, blunting of the expected rise in postseasonal allergen-specific IgE antibodies, a decline in allergen-specific IgE antibodies, rise of allergen-specific IgA and IgG antibodies in nasal secretions, development of allergen-specific suppressor T cells, decline in lymphocyte responsiveness to specific allergens, and in some patients reduction of in vitro histamine release from peripheral blood basophils when specific allergen is added [4]. In addition, other findings consistent with possible immunologic changes include suppression of immediate and late nasal reactions and late bronchial reactions after allergen challenge; reduction in conjunctival sensitivity; suppression of eosinophil migration into nasal secretions after intranasal challenge; and reduction of eosinophilic chemotactic factor and neutrophil chemotactic factor in bronchoalveolar lavage fluid after seasonal exposure to birch pollen in patients with asthma [5].

With continued allergen immunotherapy, allergen-specific IgG levels continue to rise and then a plateau is formed [6]. The blocking activity is probably of relevance in the protective effect of venom immunotherapy, but it is unlikely to be a major mechanism with inhaled allergens.

Decline in allergen specific IgE occurs after long-term allergen immunotherapy, but not, in most patients, to a level that results in the disappearance of immediate cutaneous reactivity [4].

It follows that a reduced synthesis of IgE antibody does not contribute to the clinical efficacy of immunotherapy, which occurs in the majority of patients within the first year.

Change in lymphocyte response appears to be another of the attractive hypotheses [7]. The lymphocyte response to allergen, demonstrated in vitro by, for example, lymphocyte transformation or cytokine production, is increased in allergic patients, and immunotherapy tends to return it towards normal.

Immunotherapy reduces the number of infiltrating CD4+ T lymphocytes during the late-phase response to allergen, and recent results indicate a switch from Th2 cells to Th1 cells. Th2 cells synthesize cytokines, which promote IgE formation (IL-4), as well as infiltration and activation of mast cells (IL-3), and of eosinophils (IL-3 and IL5).

Vaccination with T cell epitopes: T cells and B cells recognize different epitopes on the same antigenic molecules. B cells are capable of direct reaction with the protein molecule, T cells need the presentation of a peptide fragment by antigen-presenting cell. T cells that appear to play a direct role in allergic inflammation are probably the same cells (Th2 cells) that are involved in IgE class switching. Down-regulation of these Th2 cells could, at the same time, reduce IgE synthesis and limit inflammatory responses to

allergen. The identification of T-cell epitopes on allergens is now progressing rapidly. A number of major allergens have been cloned, sequenced and expressed. Epitope mapping with knowledge of the full peptide sequence of an allergen allows the synthesis of a series of peptides that can be used as materials for T cell specific treatment [8].

Conditions for which effectiveness of allergen immunotherapy has been demonstrated

Allergic rhinitis. Well-controlled clinical studies have demonstrated that allergen immunotherapy is beneficial in the treatment of seasonal pollinosis caused by trees, grasses and weeds [3].

Before one commences immunotherapy the following points should be evaluated: 1. duration of pollen season and number of days with symptoms, 2. severity of the disease – when nonsedative antihistamines and topical treatment reduce symptoms, physicians are left to make their own decision with regard to the commencement of immunotherapy, 3. when asthma complicates rhinoconjunctivitis, immunotherapy is favoured, and 4. may be modulated by the patient's motivation.

In rhinitis, caused by mites, immunotherapy may be started when 1. in spite of allergen avoidance patients need prophylactic treatment for a longer period, or 2. subclinical asthma (night cough, exercise-induced asthma) develops.

Bronchial asthma. The role of specific immunotherapy in asthma management is under continuous investigation. Specific immunotherapy may be considered when avoiding allergens (pollens, mites, animal danders) is not possible and when appropriate medication fails to control asthma symptoms. When immunotherapy in asthma is considered, the following points have to be evaluated: 1. the potential severity of the allergy to be treated, 2. the efficacy of available immunotherapy, 3. the cost and duration of each type of treatment, and 4. the risk incurred by the patients because of the allergic disease and the treatments.

Insect hypersensitivity. The efficacy of venom immunotherapy in the treatment of stinging insect sensitivity to honeybees, yellow jackets, hornets, and wasps has been well demonstrated [9].

An absolute indication is given by a history of severe systemic reactions with respiratory or cardiovascular symptoms and positive diagnostic tests. It is not advisable to start venom immunotherapy without documented IgE-mediated allergy.

Mild systemic reactions, such as urticaria and angioedema without respiratory or cardiovascular involvement have a favourable prognosis, especially in children, but also in adults. Therefore most European allergists recommend venom immunotherapy only for highly exposed patients in whom such reactions recur, or for those whose quality of life has bean greatly impaired by fear of flying insects. Other allergists treat all adult patients with generalized sting reactions, including those with only cutaneous symptoms, when their diagnostic tests are positive. Large local and unusual reactions, such as vasculitis, nephrosis, fever, thrombocytopenia, etc. are not indications for venom immunotherapy.

Alternative immunotherapy

To improve the safety of immunotherapy and make it more convenient to patients, routes other than the subcutaneous administration of allergen have been investigated. Placebo-controlled studies are limited and alternative procedures of immunotherapy are still controversial in many ways.

Oral immunotherapy: despite the fact that good clinical results have repeatedly been obtained with enteric-coated birch pollen, oral immunotherapy is at present not a practical option in routine work. There is still a need for more controlled trials to document the results with this method.

Sublingual immunotherapy: the principle of the low-dose sublingual immunotherapy is highly controversial. More conclusive data are required before this therapy can be recommended for routine treatment of allergic disorders.

Local nasal immunotherapy: the place of local nasal immunotherapy in the treatment of allergic patients has not been defined. The use of aqueous extracts is generally associated with significant rhinitis symptoms. A potential risk connected with nasal application is the induction of asthma by inhalation of allergens into the lungs [3].

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PHYSIOTHERAPY OF ASTHMA

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The paradigm of pulmonary rehabilitation treatment has been defined as "the art of medical practice where an individually tailored multidisciplinary programme is formulated which through accurate diagnosis, therapy, emotional support and education stabilises or reverses both the physiology and psycho-pathology of pulmonary diseases and attempts to return the patients to the highest possible function capacity allowed by his pulmonary handicap and overall life situation." [1].

Almost 30% of children with asthma had some limitation in activity, compared with only 5% of children without asthma [2]. Ten percent of children with asthma had severe disease as measured by frequency of bother and limitations in function; these children, accounted for 35% of hospitalizations for asthma and 77% of the days in the hospital.

The treatment of asthma is medical. Physiotherapy does not assist the recovery of a child from acute severe episode [3]. Rehabilitation involves all aspects of the patient's care, with the goals of expanding as much as possible the patients's potential (mental, social, and economic) while the restrictions from both disease and its treatment are being minimized.

Achieving these goals requires careful coordination of medical care, asthma education, care in the community at large (especially in the school, and workplace), physical conditioning programs, and in some cases psychologic care. This care involves a team of health professionals and is interdisciplinary, collaborative, and comprehensive. The patient should be included as a full partner. The physician is a coordinator of care.

Who needs rehabilitation?

Most asthmatic patients have mild to moderate disease and therein lies a paradox. Some patients often find their asthma frustrating and disabling precisely because of their expectations of counting in demanding jobs and their desire to live a normal life, whereas more severely disabled patients with chronic symptoms often have lower expectations. The important approach of pulmonary rehabilitation should therefore not be reserved for

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patients with end stage chronic lung disease [4]. The principles of rehabilitation should be included in the care of all patients, regardless of the severity of the diseases.

In the light of the wide variability in the severity of asthma both between patients and in a given patient at different times, we should identify the individual patient's requirements and capability, which necessitates objective measurement of exercise performance and limiting factors, and we should provide a range of options for improving exercise capacity or morbidity or both during submaximal exercise, which requires objective measures of outcome to audit the efficacy of particular programmes in individual patients.

In addition to assuring adequacy of medical care and asthma education, the physician should be aware of the patient's level of physical condition, performance in school/workplace and the presence of psychological and social problems. For each of these issues patients will present a spectrum of adaptation.

Once the patient has developed restrictions, prompt institution of rehabilitation is essential to prevent significant disability. The type of specific rehabilitation methods and the intensity of their applications will vary widely from patient to patient, with age and differences in severity of the asthma [5].

Goals of rehabilitation

1. To allow participation of the patient in age-appropriate physical conditioning activities with peers.
2. To maximize school/workplace attendance, participation and productivity
3. To promote self-esteem and self-confidence and decrease anxiety about the illness.

Aims of physiotherapy in asthma

1. To promote a relaxed pattern of breathing, or improve the pattern of breathing
2. To teach controlled breathing and reduce to a minimum the effort of breathing
3. To aid expansion of lung tissue
4. To assist in the removal of excess bronchial secretions
5. To relieve bronchospasm
6. To increase the patient's exercise tolerance
7. To make the patient as independent as possible and give advice regarding functional activities.

These aims of treatment should be adapted depending on the severity of the disease and the various needs of the patients. The following points may provide helpful information when considering the appropriate treatment for the particular patients: The patient's condition, history, lung function tests, blood gases, radiographs should be assessed before treatment begins.

Breathing pattern, rate of respiration, movement of both side of the chest should be observed. Breath sounds, cough and sputum, airway obstruction can often be detected by an audible wheeze. By regular spirometry comparisons will show the severity of the disease and the patient's response to treatment.

Respiratory physiotherapy should not be prescribed routinely. It appears to be indicated for asthmatics with continuous dyspnoea or hypersecretion who are unstable, despite medical treatment which is both correctly prescribed and properly taken.

Technics of physiotherapy. Respiratory physiotherapy: postural drainage, hatha yoga (reflex massage for relaxation, posture respiratory exercises borrowed from yoga), respiratory muscle training, physical training and sport etc. can be part of the rehabilitation program (combined often in many countries with speleotherapy).

Bronchial drainage, postural drainage. Postural drainage consists of position the patient to allow gravity to assist the drainage of secretion from specific areas of the lungs. Postural drainage can only be carried out effectively if the patient takes an active part in his treatment. The following techniques are used additionally in the clearance of bronchial secretions. Postural drainage can be carried out over a foam wedge or pile of newspaper.

Breathing exercise. The patient is encouraged to take deep breath and cough, the collateral airdrift among the alveoli can be utilised in order to remove secretion from the smaller airways. Diaphragmatic breathing must be encouraged in the relaxation positions.

Bronchial drainage on condition that certain technical precautions are taken is only useful in asthma with hypersecretion [6]. Teaching the child to cough effectively may be a useful procedure. However, regular postural coughing is disadvantageous, as coughing can induce bronchoconstriction. If an area of segmental or lobar collapse occurs, then postural coughing may be helpful, but it should always be preceded by inhalation of a beta 2 adrenergic drug.

Vibratory chest shaking. Shaking is performed only during the expiratory phase of breathing and therefore reinforces the expiratory flow of air from the lungs. When treating small children a gentle vibratory movement is carried out by the fingers of one hand.

Percussion (clapping) of the chest wall is carried out with the hands slightly cupped and by a quick flexion and extension of the wrists.

Forced expiratory technique has been shown to increase the efficiency in the clearance of bronchial secretion, without causing bronchospasm. It consists of one or two huffs from mid-lung volume to low-lung volume followed by a period of relaxed diaphragmatic breathing. Relaxed diaphragmatic period is an essential part of the technique to prevent aggravation of any bronchospasm. After taking a breath in, the patient breathes out forcefully through the mouth contracting the abdominal and chest wall muscles at the same time. It may be necessary to practise by blowing down a tube. Forced expiratory technique is combined with correct drainage position, breathing exercise, percussion and shaking.

Aerosol therapy. When secretions are tenacious or there is plugging of the airways, high humidity is helpful before postural drainage. This can be given by a humidifier or nebuliser.

Daviskas [7] investigated the effect of increasing the osmolarity of the airway surface liquid by administering an aerosol of concentrated salt solution. They compare the asthmatic groups inhaling ultrasonic nebulized 14.4% and 0.9% saline, and the control group. An increase of osmolarity of the airway surface liquid increases mucociliary clearance both in asthmatic and healthy subjects.

Speleotherapy. Speleotherapy in salt mines or cave therapy is recommended to include into combined rehabilitation treatment of asthma. Cave therapy is not a passive

use of a peculiar climate but a special complex rehabilitation adapted to circumstances of cave microclimate [8, 9].

Hyperventilation. Mucociliary clearance was increased after isocapnic hyperventilation with dry air. These findings are in agreement with the suggestion that the increase in mucociliary clearance after isotonic hyperventilation with dry air is due to a transient hyperosmolarity of the airway surface liquid.

Reflex massages, relaxation, posture, yoga. There are reflex massages for relaxation, posture and respiratory exercises which are borrowed from yoga. Techniques for correction of posture used preventively are only of value in chronic asthma of childhood. Fluge [10] compared the effects of three groups of mild asthmatic patients on the course of 3 weeks training programme. The breathing exercise group and yoga group showed a significant amelioration of the mental state but only breathing exercise induced a significant improvement of pulmonary symptom parameters compared to the individual baseline values.

By dilating the nostrils [11] the nasal breathing can be increased by nasal dilator instrument. Reduced nocturnal asthma was observed by 12 out of 15 patients.

Respiratory muscle training. Weiner [12] proved that specific inspiratory muscle training may be a complementary or alternative therapy with the aim of reducing systemic corticosteroid requirement and inhaled beta 2-agonist consumption, improving the control of asthma symptoms in patiens with asthma.

Exercise induced asthma (EIA). "If from running, gymnastic exercises, or other work the breathing becomes difficult, it is called asthma." (*Arateus*, second century AD)

In 1698, the asthmatic physician Sir John Floyer wrote: "All violent exercise makes the asthmatic to breathe short" [13]. The phenomenon of shortness of breath after vigorous exercise has been recognized for centuries and for decades it has been recognized that bronchoconstriction occurs after exercise, it most likely occurs after running. If there is no exercise there will be no exercise induced asthma, but there will be none of the benefits of exercise.

The breathlessness of asthmatic patients may be exacerbated by bronchoconstriction caused not only by airway cooling effect of large volumes of inspired air (respiratory heat loss) but also by increased osmolarity of respiratory tract epithelial fluid. The final common pathway seems to involve release of mediators from cells in the airway. Exercise induced asthma is demonstrable in about 80% of children and adolescents with asthma, especially the group of patients who would be keenest to participate in the sport [13].

Better control of asthma usually reduces the severity of any concomitant EIA and the negative effect of exercise can usually be blocked by pretreatment with beta 2 agonists, disodium cromoglycate or less commonly ipratropium bromide. In many patients EIA is less severe after repeated exercise. In 40–50% of patients with EIA, induction of a mild attack of asthma will attenuate subsequent asthma responses to identical exercise tasks performed within the next 2 hours. This is known as the refractory period. The mechanism is unknown but depletion of mediators in the airways may be a factor. Thus warm-up period may permit normal exercise subsequently.

Exercise programmes – beneficially unchanged factors. There is little evidence for the idea that regular participation in exercise improves basal lung function or that

deterioration occurs as result of not taking exercise. Non-specific bronchial responsiveness remains unchanged [14] after exercise programmes.

Benefits of exercise programmes. In healthy individuals exercise programmes and lifestyle initiatives have major benefit in reducing the risk of cardiovascular diseases. There is no reason to believe that this should not occur in patients with respiratory disease. Many patients with asthma of different severity have sufficient ventilatory reserve to allow tolerance of training routines. Improvements in fitness ranging from 10% to 92% have been reported [15].

The potential benefit of decreased ventilation at submaximal work loads after physical training may result in reduced breathlessness during exercise [14, 16, 17]. The reported improvement in exercise induced asthma after training [16] is most likely due to the reduction in minute ventilation that occurs at equivalent high intensity work loads, resulting in a decrease in the stimulus for exercise induced asthma rather than any change in the underlying pathophysiological process.

There is experimental evidence that the balance between the normal exercise induced bronchodilatation and exercise induced bronchoconstriction may shift slightly in favour of the former after training [18].

There may also be a central desensitising effect of physical training on the sensation of breathlessness. This may be due in part to familiarisation but may also be secondary to the effects of rhythmical repetitive exercise on brain endorphin concentration [17].

Physical activity. Most studies have failed to identify any significant changes in the degree of bronchial responsiveness with various aerobic conditioning programs, the potencial benefits are valuable and include

– improved fitness,
– decreased frequency and severity of acute attacks,
– decreased medication usage,
– decreased school absenteeism,
– improved self image [19].

Recommendations for evaluation of level of physical activity.

1. Educate the patients about exercise induced asthma, the prophylactic use of drugs, the overall value of exercise
2. Prescribe drugs to prevent EIA and monitor their effectiveness.
3. Question the patient concerning his/her level of activity and capacity to keep up with peers.
4. Make repeated efforts to determine that there really are no limitations of physical activity
5. If limitations in capacity to exercise are reported by the patient, consider testing
6. Design rehabilitation program based on the results of the physical fitness [5].

While physical activities are good for children with asthma, as they bring them into contact with other children and improve their physical fitness, there is little evidence that they have any specific effects on asthma. Though some studies suggest that exercise

programmes can reduce the degree of exercise induced bronchoconstriction [16, 20] this is far from constant finding [21].

This is not to denigrate the value of exercise, but rather to ensure that parents and the affected child see it as something to be enjoyed rather than as part of medical treatment [6].

Low asthmogenic sports [22]	*High asthmogenic sports*
Low minute ventilation sports:	High minute ventilation sports:
tennis, karate, football	long distance running
handball, wrestling, baseball	cycling
racquet ball, boxing, downhill skiing	basketball
gymnastic, sprinting, isometrics	football
diving, golf	rugby
Sports associated with warm	Sports associated with cool and dry climatic
and humid climatic conditions:	conditions:
swimming	ice hockey
water polo	ice skating
diving	cross-country snow skiing

It is essential to determine the patient's baseline pulmonary function, as it is unlikely that any therapeutic intervention will be successful if the underlying obstruction is not treated first.

Main questions

1. Can patients with asthma exercise normally?

When the disease is in remission and patients have been treated to prevent EIA, exercise can proceed normally.

2. Are they as fit as other people with similar exercise habits?

Maximum exercise oxygen uptake (VO_2 max) in patients with EIA was within the normal range and unaffected by beta stimulants [23]. Nevertheless, there is now good evidence that many asthmatic patients have reduced cardiorespiratory fitness, by compared with controls with a similar pattern of physical activity [24–27].

Clark [25] compared the maximum exercise capacity of 44 patients with asthma and 66 healthy matched controls. Despite exercising to a similar peak heart rate and minute ventilation as the controls, the patients had a reduced VO_2 max, reduced oxygen pulse (O_2 consumption per heartbeat) reduced anaerobic threshold and increased dyspnoea index (by expressing minute ventilation (V_E) as a percentage of estimated maximum voluntary ventilation, MVV). The study showed that patients with asthma can exercise to a similar intensity as the controls, but their cardiorespiratory fitness is usually reduced.

3. What sort of exercise is needed to make the patient fitter?

The type of activity is not critical but the exercise preferably should be conducted indoors in conditions of warmth and controlled humidity with appropriate warm up and warm down components to reduce the likelihood of breakthrough exercise induced asthma [4]. Individual exercise evaluation using progressive incremental exercise testing

may be helpful both to identify the most appropriate training intensity in the light of the patient's ventilatory response [25] and to monitor the effects of the programme on exercise capacity.

Some groups have used progressive incremental exercise around the "anaerobic threshold" but without routine a more empirical "trial and error" approach may be effective. This consists of evaluating the tolerability of submaximal exercise at the intensity that will be required during training which expressed in terms of the target heart rate. The patients either will achieve the target heart rate and tolerate steady state exercise during one of these stages or alternatively will show an inability to continue at the required work intensity owing to the breathlessness. If symptoms have remained stable with mild but tolerable breathlessness, the patients may be expected to participate in aerobic exercise programme with the achievement of improved cardiovascular fitness.

For patients who cannot sustain exercise of sufficient intensity to improve aerobic fitness because of breathlessness, they require techniques to condition peripheral muscles with the objective of improving mobility and stamina. Such patients with moderate to severe chronic airflow limitation can undertake low intensity isotonic training of individual muscle groups to improve the strength and endurance of these muscles and increase overall exercise tolerance [4]. Additional components of this second approach include callisthenics, breathing retraining exercises and walking at the maximum tolerable rate. Pulse oximetry provides a simple method of identifying the few patients who have arterial oxygen desaturation during exercise, due to exacerbation of ventilation-perfusion abnormalities. There are no guidelines for rehabilitation programmes for the subgroup of patients. Carefully supervised exercise conditioning with oxygen supplementation in a hospital gymnasium setting would seem to be an appropriate approach. They should be supervised, and the exercise prescription should be reviewed periodically

The rest with certain precautions can participate in the sport of their choice without any training in the medical milieu beforehand. However, in a minority of these patients the physiotherapists by their individualised approach and their techniques sometimes represent a useful transition towards participating in sport.

Nevertheless, strong motivation is required because variations in the disease may interrupt an exercise programme and so make the goal of increased fitness difficult to attain [28].

It is important for exercise to be habitual, it should be easily accessible and without adverse sequale. Ideally it should also be dynamic, interesting, fun, and varied [29].

Asthma: to run or not to run? [30]

Sponsored programmes, dance classes, swimming clubs provide an outlet for patients once a formal pulmonary rehabilitation programme has been completed. In general, patients with asthma can participate in most physical activities and sports.

Swimming classes for children with asthma have become very popular. They certainly are of value in improving physical fitness. Swimming was thought to be less likely to induce bronchoconstriction than other forms of activity. This is probably related to the warm, moist environment in which swimming takes place. Properly designed athletic and other dry land activities need not be more bronchoconstrictive than swimming. A few short warm-up periods combined with appropriate drugs will almost always prevent the development of exercise induced bronchoconstriction.

A widely accepted prescriptions of exercise intensity for healthy individuals to improve their fitness are: -exercise of 15–30 min duration, three times a week, to a level at which oxygen uptake (VO_2) is about 70–80% of maximum (maximum heart rate=220, age in years, Am. College of Sports Medicine, 31),

The above prescription is highly recommended for all asthmatic patients except those with moderate to severe chronic airflow limitation.

4. What effect might regular exercise have on their disease as opposed to their fitness?

Orenstein [27] in a study of exercise conditioning in 20 asthmatic children, showed a significant increase in both VO_2 and O_2 pulse after a 4-month exercise programme, indicating an improvement in physical fitness.

However neither this study nor another that evaluated swimming [32] has provided any evidence that exercise programmes alter indices of asthma severity. These results suggest that patients with asthma may underestimate their capacity and if the disease is well controlled, they can be encouraged to undertake vigorous exercise.

Low level of fitness was associated with poor psychologic function in the study of Strunk [33, 34]. A group of 90 children with asthma were tested, low level of fitness was associated with recent exacerbation, low level of FEV 1, and low specific airway conductance. Fitness did not correlate with the indicators of the most severe disease.

There is no cost benefit study available on exercise programmes.

Asthma and sports. Despite the fact that patients with asthma have often excluded themselves (or have been excluded by others) from many of the normal activities of childhood, it has long been thought that activity was likely to benefit them. Like patients with cystic fibrosis, children with asthma can increase their work capacity [27, 21, 35].

If they are treated with a bronchodilator before each exercise session, they can also increase their aerobic fitness (VO_2 max) [15]. They probably do not change their susceptibility to EIA, although improved fitness should mean a lower V_E for high-intensity exercise. This, in turn, should decrease the stimulus for EIA at a given level of exercise while not altering the reactivity of the airways.

Patients with mild to moderate asthma should not limit their athletic ability to participate in various sport programmes if appropriately treated. Patients with severe asthma should also be encouraged to participate in exercise programs and athletic events, but will require a multidisciplinary evaluation of the physician, physical therapist, exercise physiologist and physical education instructor to determine the appropriate degree of participation and achievable goals.

The 1984 Olympics saw 67 of 597 athletes on the United States team with EIA. They brought back 15 gold, 21 silver and 5 bronze medals, in events as diverse as swimming, shooting, basketball and track and field [15].

Not every youngster with asthma can achieve these levels of competitive success, but these examples should remind patients, families and physicians that the diagnosis of asthma should not deprive a youngster of the pleasure and physical benefits of exercise.

The principles of exercise rehabilitation for patients with asthma are applicable in all phases of the disease but perhaps are most useful early, when setting of appropriate

expectations can minimise restrictions from the disease. Respiratory physicians can play a leading part by incorporating exercise rehabilitation into complete management of the patient with asthma.

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PSYCHOLOGY AND RESOCIALISATION OF THE ASTHMATIC PATIENTS

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Extrinsic asthma bronchiale is a model disorder to study the interrelationship of social, psychological and physiological factors. In his original description Franz Alexander [1], the internationally acknowledged founder of psychosomatic medicine, emphasized multi-causality of asthma bronchiale. Bernát Alexander, the father of Franz Alexander, was an eminent professor of philosophy at the Pázmány Péter University here in Budapest. According to Franz Alexander the important factors from the etiological point of view are: genetic factors; perinatal traumas; organic disorders during childhood, which increase the risk of vulnerability in certain organs; the way the child was cared for or provided for (early socialization); physical traumas in infancy and childhood; traumatic emotional experiences in infancy or early childhood; the emotional climate of the family, specific personality traits of parents and siblings; physical traumas in adulthood; emotional experiences connected with professional or personal relationships.

In accordance with the social psychophysiological approach the course of the disease does not depend only on biomolecular factors but also on the person's psychological constitution, on his conflict solving strategies (coping mechanisms) and on the quality of support from the family and others.

According to numerous studies asthma patients are characterized to a greater degree by self-appraisal disturbances, depressive symptoms, anxiety and interpersonal conflicts. Leson and Gershwin [2] found by analysing the characteristics of 993 asthmatic patients between 1984 and 1994 – that the most important psychosocial risk factors correlating with severity of symptoms are: family dysfunctions; low socioeconomic status; low educational level; unemployment; smoking.

Martin et al [3] found that psychosocial factors have a central importance in 73% of severe or fatal asthma attacks.

National representative study – psychosocial characteristics of asthmatic patients in the Hungarian population.

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In 1995 12.440 persons were interviewed who represent the Hungarian population over the age of 16 according to sex, age, and place of residence. The survey was conducted in the form of personal home interviews.

107 questions referred to the socioeconomic situation, 209 questions to the health status, health behaviour and functional emotional disturbances [4–7].

Health status was quantified by OPCS (Office of Population Censuses and Surveys) Disability Questionnaire and by self-assessed quality of life and by sick days in the last year.

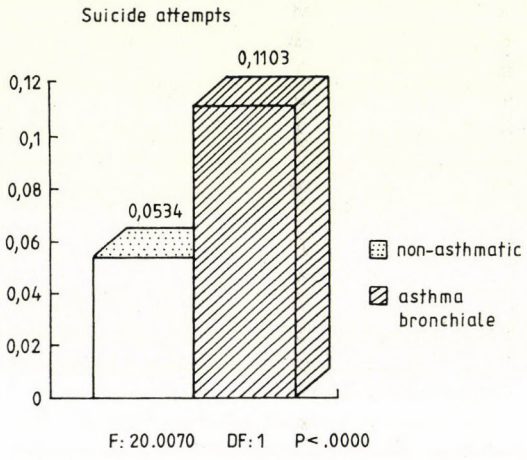


Fig. 1

The subjects answered two questions for 26 disease groups on whether they had been treated because of the given illness in their life, and how many days they were actually sick because of the given illness in the past year. We compared the psychosocial and socioeconomic characteristics of persons who were treated because of asthma bronchiale to other persons in the population.

Shortened Maastricht vital exhaustion questionnaire [8]. The shortened version consists of 9 items of the Maastricht Questionnaire with highest Cronbach alphas and has high internal consistency.

Shortened version of the Beck Depression Inventory (BDI) [4, 9]. The questions of the shortened questionnaire are related to the following characteristics of depression: social withdrawal, inability to make decisions, sleep disorder, fatigability, exaggerated anxiety over somatic complaints, inability to work, pessimism, lack of satisfaction and inability to feel pleasure, self-blame. Our shortened BDI is a slightly modified version of the original shortened BDI, which can be reliably transformed into a full score.

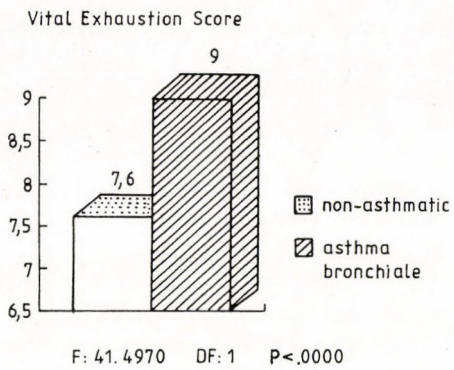


Fig. 2

Shortened Ways of Coping questionnaire (Folkman,S., Lazarus,R.S. [10]). All of the 22 items of the shortened questionnaire were included separately into the multivariate analyses.

Shortened Dysfunctional Attitude Scale (Weissman, Beck [11]). The questionnaire examines seven value systems and attitudes. They are: need for external recognition, to be loved, achievement, perfectionism, entitlement, omnipotence and external control versus autonomy. All the items of the questionnaire were included separately into the analyses.

Social support questionnaire (Caldwell et al. [12]). Ranking social support from parents, relatives, friends, official helpers. Socioeconomic characteristics: education, employment, father's employment, marital status, income, access to a car, own property, housing conditions.

The SPSS statistical program system was used for data analysis. Hierarchical log linear analysis was performed to investigate the interactive effects of social and psychological factors and sick days because of asthma bronchiale.

According to our study, 5.5% of the population reported that they were treated because of asthma bronchiale during their lifetime. According to logistic regression the most important psychosocial and socioeconomic factors connected with asthma bronchiale are as follows: (Wald statistics values proportional to the strength of connection are beside the variables)

1. Marital status (10.06) – Asthma bronchiale is most common among people who are married but actually live alone.

2. Attempted suicide is highly significantly more frequent among asthmatics (7.62) (Fig. 1)

3.Years of smoking before the onset of asthma bronchiale (5.28)

4. Inadaptive coping – lack of ability for cognitive restructuring in difficult life situations (5.13) – it can be treated by cognitive behavioural methods

5.Vital exhaustion (4.84) – sense of excess fatigue and loss of energy (Fig. 2)

6. Dysfunctional attitude – increased achievement motivation (4.04)

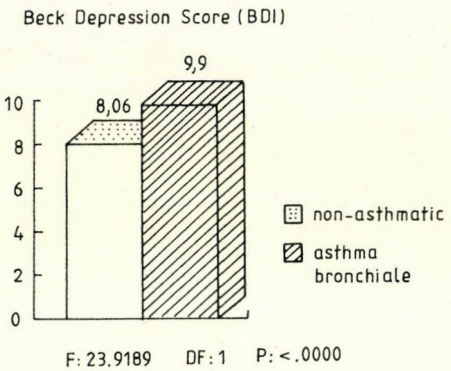


Fig. 3

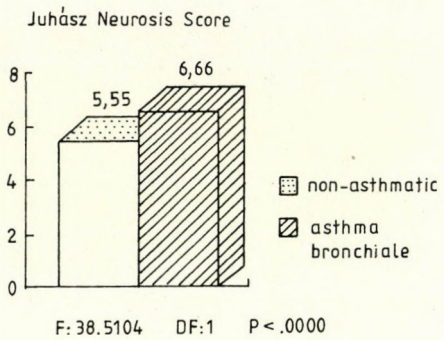


Fig. 4

In accordance with the literature, depressive symptomatology and neurosis score are significantly higher in the asthma bronchiale group (Figs 3, 4).

The burden on health care because of asthma bronchiale is considerably higher than in the non-asthmatic population (Figs 5–8).

Resocialization of asthmatic patients. On the basis of international experiences and the above data, psychosocial factors cannot be ignored to fulfil an effective treatment of asthmatic patients. In spite of more powerful, newest antiasthmatic medication, the need for hospitalization and emergency treatment did not decrease. Poor compliance is one of the central problems, which can be improved considerably by introducing cognitive and behavioural, that is behavioural medicine methods in the routine management of the disease. (Everaerd et al. [13], Maes, Schlösser [14]).

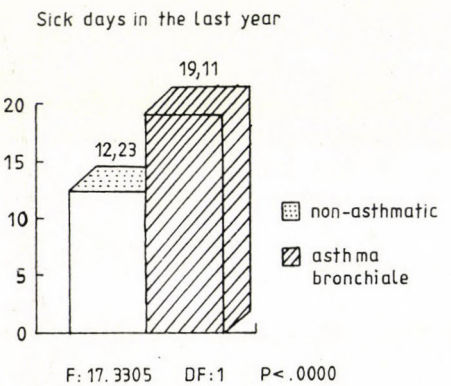
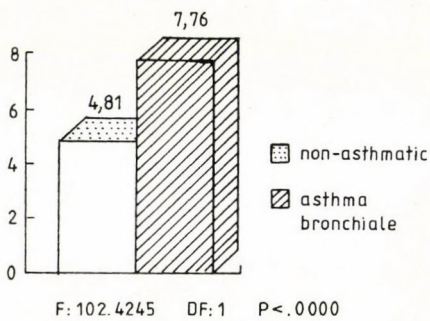


Fig. 5



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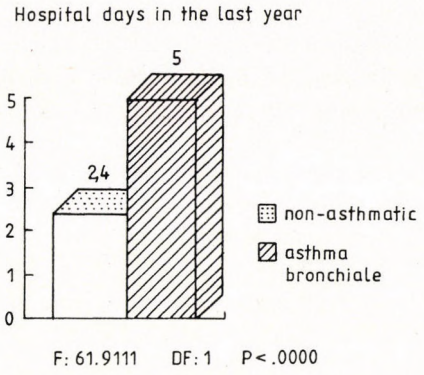

Fig. 7

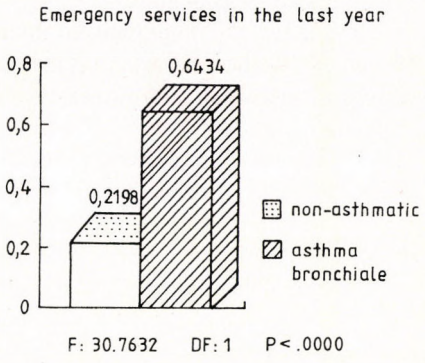

Fig. 8

The behavioural medicine approach of treatment focuses on:

– cognitive components – better understanding of the illness, triggering factors and methods of treatment, more effective active coping skills

– behavioural components – recognizing early symptoms of asthma attacks, better coping with the attacks (respiratory control, adequate self-medication, adequate help-seeking)

– maintenance of physical condition – respiratory control, swimming, other self-defense sports which do not provoke symptoms,

– decreasing anxiety and depression – relaxation training, improving problem solving strategies or expressing emotions, changing dysfunctional attitudes, strengthening social support, specially by self-help groups.

Most important elements of resocialization of asthmatic patients are:

1. *Correction of reversible pathological conditioned responses*

In the induction of asthma attacks besides the active biological factors learned mechanisms also play a very important role. The best known example of this is a patient of Dr McKenzie, in who's case asthma attacks brought on by a rose could also be induced by a paper rose. A series of researches have proven that an inhalant which did not contain any allergen could cause an attack if the patient was informed that the allergen was present. Since it was hypothesized that in this case the inhalation could in itself be a provocative factor, asthma attacks were induced whilst the patient was hypnotized by the suggestion of inhaling the allergen. So an asthma attack appears in many cases to be a conditioned, learned response. This fact can be used in the treatment of asthma bronchiale by applying cognitive behavioural treatment methods.

The increased attention given by parents and the environment, occasioned by the attacks can have a secondary affirming effect – the education of the family and possibly even hospital staff in this respect has, therefore, a significant role in the prevention of asthma bronchiale attacks.

2. *Family education*

One of the important components in the bio-psycho-social aspects of asthma bronchiale is the character of the parent-child relationship. Asthmatic children aged between 3–13 years had respiratory symptoms much more often when listening to their mothers' voices – irrespective of the tone of voice – from a tape recorder than they did when listening to an unknown woman's voice. Asthmatic children were also shown by projective tests to be dependent on their mothers to a greater degree, to have a closer relationship to them and to have an increased sensibility to separation from them – but all of these could also be the result of the illness.

Earlier, it was suggested that the child should be temporarily taken away from his parents, a "parentectomy" in other words, because this very often results in a decrease in the number of attacks.

In recent years, however, rather than the separation of the child from his parents adequate education of the family is considered to be more successful. The families of asthmatic children are usually too rigid, inhibited, too protective and have conflict solving problems. Therefore during family education real expectations of each other and the ability to express suppressed emotions are increased.

The importance of the therapeutic approach to the family as a system is emphasized when analyzing asthmatic children's psychological symptoms. 70% of them appear in very close connection with family problems such as parental marriage difficulties, or parental behavioural disorders or illnesses. In the treatment and prevention of childhood asthma bronchiale, therefore, the so-called self-management family asthma programmes have proved to be highly successful (Hindi-Alexander et al. [15], Kaszab et al. [16]). The essence of such programmes is that the parents, brothers and sisters learn the basics of how to prevent asthma attacks and have a healthy lifestyle – relaxation and respiratory control exercises, educational experiences and knowledge connected with the disorder. In this way the child and his family learn ways of preventing the attacks and thus the vicious circle caused by the patient's and parent's anxiety leading to the exacerbation of the attacks does not develop.

3. *Adaptive active coping skills*

According to Alexander asthmatic patients in alarm situations do not react with increased activity but rather with giving-up behaviour or asking for help, the physiological equivalent of parasympathetic tone. Since this condition is equivalent to psychophysiological giving up of the situation, adaptive active coping methods such as swimming or self-defence sports are basic psychophysiological factors in the prevention of asthma attacks. Active participation of the patients in controlling their condition has central importance with the help of structured self-management programmes (Wilson-Pessano [17]).

4. *Treatment of anxiety, depressive symptomatology and vital exhaustion*

The stress which precedes an asthma attack is often the experience of loss or disappointment, a real or imagined experience of losing an important person, the so-called separation anxiety.

From the point of view of the outcome of the disorder, or the probability of mortality, both the high and the low asthma specific anxiety or panic conditions are pathological – the first group because of the side effects of taking an increased quantity of

drugs, primarily steroids, and the second group is increasingly at risk because of ignoring the symptoms and ceasing to take the drugs on time.

The suicide rate of asthma patients is significantly higher than the average, corrected for age and sex, which is closely connected to higher depressive symptomatology. The most important background factors of depression and anxiety are the dysfunctional attitudes, such as irrealistically high achievement motivation and inadaptive coping strategies. Depression and anxiety can be effectively treated with adequate cognitive behavioural interventions.

In summary, cognitive-behavioural education programmes for asthmatic patients can result in improvement of health status and improve the quality of life. Well designed behavioural medicine interventions strengthen the effectiveness of the interaction between patient and physician and help patients to regulate more adaptively their physical and emotional environment by participating as active partners with their doctors in controlling their disease.

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GASTRODUODENAL INFLAMMATION ASSOCIATED WITH *HELICOBACTER PYLORI* INFECTION DIAGNOSTIC AND THERAPEUTIC IMPLICATIONS

(A REVIEW)

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1. Introduction

Early in this century there were many reports of bacteria in the gastric mucosa of a wide range of animal species, as well as humans. These organisms are phylogenetically closely related, and to date all belong to the genus *Helicobacter.* Although the traits essential for colonization of the stomach remain constant, these bacteria have coevolved with their various hosts and have acquired certain distinctive characteristics. *H. pylori* is a spiral-shaped microaerophil organism found in the stomach of patients with type B

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chronic gastritis, peptic ulcer disease, lymphoma of mucosa-associated lymphoid tissue of the stomach and gastric adenocarcinoma. This genetically diverse species has been estimated to infect the gastric mucosa of over half of the world's human population. Despite the high infection rate, symptomatic disease beyond gastritis (characterized by gastric or duodenal ulcer) is noted in a small, but nevertheless significant, fraction of this population [1–6].

The majority of bacteria are freely swimming in the gastric mucus, but about 20% have an adhesin-mediated association with the surface of the mucus neck cells of the non-acid secreting area close to the pyloric sphincter i.e. the antrum, the natural niche of *H. pylori*. *H. pylori* has been seen to adhere to epithelial cells with filamentous appendages and pedestal-like junctions, perhaps via gastric glycerolipid receptors and fibrillar hemagglutinins. *H. pylori* strains appear to be bound to surface mucous cells present in the pit region of gastric units but not to mucous neck, parietal, or chief cell lineages present in the glandular domains of these units. The bacteria thrive in intercellular junctions. In this niche, *H. pylori* appears to be of no consequence to the host. Certainly a mild inflammation has been established, but often this is not associated with symptomatology. That is, a normal balance between facultative pathogen and host seems to have been achieved. An exception to this situation may occur much later in life. After 30 to 40 years of continual inflammatory responses in the mucosa, tissue integrity is compromised and atrophic gastritis results [7–11]. *H. pylori* located in an unnatural niche may cause a serious life-threatening disease, peptic ulcer which does not normally develop in the antral mucosa, the natural habitat of *H. pylori*. Examples are gastric metaplasia in the case of duodenal ulcer and the antral-fundic junction in the case of gastric ulcer. Thus, in the duodenum, *H. pylori* is found associated only with gastric-type tissue in small islets of gastric metaplasia that may have been formed as a response to acid from the stomach. In the stomach, gastric ulcers more commonly occur at the transitional zone between the normal antral mucosa and the normal body mucosa. In both cases the underlying tissue is disorganized tissue and less uniform than normal antral mucosa. It is not difficult to imagine that changes in the gastric mucosal environment caused by *H. pylori* may be well tolerated in normal antral mucosa but less easily tolerated at a junctional site where tissue is intrinsically less stable or has been altered and acid defense mechanisms such as the proton sink capacity of the normal capillary network are severely compromised [1, 3]. This hypothesis makes it easier to understand why so many *H. pylori*-infected persons never have ulcers despite the presence of large numbers of bacteria on the antral surface.

It is now critical how *H. pylori* causes inflammation, and most importantly, to understand the immunological factors that appear to contribute to outcome of the infection. This review considers the available information concerning the immunopathogenesis of gastritis by *H. pylori* and assess the avenues for exploration and their potential for success.

2. Cell constituents of *H. pylori* that may play a part in the inflammatory responses to *H. pylori* infection

How an organism that is not basically invasive can result in inflammatory lesions must first be considered. Both intentional and accidental ingestion of these bacteria have shown that these organisms can cause inflammation in a previously healthy gastric epithelium. To achieve this, the bacteria have to penetrate the mucous layer [1, 2, 7]. Their spiral shape, unipolar flagellae, projecting filaments and capability to move in highly viscous milieu, as well as their proteolytic mucin degrading activity and lipolytic hydrophobicity lowering activity, phospholipases, chemoattractants, catalase, adhesion receptors, PAF production may be greatly advantageous in this respect. Their extremely high urease activity is believed to protect the bacteria during trip through the acidic contents of the stomach [12]. The ammonia released after the hydrolyzation of urea is likely to neutralize the hydrochloric acid. Ammonia may also be directly toxic to the gastric epithelial cells. Evidence is emerging that the more virulent strains possess well-defined segments of DNA [6]. These „pathogenicity islands" include *cagA* and *vacA* genes encoding for CagA and VacA, respectively, and encode proteins involved in signal transduction events that may facilitate intimate attachment to host cells, cytoskeletal rearrangement via actin polymerization, and host cell protein phosphorylation [6, 13–17]. Peptic ulcer formation in general correlates with the expression of CagA that is usually coexpressed with the vacuolating cytotoxin VacA, a protein that causes ulceration in the stomach of mice. Most of the strains could be classified into two major types. Type I bacteria express CagA protein and VacA. Strains of phenotype II do not express either the CagA protein or the VacA. In addition there is an intermediate phenotype, expressing CagA independently of VacA or *vice versa* [14, 18–28]. The levels of tryptase that reflects an indirect link between the action of gastrin and the function of mast cells and of cathepsin D that is relating to the phlogistic reaction of the mucosa to *H. pylori* infection are higher in patients with active peptic ulcer, whether gastric or duodenal [29]. *H. pylori* also contains superoxide dismutase providing a major defense mechanism against oxidative damage, catalyzing the breakdown of superoxide radicals to hydrogen peroxide and dioxygen [30]. A number of adhesion factors associated with *H. pylori* have been described, which may imply that adherence is a multistep process and that different adhesins mediate adherence to different sites in the gastric tissue [31–33]. *H. pylori* lipopolysaccharides have, in general, low immunological activity and this property may aid the persistence of infection. They may mimic *Lewis* blood group antigens in structure. As these antigens are present in the gastric mucosa, the expression of Lewis antigens on the bacterial surface may camouflage the bacterium and aid survival of *H. pylori.* Alternatively, since autoantibodies against human antral gastric mucosa have been observed in *H. pylori*-positive patients, the relevance of lipopolysaccharides in the development of autoimmunity in *H. pylori*-associated diseases requires further investigation. Gastric tissue lacking this antigen expression cannot bind *H. pylori* [34]. *H. pylori* lipopolysaccharides in part mediate the binding of the bacterium to laminin, and interfere with gastric cell receptor-laminin interaction, thereby potentially contributing to the loss of mucosal integrity [35–37]. Part of immunopathologic effects of *H. pylori* may

be caused by autologous and/or bacterial heat shock proteins (HSP60) which act as triggering factors in the development and persistence of the chronic inflammation in the gastric mucosa. By dot blot and immunoblotting both urease and an urease-associated heat shock protein were found to be specific and constant immunodominant *H. pylori* antigens. This class of homologous protein has been implicated in the induction of autoimmune disorders in different systems. The presence in infected patients of anti-*H. pylori* HSP60 antibodies, potential cross-reactivity between human HSP60 and a rabbit antiserum against *H. pylori* HSP60 suggest that a role of this protein in gastroduodenal diseases is possible [38–42]. As a result of this sequelae of events together with their interactions with a variety of gastrointestinal hormones, hydrochloric acid, pepsin and other ulcerogenic mediators may gain access to the epithelium and cause further damage [13].

3. The role of the host's immune reactions in mucosal damage caused by *H. pylori*

3.1. General overview

Whilst *H. pylori* affects a broad range of immune responses, chronic *H. pylori* infection persists for years. Within the gastroduodenal mucosa *H. pylori* infection stimulates activation of the antigen-transporting endocytic-endosomal system, enhances expression of the antigen-processing enzyme cathepsin E and *de novo* expression of antigen-presenting HLA-DR molecules, aberrantly induces HLA-B5, HLA-B12, HLA-BW35, local production of a range of proinflammatory and immunoregulatory cytokines, neutrophil infiltration, specific T- and B-cell responses and the development of gastric lymphoid follicles; all of these may enhance immunological damage [43–45]. Both local and circulating antibodies can regularly be demonstrated in infected patients. Local plasma cells have been shown to produce specific antibodies even *in vitro*. Macrophages and polymorphs are also involved in the inflammation. Infiltrating neutrophils are likely to be one of the major mediators of mucosal damage. Neutrophil activation, including reactive oxygen metabolite production and the release of myeloperoxidase, can be induced directly by bacterial factors and indirectly through products of complement activation, bioactive lipids and host-derived cytokines. Interleukin-8 and related peptides of the chemokine family secreted by gastric epithelial cells are likely to be important host mediators inducing neutrophil migration to sites of infection. Epithelial IL-8 is upregulated by TNF-alpha and IL-1 and directly by *H. pylori* strains expressing the CagA phenotype. The extent of mucosal injury may reflect bacterial density, the variability of different strains of *H. pylori* to induce chemokine expression in epithelial cells and the oxidative burst in neutrophils. Recent evidence from *in vivo* and *in vitro* studies shows that CagA+ VacA+ strains of *H. pylori* are associated with enhanced inflammatory responses and mucosal damage. Defining the specific bacterial mediators of mucosal inflammation will be important in elucidating the role of *H. pylori* in the pathogenesis of gastroduodenal disease [12, 44, 46–49].

The impact of immunodeficiency states upon *H. pylori* infection may shed light on the intricate interrelations between *H. pylori* and the host's immune reactions. Examples are *H. pylori* infection in HIV positive patients, persons suffering from X-linked agammaglobulinaemia and kidney transplant recipients. Conflicting results have been reported in patients with HIV infection and AIDS. Decreased susceptibility to *H. pylori* infection in HIV positive individuals [50, 53] has not been confirmed later by others [51]. Positive histological testing appeared to be directly related to the peripheral CD4+ lymphocyte count and inversely related to the frequency of antibiotic treatments performed over the six months prior to endoscopy. Serological testing was positive for anti-*H. pylori* IgG antibodies in only 39% of patients. Compared to histology, serology displayed a sensitivity of 57% and a specificity of 81%. The discrepancy between histological and serological positive results for *H. pylori* was noted to be higher in the more advanced phases of HIV infection. Based upon these results, the serological testing for anti-*H. pylori* IgG antibodies seems to require cautious interpretation in HIV-positive patients [52, 53]. The impaired functions of CD4+ cell population may account for the inadequate production of anti-*H. pylori* antibodies. Recently it was reported that two brothers with X-linked agammaglobulinaemia infected with *H. pylori* had severe active gastritis [1]. This observation suggests that immunoglobulin deficiency may permit more intense cellular interactions with *H. pylori.* While kidney transplant recipients have a high prevalence of *H. pylori* infection, active gastritis was clearly less frequently seen in these patients than in control subjects and peptic ulceration was totally absent. Current evidence suggests that prevalence of *H. pylori* colonization is higher in renal graft recipients on triple posttransplant immunosuppression (82%) than in the normal population. In dyspeptic transplant and dialysis patients, colonization with *H. pylori* do not account for development of active inflammatory lesions, an association frequently seen in subjects free from renal disease but on immunosuppressive therapy [54].

3.2. Polymorphonuclear granulocytes (PMN) and macrophages in *H. pylori*-associated inflammation

Biopsies with regenerative changes show a marked PMN infiltration, regardless of the *H. pylori* status. In biopsies without regenerative changes, however, *H. pylori* colonization is closely associated with PMN infiltration [55]. As evidenced by *in vitro* and *in vivo* studies inflammation surrounding ulcers is caused mainly by acid, but *H. pylori* is another important factor in the development of PMN infiltration. Thus, high priority is being placed on studies of this major cellular factor, focusing on understanding the mechanisms used by *H. pylori* and the stomach mucosal-associated immune system to induce disease.

Interestingly, about one-third of the *H. pylori* strains induce strong and rapid chemiluminescence in PMN even without serum opsonins; for other strains complement is required, although even then the reactions remain at a lower level. The ability of some nonopsonized *H. pylori* to activate PMN shows co-variation with their ability to produce cytotoxin, but these two properties seem to be independent markers of peptic ulcer

disease. The activating and agglutinating property is bound to the cells, heat labile, and sensitive to several enzymes but resistant to acid. Strains possessing such activity are more common in patients with peptic ulcer disease than in patients with active chronic gastritis only [56, 57].

Coating the bacteria with IgG and IgM results in complement activation liberating C5a. Consequently, there is a massive granulocyte infiltration leading to local reduction or eradication of *H. pylori* [58]. Current *in vitro* evidence suggests that in the absence of complement, remarkable amounts of *H. pylori* remain extracellularly attached even after 120 min of coincubation, as well as internalized bacteria remain morphologically largely unimpaired. If complement is present, internalization and morphological destruction of *H. pylori* are significantly enhanced. Decrease of lysosomes, in general, parallels the degree of microbial uptake. Myeloperoxydase staining experiments, furthermore, show an obviously regular consumption of lysosomal granules. However, if complement opsonization is excluded, lysosomal degranulation is not accompanied by a corresponding degradation of *H. pylori*, the latter indicating an at least partial resistance to phagocytosis caused by microbicidal mechanisms. Ingested *H. pylori* are surrounded by a rather tight phagosome. A possible explanation for this phenomenon could be a leakage of the phagosomal membrane, possibly caused by membranotoxic ammonia produced by the organism. If such an impairment of the phagocytic action would occur *in vivo*, it could lead to an impaired cellular defense, and therefore contribute to the chronic course of *H. pylori* infection [12, 59].

H. pylori synthesizes and secretes a substance, probably FMLP (N-formyl-methionyl-leucyl-phenylalanine) that may account for part of PMN accumulation that accompanies *H. pylori* infections. Immune complexes composed of *H. pylori* antigen and specific antibody potentiate the PMN oxidative burst. Significantly, mucosal recognition of the CagA protein by IgA was associated with the activity of gastritis characterized by PMN infiltration and the presence of ulceration. This finding may explain why peptic ulceration develops in only a proportion of subjects infected with *H. pylori* [60].

In contrast to this finding discrepant results have been reported as to the association between the presence of serum IgG to CagA and degree of PMN infiltration and peptic ulcer disease [28]. *H. pylori* induced chemotactic activity for PMN may also be initiated by mucosal absorption of urease [61].

Another factor that may also have a bearing on inflammation is the release from mucosal neutrophils of reactive oxygen species (ROS) that is believed to be an important part of the pathogenesis of *H. pylori*-associated gastritis and duodenal ulcer. In *H. pylori*-positive patients the neutrophil response toward the homologous strain is in general absent. In contrast, a significant response can be observed toward the reference strain. In addition, on stimulation of neutrophils from patients without *H. pylori* infection, ROS release is significant. The stimulatory effect on neutrophils by FMLP was comparable in the two groups. It is concluded that a specific neutrophil hyporesponsiveness in ROS release toward the homologous *H. pylori* strain exists. This feature appears to be unique to *H. pylori*, not being described previously for neutrophil responses to any human pathogen [62, 63].

A recent article shows that the *in vitro* property of *H. pylori* reported may have relevance to the *in vivo* disease process. As evidenced by intravital microscopy *H. pylori* extract both increases leukocyte adherence and emigration and microvascular albumin leakage and elicits perivenular mast cell degranulation and the formation of platelet-leukocyte aggregates within post-capillary venules. The early phase of albumin leakage can be attenuated by pretreatment with a mast cell stabilizer. The late phase of albumin leakage can be reduced by monoclonal antibodies against P-selectin, CD11b/CD18 or ICAM-1 [64].

As it is clear from the aforementioned combinations of *H. pylori* derived products, host PMN and antibodies are involved in the mucosal damage observed in *H. pylori*-associated gastritis.

Recent comparative studies have confirmed that eosinophils appear to comprise part of the inflammatory reaction both in acute and chronic *H. pylori* gastritis. In antral gastritis significantly greater eosinophil infiltration and degranulation can be found compared to the normal group. The severity of chronic gastritis is significantly correlated with the eosinophil score. Eosinophil degranulation does not appear to be greater at or near sites of bacterial colonization in the *H. pylori* gastritis specimens. Furthermore, geometric mean of eosinophil cationic protein concentrations are more than 13 times higher in gastric juice from *H. pylori*-positive than from *H. pylori*-negative subjects [7]. The release of toxic cationic proteins from eosinophils may contribute to the inflammatory changes present in *H. pylori* gastritis [65]. Another study has indicated that eosinophil infiltration into the mucosa correlate with the extent of mucosal atrophy [8, 66, 67].

Observations of macrophage system suggest that whole *H. pylori* and the extracted LPS induce expression of the macrophage surface antigen HLA-DR and Il-2 receptors, production of the inflammatory cytokines IL-1 and tumor necrosis factor and secretion of the reactive oxygen intermediate superoxide anion. Thus, activation of resident lamina propria macrophages and monocytes trafficking through the mucosa could play an important role in mediating the inflammatory response associated with *H. pylori* gastritis and duodenitis. Also, *H. pylori* is capable of activating human monocytes by an LPS-independent as well as an LPS-dependent mechanism. [68].

3.3.The putative role of cytokines in *H. pylori*-associated inflammation

Chronic antral gastritis following *H. pylori* infection is characterized by a cellular inflammatory infiltrate, the cytokines of which may represent a host-dependent factor influencing the outcome of the infection. The nature of the mucosal cytokine response varies with the immunohistology of the disease [69, 70].

Although tumour necrosis factor (TNF) production by peripheral blood mononuclear cells in response to *H. pylori* stimulation is depressed in *H. pylori* positive individuals, its production by cells isolated from gastric mucosa during short-term culture is significantly higher in *H. pylori* positive patients than in negative patients, reflecting infiltration of T lymphocytes and macrophages within the local mucosa [71]. Levels of

interferon (INF) gamma do not differ significantly in the gastric explant culture from the two groups. Antral biopsies from *H. pylori*-infected patients show IFN-gamma, TNF-alpha, and IL-12, but not IL-4 mRNA expression, whereas no cytokine mRNA signal can be found in the mucosa of controls [72].

Platelet activating factor (PAF) is known as a potent ulcerogenic agent. In a study it has been revealed that lyso PAF, the immediate PAF precursor, is produced by gastric mucosa in basal condition. *H. pylori* seems to metabolize lyso PAF to produce PAF which in turn stimulates gastric acid secretion via specific receptor on the parietal cells. In addition to that, *H. pylori* also appears to produce PAF by itself [73].

Studies with *H. pylori* on biopsies and cell cultures have shown that there is a site dependent variation in epithelial IL-6 and IL-8 expression and IL-8 concentrations in active gastritis are significantly correlated with the extent of epithelial surface degeneration. *H. pylori* directly increases gastric epithelial IL-8 mRNA expression in a strain specific manner. Induction of epithelial IL-8 by CagA/cytotoxin positive strains are likely to result in neutrophil chemotaxis and activation and thus mucosal damage. This observation on epithelial IL-8 may explain the association between CagA/cytotoxin positive strains and gastroduodenal disease. *H. pylori* stimulates the gastric epithelium to initiate inflammation and PMN recruitment. In *H. pylori* positive patients with active gastritis and neutrophil infiltration into the epithelium IL-8 secretion is significantly increased relative to subjects with normal mucosa, inactive gastritis and reactive gastritis. IL-8 concentrations in active gastritis are significantly correlated with the extent of epithelial surface degeneration. It is of a note that IgA autoantibodies are present in most patients and their concentrations are significantly correlated with IL-8 production. The local production of IgA antibodies to IL-8 may represent a down-regulatory response of the host to limit mucosal damage associated with chronic bacterial infection [74].

As it is clear from previous observations, IL-4 is a type 2 cytokine which has a negative immunoregulatory role in human infection. The increased IL-4, however, does not seem to directly contribute to suppressed lymphocyte proliferation in *H. pylori* infection. Further studies will determine the role of IL-4 in other aspects of down-regulation of immune response in *H. pylori* infection [69, 75].

Another important cytokine, IL-1beta mainly released from activated macrophages may be involved in the hypergastrinaemia associated with *H. pylori*-associated gastritis [76].

Evidence suggests that there is a significant correlation between the LTB4 level in the mucosa and the degree of gastritis evaluated histologically. The LTB4 level in mucosa infected with *H. pylori* is higher than that in noninfected mucosa [77].

Recent studies have confirmed that human monocytes and lymphocytes cultivated in the presence of *H. pylori* porins release cytokines. Release of the various cytokines such as TNF alpha, IL-3, IL-4, IL-6, IL-8, GM-CSF, gamma INF can be obtained with differentiated kinetics and at various porin concentrations. Thus, during *H. pylori* infection, surface component porins are able to induce a series of chain reactions ranging from the inflammatory to the immunological responses [78].

3.4. Cellular immunity in *H. pylori* infection

In the chronic stage of *H. pylori* infection an increase in the number of lymphocytes, plasma cells and macrophages is evident. The infection is also associated with both a local and systemic specific cellular immune response [1, 79]. However, the contribution of T cell responses to host defenses to *H. pylori* is little understood.

Many studies have shown that both B and T lymphocytes are present in *H. pylori* infection, but among T cells those bearing the CD8 marker appear to be overrepresented [80]. Data also show that CD4+ mucosal lymphocytes can, however, selectively accumulate in *H. pylori*-associated chronically active antral gastritis. In addition, there is a strong increase in the number of activated CD3DR, CD22, and CD25 lamina propria lymphocytes in the majority of biopsies. Furthermore, T cells in the biopsies and in the lines obtained from the biopsies comprises a peculiar double positive CD4+CD8+ subpopulation [81]. Examinations have also revealed that CD4+ T cell clones specific for *H. pylori* from peripheral blood samples and gastric mucosa of infected patients are MHC class II restricted and discriminate between several cytotoxic and noncytotoxic bacterial strains. Localization of these cells at the site of disease suggests they are effectors of the immune response to the bacteria [82, 83].

It has been demonstrated that *H. pylori* bacteria contain both stimulatory and inhibitory components for T cells of healthy individuals. The surface haemagglutinin as well as crude surface fractions can induce proliferative response of the T lymphocytes. By contrast, cytoplasmic fractions can inhibit, in dose dependent manner, the proliferation of T cells in the cultures stimulated with *H. pylori* or PHA. It is supposed that *in vivo* a dominance of activation or immunosuppression could depend on the concentration of the bacteria and their products in infective foci [84–86]. A recent study has shown that CD4+ T cell clones derived from *H. pylori*-infected patients well proliferate in response to a *H. pylori* lysate; some clones also react with CagA, VacA, and urease. The majority of *H. pylori*-reactive clones produce IFN-gamma, but not IL-4 or IL-5. All studied *H. pylori*-specific CD4+ clones secrete high levels of TNF-alpha. At low T:B cell ratio, *H. pylori*-specific clones express antigen-dependent helper function for B cell proliferation and immunoglobulin production, whereas at higher T:B cell ratios, some Th1 and Th0 clones can lyse antigen-pulsed autologous EBV-transformed B cells. The results provide evidence for *H. pylori*-specific Th1 effectors in the gastric antrum of *H. pylori*-infected patients, where they may play a role in the genesis of either peptic ulcer or *H. pylori*-associated gastric B cell lymphoma [83]. In concert with these observations *H. pylori* infection and/or immunization stimulate a predominantly Thl-type, antigen-specific response and promote a local delayed-type hypersensitivity in the stomach that may be inhibited by depletion of gamma INF [82]. Furthermore, it has been reported that while the production of INF-gamma and IL-2, which are type 1 cytokines, is decreased in *H. pylori* infection, an enhanced production of type 2 cytokines (IL-4 and IL-6) is observed in individuals with *H. pylori* infection. Suppressed proliferative responses of peripheral blood and gastric lymphocytes have also been demonstrated in patients with *H. pylori* colonization, suggesting that specific T-cell responses may be down-regulated by an enhanced Th2 reaction [87]. Current evidence suggests that there is a significant reduction

in peripheral blood mononuclear cell (PBMC) proliferation and a reduced INF secretion in response to *H. pylori* antigen in patients with *H. pylori* colonization compared to *H. pylori*-negative individuals. These findings indicate a defective T cell response to *H. pylori* antigens in *H. pylori*-positive individuals. These data might suggest prior encounter with and elimination of *H. pylori* in *H. pylori*-negative individuals. These findings could also reflect antigen-specific suppression of T cell proliferative responses in the peripheral circulation [69, 82]. A finding consistent with the latter shows that the *in vitro* proliferation of PBMC to PPD, PHA, and ConA can be reduced by inactivated *H. pylori* as well as by a soluble cytoplasmic fraction of *H. pylori*. The suppressor activity was non-dialyzable, heat-labile and sensitive to trypsin. An influence on monocytes rather than on T cells is the major cause of the observed suppressive effect. More recently it has been demonstrated that fractionated peripheral mononuclear cells (MNC) from individuals seronegative for *H. pylori* antibodies respond well to low „doses" of live *H. pylori* or cytoplasmic antigens. However, no proliferation is observed in MNC cultures containing higher doses of live *H. pylori* or cytoplasmic antigens. Moreover, higher „doses" of the bacteria or cytoplasmic antigens entirely inhibited the response of T cells to PHA [88].

Data on gastric gamma/delta T cells in *H. pylori*-associated gastritis are controversial. Intraepithelial gamma/delta T cells may play a role in host defense against invading *H. pylori*, and the bacteria may trigger an autoimmune response to stress proteins expressed by the gastric epithelial cells [84, 89, 90].

Interestingly, cells of the immune system in the lamina propria proved to be the only cells that showed detectable mRNA for histamine, muscarinic, gastrin, and dopamine receptors by *in situ* hybridization histochemistry. None of the epithelial cells express any of these mRNAs. Thus, the targets of antiulcer drugs seem to be cells of the immune system in the stomach and not parietal cells. These findings indicate that the agents used to treat ulcer disease may act on immunocytes in the lamina propria ant not on parietal cells [91].

3.5. Humoral immune response to *H. pylori*

Specific serum antibodies, chiefly IgG, directed against *H. pylori* are nearly universal during infection. After antimicrobial treatment of *H. pylori* infections, levels of specific serum antibodies decline, reflecting elimination of the antigenic focus [43, 92, 93]. Specific immunoglobulins in gastric juice provide further evidence of their pathogenetic role.

A serologic study has shown that the presence of seropositivity against *H. pylori* is strongly correlated with the presence of autoantibodies against human antral gastric mucosa. IgG autoantibodies can be detected in the majority of infected patients but in none of the patients without infection and gastritis. The autoimmune reaction involves mainly the luminal surface of glandular cells and secretory canaliculi of parietal cells. The autoantibody status correlates with the presence and degree of inflammation and atrophy of the glands. The grade of antigenic mimicry of the infecting *H. pylori* strain appears to play a role in the progression of chronic gastritis to atrophy. Thus, *H. pylori* appears to

stimulate antibodies cross-reacting with gastric autoantigens and that this immunologic mechanism may represent a pathogenic link between *H. pylori* and gastritis [94, 95].

Evidence of IgA, IgG and IgM antibodies as well as of lysozyme in the mucosa are demonstrated by immunohistochemical methods. *H. pylori* is coated by antibodies and a significant correlation between extent of opsonization and the number of plasma cells in the connective tissue of the lamina propria could be stated. In the depths of gastric pits the antibody-coating of bacteria is faint. Instead, lysosome and lactoferrin are produced there. Lagging behind, the inflammation follows the motile bacteria resulting in a patchy distribution of inflamed areas in the mucosa. At the peak of local inflammation-waves the production of antibodies and lysozyme is intensified [58]. Serum anti-*H. pylori* IgG antibody titers are significantly correlated with the severity of inflammation in both the antrum and body. Significant associations are found between serum anti-*H. pylori* IgG and IgA antibody titers and the development of atrophic gastritis [96].

A number a studies have pointed out that the decreasing IgG, IgA and IgM antibody titres after eradication of *H. pylori* have diagnostic value. A long-term serological surveillance after treatment of *H. pylori* infection showed that six weeks after treatment IgG titres fall by 20–30%. In the bacteria-negative patients the decrease continued and 6 and 12 months after treatment the titre was 50% or less of the pretreatment value in 97% of these patients. The high sensitivity of the IgG antibody tests and a consistent fall within 6 months after eradication of *H. pylori* infection make IgG the most useful Ig class for follow-up of antimicrobial therapy in individual patients. Furthermore, duodenal ulcer patients compared with non-ulcer dyspepsia patients were tended to have higher serum IgG anti-*H. pylori* and significantly higher gastric juice IgA anti-*H. pylori*. Concentrations of both serum IgG, anti-IgG, anti-*H. pylori* and gastric juice IgA anti-*H. pylori* tended to be higher in patients with positive ulcer history but no present ulcer compared with patients with florid ulcer disease [92].

Antibody titers against CagA correlate with severity of disease, suggesting a role for the CagA protein and the coexpressed cytotoxin in the pathogenesis of *H. pylori* infections [23, 24, 98]. Furthermore, circulating IgG antibodies to CagA are associated with gastric atrophy, as well as peptic ulcer disease [19, 99].

Since the early 1970s there has been increasing clinical and experimental evidence of the capacity of allergic reactions to evolve into chronic inflammatory responses. In fact, the antigen and mast cell-bound IgE leads not only to the rapid release of vasoactive mediators but also to the so-called late-phase reaction, characterized by the involvement of inflammatory cells. The infiltrate consisting of PMN is an early event in such a reaction, followed by a later mononuclear cell infiltration. The histologic picture of *H. pylori*-associated gastritis is quite consistent with that observed in allergic reactions. Indeed, the biopsy specimens containing the bacterium are characterized by the presence of a PMN infiltrate in the context of a diffuse mononuclear cellularity, and eosinophil leukocytes have been observed in association with the bacterium [100]. Recent data have revealed that the majority of *H. pylori* positive patients are positive for basophil-bound specific IgE and serum specific IgE. Histamin release occurs through an IgE-dependent mechanism. When normal basophils, passively sensitized with serum from IgE-positive patients, are exposed to the *H. pylori* antigen, a significant release is observed, confirming the class specificity. The ability of *H. pylori* to induce a specific IgE immune response

could answer key questions regarding the mechanisms inducing gastric inflammation. In addition to this, another paper has shown that one fifth of the patients present total IgE serum levels above 200 kU/l. A relapse of the ulcer after 6 months of maintenance therapy was observed in 39.5% of the patients with ulcer and high total IgE as against the 11.9% observed in patients with normal IgE [101]. These data lend support to the hypothesis of an underlying immuno-allergic reaction in some forms of gastric or duodenal ulcer. It is of importance that some features of *H. pylori*-associated gastritis such as edema, vasodilation, inflammatory cell infiltration are typical of the histamine-mediated response. In *in vitro* systems *H. pylori* really potentiates histamin release induced by bile acids or calcium ionophore, or compound 48/80 [102]. On the contrary, no significant histamine release was obtained when incubating any *H. pylori* preparations alone with mast cells [103]. Another proof in this line is that specific IgE to food and inhalants allergens have been found in 28% of peptic ulcer patients and in 4% of controls. The duodenal bulb was the site in which most frequently IgE have been found. No relevant effects were observed after incubation of specific allergens with gastric or duodenal mucosa biopsies containing specific IgE. A defect of the gastric or duodenal epithelial barrier, which permit a passage way for proteins with subsequent IgE production in the submucosa, appears to be the cause of localization of specific IgE in stomach and duodenum [104].

4. Vaccination

H. pylori infection persists for life if not treated, and is responsible for major morbidity and mortality throughout the world. Considering the high cost of drug therapy, the appearance of antibiotic resistant strains, and the failure of drug therapy to prevent reinfection, preventive immunization is now considered by many to be the only practical approach to large-scale elimination of the bacterium from the population. Success hinges on determining the extent to which immunologic memory can be evoked in the stomach, and the ability to identify antigens and delivery systems which stimulate protective immunity rather than disease-promoting responses. High rates of protection have been achieved in the *H. felis* mouse model, utilizing antigens ranging from whole cells to purified recombinant proteins. Probably the most significant finding in the early vaccine studies was immunization of already infected mice resulting in a cure of *H. pylori* infection [105, 106].

Recently, fresh clinical isolates of *H. pylori* was adapted to colonize the stomach of mice. A gastric pathology resembling human disease was observed in infections with cytotoxin-producing strains but not with noncytotoxic strains. Oral immunization with purified *H. pylori* antigens protected mice from bacterial infection. This mouse model will allow the development of therapeutic agents and vaccines against *H. pylori* infection in humans [107]. Furthermore, twenty-six per cent of mice immunized with *H. pylori* antigen plus nontoxic cholera toxin were protected against *H. felis* challenge, confirming the value of the model in predicting effects of immunization in humans. This observation opens the way for human studies with *H. pylori* vaccines [108]. Purified or recombinant

urease enzyme [109, 110] remains the favourite antigen but other *H. pylori*-derived antigens and adjuvants have been used in animals [79, 105, 106, 111]. New combinations will most likely be required in the future. In most studies where single antigen was used, protection rates ranged from 50–90% which were nearing 100% by using combined vaccines [112]. Furthermore, recently it was reported another novel approach that harnessed adhesin analogues for preventing *H. pylori* from colonization. Characterization of the adhesins of *H. pylori* could lead to the development of adhesin analogues for use in the inhibition of colonization and improved therapy for ulcer disease. *In vivo* studies with isogenic mutants which are incapable of adhering to the gastric mucosa would greatly clarify the significance of adherence. Such mutants could possibly be useful as a vaccine against infection with wild-type organisms [113].

A priority is to define alternate mucosal adjuvants, as some used in the animal models may be too toxic for use in humans. Also, there is a need to understand the basis of immunization. Why does the natural immune response to *H. pylori* fail while the artificially stimulated response succeeds? Taking into account that over the next few years antimicrobial resistance is likely to be an increasing problem in the treatment of *H. pylori* infections, the time appears to be ripe for the completely new approach of therapeutic immunization [114–119].

5. Speculations about the role of *H. pylori* in the immunopathogenesis of specific upper gastrointestinal pathology

Chronic superficial gastritis may progress over decades to atrophic gastritis. *H. pylori* infection is highly associated with each stage of this progression leading to the hypothesis that gastric inflammation may be advantageous for *H. pylori*. Inflammation, with disruption of mucosal barriers, may facilitate the release of nutrients into the mucus gel. The apparent predominance of CD8 lymphocytes, eosinophil cells, anti-cytokine antibodies suggest that the host may be attempting to down-regulate the exuberance of the inflammatory response. Successful down-regulation could be defined as occurring in infected persons who no longer have neutrophils in the lesions because pyogenic infection is in general a most destructive host-pathogen interaction. However, even a mild inflammation without a major contribution of PMN disrupts epithelial function and therefore is deleterious to the host. Superficial gastritis may persist for years and therefore may represent a long-term equilibrium between a host's inability to remove a noxious stimulus and ability to contain the damage. In the presence of the chronic *H. pylori* infection, antibodies to host-specific epitopes may develop and contribute to the pathological process. Clearly the presence of activated T cells in the host unable to eradicate a particular antigenic focus may be deleterious. It is probably important to take into consideration that some of the T cell abnormalities that are reported in *H. pylori*-associated diseases (and other inflammatory processes) might be related to an interplay of many inflammatory cytokines initiated by antigen-antibody reactions. Thus, in this peculiar chronic inflammation caused by *H. pylori* where antibodies to *H. pylori* and autoantibodies have been detected, it might be the B cell that is playing a pivotal role in

initiating inflammation and leading to a host of downstream events such as participation of T cells introducing new inflammatory pathways and further escalating pathogenic mechanisms [120].

Regarding the possible part of inflammatory and immune reactions against *H. pylori* in the development of peptic ulcer there are two theories. According to the mediator washdown theory cytokines and other inflammatory mediators wash down to the duodenum during gastric emptying damaging duodenal mucosa. This theory is compatible with the fact that *H. pylori* is a noninvasive bacteria, and that *H. pylori* gastritis results in the release of a variety of mediators. However, mediator washdown alone may be insufficient for ulcerogenesis. Thus acid-induced mucosal injury may be a prerequisite to *H. pylori*-associated ulcerogenesis. The immunologic damage theory states that mucosal injury is caused by a continued immune response that fails to eradicate organisms. In support of this hypothesis is the presence of increased number of PMNs, T and B cells within the mucosa and evidence of local Ig production [121].

In conclusion, the regulation of inflammation after *H. pylori* infection may have important consequences with respect to gastric function. Two key factors may be (i) the adequacy of the host to suppress the inflammatory response to *H. pylori* and its products and (ii) the effect of the residual inflammatory process on the physiology of gastrin-mediated production of hydrochloric acid. Careful characterization of the immunopathology of *H. pylori* infection and the pathophysiology of the disordered gastric acid regulation may enable prediction in individual hosts of the natural history of this chronic infection.

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SAPROPHYTIC AND CYCLOHEXIMIDE RESISTANT FUNGI ISOLATED FROM GOLDEN HAMSTER

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Healthy hair samples from golden hamsters were examined for the presence of dermatophytes and non-dermatophytes using baiting technique and direct inoculation. Thirty-four species and 2 varieties attributed to 17 genera were recovered. *Paecilomyces variotii* (isolated from 84.4% of the examined hair) and *Aspergillus niger* (81.3%) were the more frequent isolates on Sabouraud's dextrose agar (SDA) without cycloheximide. Our results have clearly demonstrated that the hair of hamster was free from true dermatophytes. Using the dilution plate method many fungal species were isolated from cage material (7 genera and 10 species+1 variety); from faeces (10 genera and 17 species); from standard chow (3 genera and 6 species) of hamster. *P. variotii* which was the most frequent fungus in the preceding 3 substrates was completely absent in the presence of cycloheximide in SDA. The present study has demonstrated for the first time the isolation of *Trichophyton rubrum* from hamster faeces. Also, several saprophytic and cycloheximide resistant fungi were isolated. In the air of hamster cage *Cladosporium cladosporioides, Penicillium chrysogenum, Alternaria alternata* and *Scopulariopsis brevicaulis* were the most dominant species on SDA with or without cycloheximide.

Using the agar diffusion method, Aloe sap, onion oil, garlic bulb extract and aqueous leaf extracts of *Andropogon citratus, Euphorbia* sp. and *Ruta graveolens* were tested for their antifungal activity on 10 fungal species. It was observed that onion oil exhibited a high inhibitory effect against most of the tested fungi.

The skin of animals is contaminated by numerous fungi, some of which are opportunistic pathogens or allergens. Several investigations have reported the occurrence of dermatophytes on the apparently healthy skin of domestic and wild animals [1–3].

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Also, animal pens and animal faeces represent a good habitat for keratinophilic and saprophytic fungi [4, 5].

The objective of this work was to determine:

1. The existence of dermatophytes and non-dermatophytes in the hair of hamster used for biomedical research.

2. Occurrence of these fungi in cage material, faeces and standard chow of hamster.

3. Incidence of these fungi in air of hamster's cage.

4. Antifungal activity of the extracts of six plants against some fungal species.

Materials and methods

Animals. Adult male golden hamsters *(Mesocricetus auratus)* weighing approximately 110 g were obtained from Schistosoma Biological Supply Program, Theodore Bilhars Research Institute, Imbaba, Egypt. The animals were maintained for at least 4 weeks before experiments in temperature controlled room (24±3 °C). Light was provided by Philips 40 watt fluorescent tubes between 06.00 and 20.00 h (light:dark 14:10). The hamsters received standard laboratory chow and tap water.

Isolation medium. Sabouraud's dextrose agar [6] supplemented with antibiotics (Chloramphenicol - 0.05 mg/ml and cycloheximide - 0.05 mg/ml and without cycloheximide) were used.

Isolation of fungi from hamster's hair. Thirty-two samples of hamster hair were collected. These samples were placed separately in clean polyethylene bags and then transferred directly to the laboratory and kept in a cool place (3–5 °C) till fungal assay. All samples were tested with 10% KOH for direct examination. Two different techniques were used: Hair baiting as recommended by Vanbreuseghem [7]. Pieces of hair were sprinkled on the surface of double sterilized soil. The soil was moistened with sterilized distilled water and remoistened whenever necessary and incubated at room temperature for up to 4 weeks. The mould which appeared on the baits were transferred onto agar medium without cycloheximide.

The other technique was direct plating of the hair onto the agar media. Plates were incubated at 28 °C for 10–21 days and the cultures were examined periodically for fungal growth.

Isolation of fungi from cage, faeces and chow of hamster. Samples of cage material (wood shavings was the sole constituent of hamster cage), faeces and chow were collected. The dilution plate method [8] was employed. Ten plates (five plates for each medium) were used for each sample and were incubated for 7–15 days. The developing fungi were identified and counted per gram dry samples.

Determination of air borne fungi in hamster's cage. Ten plates (five for each medium) of 9 cm diameter were used for each exposure. The plates were exposed to the air in hamster's metal cage weekly at 11 a.m. for 10 min. The plates were incubated at 28 °C for 7–15 days during which the developing fungi were calculated per 20 plates in 4 exposures. The following literatures were used for identification: Raper & Thom [9] for *Penicillium,* Carmichael [10] for *Chrysosporium,* Raper & Fennell [11] for *Aspergillus,* Ellis [12] for *Dematiaceous hyphomycetes,* Frey et al. [13] for pathogenic fungi and Domsch et al. [14] for other genera.

Antifungal preparations. For the in vitro studies, *Aloe* leaf sap, onion *(Allium cepa* L.*)* oil (from El-Nasr Company, Egypt), garlic *(Allium sativum* L.) bulb extract and aqueous leaf extracts, prepared as mentioned by Hasan & Abdel-Mallek [15] of each of *Andropogon citratus* Hort, *Euphorbia* sp. and *Ruta graveolens* L. were screened for their activities against 10 fungal species *(Trichophyton rubrum, Chrysosporium keratinophilum, Scopulariopsis brevicaulis, Acremonium strictum, Alternaria*

alternata, Aspergillus niger, A. flavus, A. fumigatus, Paecilomyces variotii and *Penicillium chrysogenum).* The agar diffusion technique [16] was employed with some modification. One ml portions of spore suspension of each tested fungus were pipetted in sterilized plates followed by the addition of 20 ml of Sabouraud's dextrose agar. After solidification similar holes (5 mm) were made in the agar plates with cork borer. 0.2 ml extract of each material to be tested was placed inside the holes. The antifungal agent trosyd (1%, manufactured by Pfizer, Egypt) was used as standard. Three plates were used for each plant and for trosyd per fungus species. Cultures were incubated at 28 °C for 10–15 days, after which the inhibition zones around the holes were measured. The relative inhibitory effect of the extracts was calculated as % of the inhibition exerted by trosyd.

Results

Fungi isolated from hamster's hair. Thirty-four fungal species and 2 varieties which belong to 17 genera were isolated using hair baiting and direct plating techniques (Table I).

Results by hair baiting technique. A total of 9 genera, 15 species and 1 variety were collected. *Aspergillus niger* (59.4% of the examined hair), *Paecilomyces variotii* (56.3%) and *A. flavus* (50.0%) were highly frequent. Three species showed low incidence viz: *A. fumigatus* (18.8%), *Penicillium citrinum* and *Rhizopus stolonifer* (12.5%, each). The remaining species (9 species+1 variety) were less frequent as listed in Table I.

Results by direct plating technique. On Sabouraud's dextrose agar (SDA) without cycloheximide 21 species and 1 variety were isolated (Table I). *Paecilomyces variotii* and *A. niger* were isolated in high frequency. They emerged from 27 and 26 out of 32 hair samples, respectively. *A. fumigatus* was isolated from 8 samples. Four fungal species showed low incidence viz: *Cladosporium cladosporioides* (6 samples, 18.8% of the samples), *A. flavus* (5 samples, 5.6%), *Alternaria alternata* and *Penicillium corylophilum* (4 samples, 12.5%, each). The remaining fungal species (15 species+1 variety) occurred rarely in hamster's hair.

Twenty-three cycloheximide resistant species were recovered. *A. niger* was the most common fungus species, occurring in 50% of the hair samples. *A. flavus, P. chrysogenum* and *C. cladosporioides* were recovered in moderate frequency, (43.8%, 34.4% and 31.3% of the hair samples, respectively). *A. sydowii* (21.8%) and *P. citrinum* (18.8%) were low frequent. Sixteen species were isolated with rare frequency including *Chrysosporium keratinophilum, C. inops, Paecilomyces variotii, Acremonium strictum* and *Alternaria alternata* (3.1% of the examined hair).

Fungi isolated from hamster's cage. Wood shavings was the sole constituent of the hamster's cage. The results of Table II show that the total fungal count isolated on SDA without cycloheximide was 1191.9 colonies (calculated per g dry sample) whereas on SDA with cycloheximide was 244.7 colonies. Seven genera and 10 species in addition to 1 variety were recovered on SDA without cycloheximide. *Paecilomyces variotii* was the most common fungus. *A. niger* was the second frequent species accounting for 17.2%.

Table I

Incidence of fungi in hamster's hair on Sabouraud's dextrose agar at 28 °C

Species	Hair baiting technique			Direct plating technique					
	S			S			S+Cy		
	NCI	%	OR	NCI	%	OR	NCI	%	OR
Acremonium strictum W. Gams	–	–	–	–	–	–	1	3.1	R
Alternaria alternata (Fr.) Keissler	–	–	–	4	12.5	L	1	3.1	R
A. tenuissima (Kunze ex Pers.) Wiltshire	–	–	–	1	3.1	R	3	9.3	R
Aspergillus candidus Link	–	–	–	–	–	–	1	3.1	R
A. flavus Link	16	50.0	H	5	15.6	L	14	43.8	M
A. flavus var. *columnaris* Raper & Fennel	1	3.1	R	–	–	–	–	–	–
A. fumigatus Fresenius	6	18.8	L	8	25.0	M	–	–	–
A. niger van Tleghem	19	59.4	H	26	81.3	H	16	50.0	H
A. ochraceus Wilhelm	1	3.1	R	–	–	–	–	–	–
A. sydowii (Bain. & Sart.) Thom & Church	1	3.1	R	3	9.3	R	7	21.8	L
A. ustus (Bain.) Thom & Church	–	–	–	–	–	–	2	6.2	R
A. versicolor (Vuill.) Tirab.	–	–	–	–	–	–	2	6.2	R
Candida spp.	–	–	–	1	3.1	R	3	9.3	R
Chaetomium globosum Kunze ex Fries	–	–	–	–	–	–	1	3.1	R
Chrysosporium inopes Carmichael	–	–	–	–	–	–	1	3.1	R
C. keratinophilum (Frey) Carmichael	1	3.1	R	1	3.1	R	1	3.1	R
Cladosporium cladosporioides (Fres.) De Vries	1	3.1	R	6	18.8	L	10	31.3	M
C. herbarum (Pres.) Link ex Gray	–	–	–	1	3.1	R	–	–	–
C. sphaerospermum Penz.	–	–	–	1	3.1	R	–	–	–
Drechslera spicifera (Bain.) von Arx	–	–	–	–	–	–	1	3.1	R
Fusarium moniliforme Scheldon	1	3.1	R	–	–	–	–	–	–
Geotrichum candidum Link	–	–	–	1	3.1	R	–	–	–
Humicola grisea Traaen	1	3.1	R	–	–	–	–	–	–
Paecilomyces variotii Bain.	18	56.3	H	27	84.4	H	1	3.1	R
Penicillium brevicompactum Dierckx	–	–	–	–	–	–	1	3.1	R
P. chrysogenum Thom	3	9.3	R	1	3.1	R	11	34.4	M
P. citrinum Thom	4	12.5	L	3	9.3	R	6	18.8	L
P. corylophlilum Dierekx	3	9.3	R	4	12.5	L	1	3.1	R

(Continued on p. 199.)

Table I (continued)

P. decumbens Thom	–	–	–	–	–	–	1	3.1	R
P. duclauxii Delacrolx	–	–	–	2	6.2	R	–	–	–
P. frequentans Westling	–	–	–	2	6.2	R	–	–	–
P. funiculosum Thom	–	–	–	1	3.1	R	–	–	–
P. goldewskii Zaleski	–	–	–	1	3.1	R	–	–	–
Penicillium spp.	2	6.2	R	2	6.2	R	3	9.3	R
Rhizopus stolonifer (Ehrenb. ex Fries) Lind	4	12.5	L	2	6.2	R	1	3.1	R
Scopulariopsis brevicaulis (Sacc.)	1	3.1	R	2	6.2	R	–	–	–
Sterile mycelium	–	–	–	1	3.1	R	1	3.1	R
Talaromyces flavus var *flavus* (Klocker) Stolk & Samson	–	–	–	2	6.2	R	–	–	–
Trichothecium roseum (Pers.) Link	–	–	–	1	3.1	R	1	3.1	R
Number of species	15+1 varie			22+1 Varie			23		
Total number of species	34+2 variety								

S=Sabouraud's dextrose agar medium, S+Cy=Sabouraud's dextrose agar medium + cycloheximide (Actidione), NCI=Number of cases of isolation (out of thirty-two hair samples, %=Percentage of occurrence (calculated/32 samples), OR=Occurrence remarks, H=High occurrence (more than 16 samples), M=Moderate occurrence (between 8–15 samples), L=Low occurrence (between 4–7 samples), R=Rare occurrence (less than 4 samples)

The following five fungal species could not be isolated on SDA without cycloheximide but appeared on SDA supplemented with cycloheximide: *Chrysosporium keratinophilum* (77.8 colonies, 31.8% of total fungal count), *Geotrichum candidum, P. citrinum, P. jensenii* (11.1 colonies, 4.5%, each) and *F. monilliforme* (3.6 colonies, 2.3%).

Fungi isolated from hamster's faeces. A total of 10 genera, 17 species of saprophytic fungi were isolated from hamster's faeces on SDA (Table II). These numbers were higher than those isolated on SDA with cycloheximide (7 genera and 8 species). *Paecilomyces variotii* was the most frequent fungus, accounting for 67% of total fungi on SDA without cycloheximide, whereas it was completely absent on SDA with cycloheximide. *Candida* was isolated in 19.7% and 63.2% of total fungal count on the above mentioned media.

Six cycloheximide resistant species were isolated from hamster's faeces namely: *Chrysosporium keratinophilum* (6.7%), *Trichophyton rubrum, Fonseacae compactum, Gymnoascus reessii, Aspergillus ustus* and *Penicillium citrinum* (3.4%, each).

Fungi isolated form hamster's chow. From chow of hamster, six fungal species were recovered on Sabouraud's agar viz: *Paecilomyces variotii* (44.4 colonies/g dry sample, 30.7% of total fungal count), *A. niger* (33.3 colonies, 23%), *A. flavus, A. fumigatus, A. ustus* and *Cladosporium cladosporioides* (16.7 colonies, 11.6%; each).

Whereas *P. chrysogenum, A. flavus, P. citrinum* and *Rhizopus stolonifer* were isolated on SDA with cycloheximide. They occurred in 56.6%, 23.3%, 10.0% and 10.0% of total fungal count, respectively.

Table II

Count (per g dry sample) and percentage count (calculated to the total count) of fungal species isolated from cage material, faeces and chow of hamster

Species	Cage				Faeces				Chow			
	S		S+CY		S		S+CY		S		S+CY	
	C	%	C	%	C	%	C	%	C	%	C	%
Alternaria alternata	–	–	–	–	5.6	0.5	–	–	–	–	–	–
Aspergillus flavipes (Bain. & Sart.) Thom & Church	–	–	–	–	11.1	1.0	–	–	–	–	–	–
A. flavus	27.8	2.3	27.8	11.4	11.1	1.0	16.7	10.0	16.7	11.6	38.9	23.3
A. flavus var. *columnaris*	5.6	0.5	16.7	6.8	–	–	–	–	–	–	–	–
A. fumigatus	–	–	–	–	5.6	0.5	5.6	3.4	16.7	11.6	–	–
A. niger	17.2	–	–	10.6	0.9	–	–	33.3	23.0	–	–	6
A. terreus Thom	5.6	0.5	–	–	5.6	0.5	–	–	–	–	–	–
A. ustus	–	–	–	–	–	–	5.6	3.4	16.7	11.6	–	–
A. versicolor	–	–	–	–	5.6	0.5	–	–	–	–	–	–
Candida spp.	–	–	–	–	19.7	63.2	–	–	–	–	3	5
Chrysosporium keratinophilum	–	–	77.8	31.8	–	–	11.1	6.7	–	–	–	–
Cladosporium cladosporioides	5.6	0.5	16.7	6.8	5.6	0.5	–	–	16.7	11.6	–	–
Drechslera spicifera	5.6	0.5	–	–	–	–	–	–	–	–	–	–
Fonsecae compactum Carrlon	–	–	–	–	–	–	5.6	3.4	–	–	–	–
Fusarium moniliforme	–	–	5.6	2.3	5.6	0.5	–	–	–	–	–	–
Geotrichum candidum	–	–	11.1	4.5	5.6	0.5	–	–	–	–	–	–
Gymnoascus reessii Baran	–	–	–	–	–	–	5.6	3.4	–	–	–	–
Mucor hiemalis Wehmer	5.6	0.5	–	–	–	–	–	–	–	–	–	–
M. racemosus Fresenius	–	–	–	–	5.6	0.5	–	–	–	–	–	–
Paecilomyces variotii	71.5	–	–	67.0	–	–	44.4	30.7	–	–	8	6
Penicillium chrysogenum	11.1	0.9	66.7	27.3	5.6	0.5	–	–	–	–	94.4	56.6
P. citrinum	–	–	11.1	4.5	–	–	5.6	3.4	–	–	16.7	10.0
P. corylophilum	38.8	3.3	–	–	11.1	1.0	–	–	–	–	–	–
P. frequentans	–	–	–	–	38.9	3.4	–	–	–	–	–	–

(Continued on p. 201.)

MAGYAR TUDOMÁNYOS AKADÉMIA KÖNYVTÁRA

Table II (continued)

P. funiculosum	–	–	–	–	–	5.6	–	–	–	–	–	–
P. jensenii Zaleski	–	–	5.6	4.5	5.6	0.5	–	–	–	–	–	–
Penicillium spp.	16.7	1.4	–	–	–	–	–	–	–	–	–	–
Rhizopus stolonifer	–	–	–	–	5.6	0.5	–	–	–	–	16.7	10.0
Syncephalastrum racemosum (Cohn) Schroeter	11.1	0.9	–	–	–	–	–	–	–	–	–	–
Sterile mycelium	–	–	5.6	4.5	5.6	0.5	–	–	–	–	–	–
Trichophyton rubrum (Castenani) Sabouraud	–	–	–	–	–	–	5.6	3.4	–	–	–	–
Gross total count	1191.9		244.7		1127.9		166.9		144.5		166.7	
Number of species	10+1 variety		8+1 variety		17		8		6		4	
Total number of species	27+1 variety											

C=Count of fungi
%=Percentage count of fungi
S=Sabouraud's dextrose agar medium
S+Cy=Sabouraud's dextrose agar medium + cycloheximide

Table III

Count (per 20 plates in 4 exposures) and percentage count of air borne fungi hamster's cage

Species	S		S+C	
	C	%	C	%
Acrophialophora fusispora (Saksena) Samson	1	0.5	–	–
Alternaria alternata	8	3.7	16	26.2
Aspergillus flavus	7	4.8	6	9.8
A. flavus var. *columnaris*	1	0.5	1	1.6
A. niger	50	22.9	–	–
A. ochraceus	1	0.5	–	–
A. sydowii	3	1.4	–	–
A. ustus	1	0.5	1	1.6
A. versicolor	5	2.3	–	–
Botryotrichum piluliferum Sacc. & Marcb	6	2.8	–	–
Candida spp.	3	1.4	–	–

(Continued on p. 202.)

Table III (continued)

Cladosporium cladosporioides	66	30.3	8	13.1
C. herbarum	1	0.5	–	–
Drechslera spicifera	1	0.5	–	–
Fusarium moniliforme	2	0.9	1	1.6
F. solani (Mart.) Sacc.	1	0.5	–	–
Gliocladium roseum Bain.	1	0.5	–	–
Mucor racemosus	1	0.5	–	–
Paecilomyces variotii	19	8.7	–	–
Penicillium brevicompactam	–	–	4	6.6
P. chrysogenum	14	6.4	11	28.8
P. citrinum	–	–	7	11.5
P. corylophilum	2	0.9	–	–
P. duclauxii	1	0.5	–	–
P. funiculosum	2	0.9	–	–
Penicillium spp.	4	1.8	1	1.6
Rhizopus stolonifer	6	2.8	–	–
Scopulariopsis brevicaulis	1	0.5	4	6.6
Sterile mycelium (dark colour)	7	4.8	–	–
Torula herbarum pers. ex gray	2	0.9	–	–
Trichothecium roseum	1	0.5	–	–
Ulocladium alternariae (Cke.) Simnons	–	–	1	1.6
Gross total count	218		61	
Number of species	25+1 variety		10+1 variety	

C=Count of fungi
%=Percentage count of fungi
S=Sabouraud's dextrose agar medium
S+Cy=Sabouraud's dextrose agar medium + cycloheximide

Air borne fungi in hamster's cage. Air borne fungi were determined using two agar media (Sabouraud's dextrose agar with or without cycloheximide). The results of Table III reveal that the number of genera and species obtained on SDA with cycloheximide (7 genera, 10 species and 1 variety) was markedly lower than that obtained on SDA free from cycloheximide (16 genera, 25 species and 1 variety). Similarly the count of total fungi on SDA with cycloheximide (61 colonies/20 plates for 4 exposures) was lower than that on SDA without cycloheximide (218 colonies). However, the most dominant species on both media are alike e.g. *Cladosporium cladosporioides, Penicillium chrysogenum, Alternaria alternata, Aspergillus flavus* and *Scopulariopsis brevicaulis.* In addition to the above-mentioned species 19 fungal species were isolated only on SDA without cycloheximide including *A. niger* (22%), *Paecilomyces variotii* (8.7%) and *Botryotrichum piluliferum* (28%).

Antifungal activity. The results of Table IV show the in vitro antifungal activity of some natural compounds against 10 fungal species. It was noticed that onion oil was the most active against *Paecilomyces variotii* (144.3% inhibition compared with trosyd), *Scopulariopsis brevicaulis* (141.5%), *Aspergillus fumigatus* (139.5%), *Alternaria alternata* (133.3%), *Aspergillus flavus* (121.9%) and *A. niger* (111.4%). However, *Acremonium strictum* and *P. chrysogenum* were inhibited with onion oil less than trosyd. Also, it was observed that *Aloe* sap and garlic extract showed better antifungal activity against *Acremonium strictum, A. niger* and *Scopulariopsis brevicaulis.* On the other hand, *Paecilomyces variotii* was affected with garlic extract more than trosyd, whereas it was not affected by *Aloe* sap. The above 3 mentioned plants showed less antifungal activity against *Trichophyton* and *Chrysosporium keratinophilum.*

The remaining aqueous leaf extracts were ineffective against all tested fungi.

Table IV

Average diameter of inhibition zone (mm) of Aloe leaf sap, aqueous garlic bulb extract and onion oil on ten fungal species[a]

Species	Diameter mm of inhibition zone			
	Aloe sap extract	Garlic	Onion oil	Trosyd
Trichophyton rubrum	22 (69.8)[b]	22 (69.8)	23 (73)	31.5
Chrysosporium keratinophilum	8.2 (33.3)	20 (81.6)	22 (89.8)	24.5
Scopulariopsis brevicaulis	22 (135)	22 (135)	23 (141)	16.25
Acremonium strictum	23 (104.5)	23.3 (106)	20 (90.9)	22
Alternaria alternata	10 (33.3)	14 (46.7)	40 (133.3)	30
A. niger	20 (101.3)	21 (106)	22 (111.4)	19.75
A. flavus	–	8 (39)	25 (121.9)	20.5
A. fumigatus	–	–	30 (139.5)	21.5
Paecilomyces variotii	–	22 (144.3)	22 (144.3)	15.25
Penicillium chrysogenum	–	–	12.5 50	25

[a] None of the aqueous extract of *Andropogon citratus*, *Euphorbia* sp. and *Ruta graveolens* inhibited growth of test fungi

[b] The number between parentheses indicates the percentage inhibition of growth comparable with that of trosyd

Discussion

In the present study, three different techniques (hair baiting, direct inoculation and dilution plate) and Sabouraud's dextrose agar with or without cycloheximide (Actidione) were employed. This indicates that a careful examination of the hair, cage material, faeces

and chow of hamster by the use of different types of techniques and media gives a better idea about the real fungal content of these samples and allows the isolation of a wide spectrum of fungi. The mycological analysis of hamster hair revealed the isolation of 34 species and 2 varieties which belong to 17 genera of saprophytic fungi. Bagy & Abdel-Mallek [17] isolated 23 genera and 53 species from the hair of small mammals (rabbits, guinea pigs, mice, cats and rats). In this respect, Aho [18] isolated the saprophytic fungi with suspected dermatophytes from the hair of domestic and laboratory animals (dog, cat, horse, cow, guinea pig, parakeet, goat, rat, lesser panda and mink). The commonest isolated genera in order of frequency were *Penicillium, Cladosporium, Aspergillus, Mucor, Aureobasidium, Alternaria, Scopulariopsis* and *Trichothecium.* He also suggested that the presence of saprophytic fungi on hair and skin creates an opportunity for them under special circumstances to become invasive to the skin or hair and thus cause primary or secondary infection. In the present study, *Paecilomyces variotii* and *Aspergillus niger* were recovered with high frequent either by using the hair baiting or direct inoculation techniques. They occurred in 84 and 81% of the examined hair, respectively. Twenty-three cycloheximide resistant fungal species were obtained from hamster's hair of which *A. niger* (50% of the samples) was the most common one, *A. flavus, P. chrysogenum* and *C. cladosporioides* were moderately isolated. Whereas, *Chrysosporium inops, C. keratinophilum, Paecilomyces variotii, Acremonium strictum* and *Alternaria alternata* were recovered with less frequently. Moubasher et al. [19] isolated 47 species and twenty-five genera of non-dermatophyte cycloheximide resistant fungi from patients of skin diseases. Of which *Penicillium* and *Aspergillus* were the most abundant followed by *Scopulariopsis, Alternaria, Thermoascus, Chrysosporium* and *Cladosporium.* Our results have clearly demonstrated that hamster's hair was free from true dermatophytes. However, Stenwig [20] isolated *Microsporum canis* from hamster but he reported that *M. canis* infection in the hamster should not be unexpected. In a review by Dvorak and Otcenasek [21] *Trichophyton mentagrophytes* was listed as the only dermatophyte isolated from this species. In the same review, more than 30 animals, hosts, among them rodents, were considered to be susceptible to *M. canis* infection. In Egypt, most of the preceding genera and species were isolated previously from healthy hair of dog, donkey and cow [22], from camel and goat [23], from small mammals [17], from human axillary hair [24] and from diseased camels [25].

From metal cage material (wood shavings) of hamster 7 genera, 10 species and 1 variety were recorded on SDA without cycloheximide. *Paecilomyces variotii* (71.5% of total fungi) was the most common fungus followed by *A. niger* (17.2%). Whereas Moharram et al. [26] isolated *Paecilomyces variotii* in rare frequency from animal and bird pens. They also reported that *A. niger, A. fumigatus, A. flavus, A. terreus, A. sydowii, S. brevicaulis, P. chrysogenum* and *P. funiculosum* were the most common species in animal and bird pens. Also, Hubalek et al. [27] isolated *A. flavus, A. fumigatus, A. niger, A. sydowii, A. versicolor* and *Penicillium* spp. from bird's nests in the nest boxes.

Five fungal species were recovered only on SDA plus cycloheximide viz: *Chrysosporium keratinophilum* (31.8% of total fungal count), *Geotrichum candidum, P. citrinum, P. jensenii* (4.5%, each) and *F. moniliforme* (2.3%). Hubalek et al. [27] isolated, *C. keratinophilum* (22.8%) from birds' nest in boxes. Pugh & Evans [28] reported that

Chrysosporium spp. collectively represented 32% of the isolates from 59% of the nests. They also reported that *Chrysosporium* spp. were much more common in the nests than in the soils. These fungi were also isolated from animal and bird pens after baiting the samples with sterile camel wool [29] and from chicken and wild sparrow nests [30].

A total of 10 genera, 17 species of saprophytic fungi were isolated from hamster's faeces on SDA without cycloheximide. *Paecilomyces variotii* was also the most frequent species (67% of total fungal count) followed by *Candida* (19.7%). Bagy et al. [31] isolated 14 genera and 38 species from camel dung on glucose agar, among them *Paecilomyces variotii* was less frequent. Seven cycloheximide resistant fungi were isolated viz. *Candida* (63.2%), *Chrysosporium keratinophilum* (6.7%), *Trichophyton rubrum, Fonsecae compactum, Gymnoaseus reessii, A. ustus* and *P. citrinum* (3.4%, each).

It is worth mentioning that the demonstration of *T. rubrum* from hamster's faeces does not seem to have been reported earlier. Currah [32] reported that *Arthroderma* and *Nannizzia* contain both saprophytic species found on dung and in soil enriched with keratin, and species that cause ringworm.

The following fungal species were recovered from hamster's chow on SDA: *Paecilomyces variotii* – the most common one (30.7% of total fungal count) – *A. niger* (23%), *A. flavus, A. fumigatus, A. ustus* and *Cladosporium cladosporioides* (11.6%, each). Ogundero [33] isolated *A. candidus* (16% of the samples) and *A. fumigatus* (50%) from poultry feeds. On the other hand, most of these fungi were isolated from poultry feedstuff ingredients [34].

Five cycloheximide resistant species were the most dominant species in air of hamster's cage and these were *Cladosporium cladosporioides, P. chrysogenum, Alternaria alternata, Aspergillus flavus* and *Scopulariopsis brevicaulis.* Della Franca & Caretta [35], isolated *A. flavus, Alternaria alternata, Cladosporium* (6 species), *Penicillium* (3 species) and many others which were resistant to antibiotic from the air at pavia, Italy. In addition to the previous species 19 fungal species were isolated only on SDA without cycloheximide among them *A. niger, Paecilomyces variotii* and *Botryotrichum piluliferum.* Bagy [36] in Egypt also recovered these fungi from the air of chicken's pens.

From the preceding results and discussion it can be concluded that *Paecilomyces variotii* was the most dominant fungus species in the air and in the hair, cage faeces and chow of hamster on SDA without cycloheximide. However, it was completely absent in the presence of cycloheximide. This clearly indicate that this species was more sensitive to the antibiotic cycloheximide.

When the antifungal activity of *Aloe* sap, onion oil, garlic bulb extract and the aqueous leaf extracts of 3 plants was tested it was noticed that onion oil exhibited a high inhibitory effect (comparable with trosyd) on the in vitro growth of *Paecilomyces variotii* (144.3%), *Scopulariopsis brevicaulis* (141.5%), *Aspergillus fumigatus* (139.5%), *Alternaria alternata* (133.3%), *Aspergillus flavus* (121.9%) and *A. niger* (11.4%). El-Shanawany [37] reported that the mycelial growth of *Scopulariopsis brevicaulis* was greatly suppressed by 100, 200, 400 ppm of onion oil. However, *A. niger* was not significantly affected at any level of onion oil.

Aloe sap and garlic extract show better antifungal activity against *Acremonium strictum, Aspergillus niger* and *Scopulariopsis brevicaulis.* Yashida et al. [38] observed

that the growth of both *A. niger* and *Candida albicans* was inhibited by ajaene (derived from garlic) at <20 µg/ml. On the other hand El-Shanawany [37] noticed that *Scopulariopsis brevicaulis* was not significantly affected by any concentration (1000, 2000, 4000 ppm) of garlic extract. In the present study, *Paecilomyces variotii* was affected with garlic extract more than trosyd, whereas it was not affected with *Aloe* sap.

The above 3 mentioned plants showed less antifungal activity against *Trichophyton rubrum* and *Chrysosporium keratinophilum.*

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FOLLOW UP OF CLINICAL, LABORATORY, AND SEROLOGICAL FINDINGS OF ADULT HUNGARIAN HOSPITALIZED ACUTE HEPATITIS PATIENTS AND CHARACTERISTICS OF RECOVERY

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Follow-up features of acute viral hepatitis were evaluated of 210 hospitalized adult patients. HA, HB, HC, and NON A-C H were diagnosed in 68 patients, 84 patients, 22 patients, and 36 patients, respectively.

Post-hepatitis syndrome was shown in about 20.6%, 19%, 45.5%, and 25% of patients with HA, HB, HC, and NON A-C H, respectively.

At three months, the recovery was shown in about 61.7%, 69%, 13.6%, and 63.9% of patients with HA, HB, HC, and NON A-C H, respectively. Factors of sex, age, and severity of acute phase had no effect on the protracted rate in all types, except in HB.

After six months, the disease remained active in 1.5%, 6%, 69%, 8.3% of patients with HA, HB, HC, NON A-C H, respectively.

Factors of sex, age, severity of acute phase had no effect on the chronicity rate in different types, except HB. It was more significantly more frequent in males, elderly persons, and mild acute phase in chronic HB cases. Diabetes mellitus was also significantly more frequent in chronic HB cases.

Flat pattern of ALT elevation was significantly more frequent in chronic HB and NON A-C H cases.

The prognostic features of different types of acute viral hepatitis are differing.

Hepatitis A and E are self-limited diseases with good prognosis, despite several reports have been shown that, type A hepatitis may occasionally present a prolonged and polyphasic clinical course characterized by two or more relapse of aminotransferase values [1, 2].

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Hepatitis B, D, C and non-C, sometimes, may develop to chronic infection in different rates [3–6].

Acute phase in all types of hepatitis usually resolves in about 2–3 months, only in a small proportion may take a protracted course (>3 months) [7, 8].

In this study, age, sex, level of ALT and S.T.B elevation, pattern of ALT elevation, underlying diseases (when exist) were evaluated in order to determine whether these factors could be helpful in predicting the course or outcome of the disease.

Patients and methods

Patients. 210 adult patients were studied, who were admitted consecutively to Szent László hospital from 1 January 1993 till 1 July 1994 and diagnosed as acute viral hepatitis according to the diagnostic criteria of acute viral hepatitis (clinical, biochemical, serological) with absence of alcohol or drug consumption, biliary disease, and previously documented chronic hepatitis. Hepatitis A were diagnosed in 68 (34 males), hepatitis B in 84 (28 males), Hepatitis C in 22 (13 males), and NON A-C hepatitis in 36 (20 males).

Biochemical methods. Alanine aminotransferase (ALT), and serum total bilirubin (S.T.B) were measured by standard techniques. N.R.: ALT<40 U/L, AST<37 U/L, S.T.B<17.1 μmol/L

Serological methods. Acute hepatitis A was confirmed by the detection of the anti-HAV IgM, by use of Organon Teknika Hepanostika and Roche Cobas-Core HAV IgM tests.

Acute hepatitis B was defined by the concomitant presence of HBsAg and Anti-HBc IgM or anti-HBc IgM alone, using the Roche Cobas-Core, Automated HBsAg, and Organon Teknika Hepanostika, and Uniform II.

Acute hepatitis C was defined by detection of anti-HCV IgG, by use of second generation ELISA (Ortho and Ubi tests), acute Hepatitis C was confirmed by ratio of Optical Density/Cut Off (OD/CO)>2, and by exclusion of HAV, HBV, cytomegalovirus (CMV), Epstein-Barr virus (EBV), toxoplasmosis, and leptospirosis.

NON A-C hepatitis was diagnosed if there was no serological evidence of acute hepatitis A, B, C, and other likely causes of acute viral hepatitis.

Follow-up form. All the patients who had been discharged from the hospital were asked to contact the outpatient consulting department monthly (4–6 weeks) until normalization of liver functions. All follow up data (clinical, serological, and biochemical) were recorded according to a special form for six months. The first day of the follow-up study was the day of the release from the hospital.

Patterns of ALT elevation were classified according to the description of Tadeda and co-workers [9] as follows:

„Monophasic type" a rapid increase of the level of ALT to a maximum and then immediate decrease.

„Biphasic type" a rapid increase of the level of ALT to a maximum, rapid decrease, another increase , and then a gradual decrease.

„Flat type" a slight elevation of the level of ALT continuing for a long period.

„Typical course" was referred to acute viral hepatitis, which resolved within 3 months.

„Protracted course" was applied to infection with acute viral hepatitis persisting for more than 3 months.

„Relapsing course" was applied to infection with acute viral hepatitis which had a second elevation in values of liver function tests after discharge from the hospital.

„Resolving infection" was used for acute viral hepatitis that resolved within 6 months.

„Ongoing infection" (Chronic state) was used for acute viral hepatitis that persisted for >6 months by abnormal liver function tests.

Statistical analysis. The Chi-Square test was used to calculate P values for the differences between groups, and Student's t-test to calculate P values for the difference between means. A P value of 0.0.5 or less was considered as statistically significant for both tests.

Results

Hepatitis A. Follow-up features of HA patients are shown in Table I.

42 of 68 patients (61.8%) had a typical infection, as the infection entirely resolved within 3 months. The remaining patients 26 (38.2%) had a protracted infection, as the infection needed more than three month to resolve; of them 15 (22.1%) by prolonged course with abnormal liver function tests, 9 (13.25) by relapsing course, 2 (2.9%) by cholestasis course.

When sex and age, severity of acute phase (represented by maximum level of ALT and S.T.B), pattern of ATL elevation between the groups of typical infection and protracted infection were compared, only one significant difference was found in frequency of pattern of ALT elevation, as flat and biphasic patterns were significantly more frequent in the protracted infection group than in the typical infection group ($p<0.008$, and <0.00001, respectively).

Only, in one (1.5%) patient (female), the persisted infection (abnormal aminotransferase level) for more than six months. But entire recovery was observed even in this case within one year; anti-HAV IgM was negative after six months, other viral causative agents, alcohol, and drugs had been excluded.

Hepatitis B. Follow-up features of HB patients are shown in Table II.

Of 84 patients 58 (69%) had a typical infection, as the infection entirely resolved within 3 months, the remaining 26 (31%) had a protracted infection.

In 5 followed-up HB patients the infection remained ongoing for more than 6 months proved by abnormal liver function tests and persisting of HBsAg in the blood.

As shown in Table II, male, higher age, mild acute phase (by level of ALT), underling disease (diabetes mellitus), and flat pattern were significantly more frequent in ongoing infection than in the resolving infection group ($p<0.02$, $p<0.0003$, $p<0.05$, $p<0.00001$, and $p<0.0009$, respectively).

Hepatitis C. Follow-up features of HC patients are shown in Table III.

Of 22 patients only 3 (13.6) patients had a typical infection, as the infection resolved within 3 months by the recovery of liver function tests only, the remaining 19 (86.4%) had a protracted infection, shown by the persistence of pathologic liver function tests for more than three month.

In 15 (68.2%) patients the infection remained ongoing by abnormality of liver function and positivity of anti-HCV test for more than six months.

Table I

Follow-up features of hepatitis A in 68 patients

	Typical infection (≤3 months)	Protracted infection (>3 months)			
		Prolonged	Relapsed	Cholestatic	Total
No. (%)	42 (61.8)	15 (22.1)	9 (13.2)	2 (2.9)	26 (38.2)
M/F	(19/23)***	10/5	4/5	1/1	15/11
Mean age (Yrs)±SD*	(28.8±11.4)***	28.8±3.6	33±7.7	36.2±3.5	30.8±8.1
M.Mx.** ALT of acute phase (U/l)±SD*	(1759.1±1062)***	1529±1417.5	1644.1±1063.3	1766±106.7	1587.1±1221.9
M.Mx.** S.T.B of acute phase (μmol/l)±SD*	(122.5±88.9)***	133.6±70.8	111.3±53.7	392±166.9	151.6±101.1
ALT pattern no. (%)					
monophasic	42 (100)[a]	9 (60)	0 (0)	2 (100)	11 (42.3)
Biphasic	0 (0)	2 (13.3)	9 (100)	0 (0)	11 (42.3)[b]
Flat	0 (0)	4 (26.7)	0 (0)	0 (0)	4 (15.4)[c]

*: standard deviation

**: mean maximum

***: p=not significant if compared with relapsed and total protracted groups

a: $p<0.00001$ vs. total protracted group

b: $p<0.00001$ vs. typical group

c: $p<0.008$ vs. protracted group

When factors of sex and age, severity of acute phase (by level of ALT and S.T.B), and pattern of ALT elevation between the groups of resolving infection and ongoing infection were compared, no significant differences were found, except the pattern of ALT elevation, as monophasic pattern was significantly more frequent in the resolving infection group than in the ongoing infection group ($p<0.05$).

NON A-C hepatitis. Follow-up features of NON A-C H patients are shown in Table IV.

Of 36 patients 23 (63.9%) had typical infection according to the abnormality of liver function tests within 3 months, the remaining 13 (36.1%) had a protracted infection by persistence of liver function tests for more than three months.

In 3 (8.3%) patients the infection remained ongoing shown, by abnormality of liver function tests for more than six months.

When factors of sex and age, severity of acute phase (by levels of ALT and S.T.B), and pattern of ALT elevation were compared between the groups of resolving infection and ongoing infection, no significant differences were found, except distribution of the pattern of ALT elevation. Monophasic pattern was significantly more frequent in the

resolving infection group than in the ongoing infection group ($p<0.0001$). While flat pattern was significantly more frequent in the ongoing infection group than in the resolving infection group ($p<0.0001$).

Comparison of follow-up features among different types of acute viral hepatitis.

Follow-up features of different types of acute viral hepatitis are shown in Tables V and VI.

In the monthly normalization rate in liver function tests. Hepatitis C patients showed slow rate by significantly degree if compared with HA, HB, NON A-C H in second month, $p<0.00006$, $p<0.0001$, and $p<0.00008$, respectively. In third month, $p<0.00008$, $p<0.00001$, and $p<0.0001$, respectively, in fourth month, $p<0.00001$, $p<0.00001$, and $p<0.0001$, respectively, in fifth month, $p<0.00001$, $p<0.00001$, and $p<0.00003$, respectively. In sixth month, $p<0.000001$ versus all.

Table II

Follow-up features of hepatitis B in 84 patients

Time of recovery	(≤ 3 months)	(≤ 6 months)	(> 6 months)
No. (%)	58 (69)	79 (94)	5 (6)
M/F	14/44	24/55	(4/1)[a]
Mean age (Yrs)±SD*	45.1±9.4	45.5±4.7	(73±7.4)[b]
M.Mx.** ALT of acute phase (U/l)±SD*	2231±1257.7	2102.7±1188.6	(906±300.2)[c]
M.Mx.** S.T.B of acute phase (μmol/l)±SD*	219.6±124.2	277±119	(305.6±244.8)[NS]
Frequency of underlying disease (diabetes) no. (%)	0 (0)	5 (6.3)	4 (80) [d]
ALT pattern no. (%)			
monophasic	54(93.1)	64 (81)	3 (60)[NS]
Biphasic	4 (6.9)	12 (15.2)	0 (0)[NS]
Flat	0 (0)	3 (3.8)	2 (40) [e]

*: standard deviation
**: mean maximum
NS: p=not significant
a: $p<0.02$
b: $p<0.0003$
c: $p<0.05$
d: $p<0.00001$
e: $p<0.0009$ when compared with ≤ 6 months group

Table III

Follow-up features of hepatitis C in 22 patients

Time of recovery	(≤3 months)	(≤6 months)	(>6 months)
No. (%)	3 (13.6)	7 (31.8)	15 (68.2)
M/F	2/1	5/2	$(8/7)^{NS}$
Mean age (Yrs)±SD*	51.7±7.5	40.3±12	$(54.9±7.6)^{NS}$
M.Mx.** ALT of acute phase (U/l)±SD*	1007.7±449.1	1362.4±787	$(969.7±660.6)^{NS}$
M.Mx.** S.T.B of acute phase (μmol/l)±SD*	285.3±251.8	208.7±180.8	$(138.2±114.9)^{NS}$
ALT pattern no. (%)			
monophasic	7(100)	6 (100)	9 $(60)^{a}$
Biphasic	0 (0)	0 (0)	$3 (20)^{NS}$
Flat	0 (0)	0 (0)	$3 (20)^{NS}$

*: standard deviation
**: mean maximum
NS: p=not significant
a: $p<0.05$ when compared with ≤6 months group

Table IV

Follow-up features of non A-C hepatitis in 36 patients

Time of recovery	(≤3 months)	(≤6 months)	(>6 months)
No. (%)	23 (63.9)	33 (91.7)	3 (8.3)
M/F	15/8	18/15	$(2/1)^{NS}$
Mean age (Yrs)±SD*	51.5±19.5	50.6±18.1	$(49.7±20.8)^{NS}$
M. Mx.** ALT of acute phase (U/l)±SD*	1099.4±969.3	1159.3±861.6	$(820±401.1)^{Ns}$
M. Mx.** S.T.B of acute phase (μmol/l)±SD*	130.8±99.6	149.5±116	$(52.3±30.7)^{NS}$
ALT pattern no. (%)			
monophasic	23 (100)	32 (97)	1 $(33.3)^{a}$
Biphasic	0 (0)	0 (0)	$0 (0)^{NS}$
Flat	0 (0)	1 (3)	2 $(66.7)^{a}$

*: standard deviation
**: mean maximum
NS: p=not significant
a: $p<0.0001$ when compared with ≤6 months group

In the readmission rate, Hepatitis C patients group shown significantly higher rate than HB, $p<0.02$.

In the symptomatic rate, Hepatitis C patients group has shown significantly higher rate than HA and HB $p<0.02$ and $p<0.01$, respectively.

In the frequency of ALT pattern, NON A-C H group showed higher frequency in distribution of monophasic pattern by significantly degree if compared with HA, HB, HC groups, $p<0.03$, $p<0.04$, $p<0.01$, respectively, and shown lower frequency in distribution of biphasic pattern by significantly degree if compared with HA, HB and HC, $p<0.01$, $p<0.01$, $p<0.02$, respectively.

Table V

The monthly normalization rate of liver function tests in patients with different types of acute viral hepatitis during a 6 months period

	1st month no. (%)	2nd month no. (%)	3rd month no. (%)	4th month no. (%)	5th month no. (%)	6th month no. (%)
Hepatitis A (n=68)						
monthly rate	1 (1.5)	35 (51.4)	6 (8.8)	11 (16.2)	4 (5.9)	10 (14.7)
Cumulative rate	1 (1.5)	36 (52.9)	42 (61.7)	53 (77.9)	57 (83.8)	67 (98.5)
Hepatitis B (n=84)						
monthly rate	1 (1.2)	41 (48.8)	16 (19)	10 (11.9)	6 (7.1)	5 (6)
Cumulative rate	1 (1.2)	42 (50)	58 (69)	68 (80.9)	74 (88)	79 (94)
Hepatitis C (n=22)						
monthly rate	0 (0)	1 (4.5)	2 (9.1)	2 (9.1)	0 (0)	2 (9.1)
Cumulative rate	0 (0)	1 (4.5)[a]	3 (13.6)[b]	5 (22.7)[c]	5 (22.7)[d]	7 (31.8)[e]
Non A-C hepatitis (n=36)						
monthly rate	0 (0)	20 (55.6)	3 (8.3)	4 (11.1)	1 (2.8)	5 (13.9)
Cumulative rate	0 (0)	20 (55.6)	23 (63.9)	27 (75)	28 (77.8)	33 (91.7)

a: $p<0.00006$ vs. HA, $p<0.0001$ vs. HB, $p<0.00008$ vs. NON A-C H
b: $p<0.00008$ vs. HA, $p<0.00001$ vs. HB, $p<0.0001$ vs. NON A-C H
c: $p<0.00001$ vs. HA, $p<0.00001$ vs. HB, $p<0.0001$ vs. NON A-C H
d: $p<0.00001$ vs. HA, 0.00001 vs. HB, 0.00003 vs. NON A-C H
e: $p<0.000001$ in all, vs. HA, vs. HB, and vs. NON A-C H

Table VI

Follow-up features to 210 patients with different types of acute viral hepatitis

	HA patients (n=68) no. (%)	HB patients (n=84) no. (%)	HC patients (n=22) no. (%)	NON A-C H patients (n=36) no. (%)
Number of readmission to hospital	2 (2.9)	2 (2.4)	3 (13.6)[a]	2 (5.6)
Number of symptomatic patients	14 (20.6)	16 (19)	10 (45.5)[b]	9 (25)
ALT pattern				
monophasic	53 (77.9)	67 (79.8)	16 (72.8)	34 (94.4)[c]
Biphasic	11 (16.2)	12 (14.3)	3 (13.6)	0 (0)[d]
Flat	4 (5.9)	5 (5.9)	3 (13.6)	2 (5.6)

a: $p<0.02$ vs. HB.
b: $p<0.02$ vs. HA, $p<0.01$ vs. HB.
c: $p<0.03$ vs. HA, $p<0.04$ vs. HB, $p<0.01$ vs. HC.
d: $p<0.01$ vs. HA, $p<0.01$ vs. HB, $p<0.02$ vs. HC.

Discussion

Hepatitis A is usually a self-limited disease, benign, and resolves in six months. Similar to other studies [2, 8], majority of cases recovered within 3 months.

A repeated increase of aminotransferase levels was observed in 13% of HA patients. Therefore, the present study confirmed that relapse in HAV infection is a common feature and its occurrence is somewhat higher than observed in other studies [1, 2, 10]. Pathogenesis of this phenomenon remains unknown, despite many hypotheses, as a supposed defect in cell-mediated immune mechanism, or mutation in HAV, or superimposed infection with other viral agents [7, 11, 12].

Factors of sex, age, and severity of acute phase were found to have no effect on the course and outcome of the infection. However, patterns of ALT elevation, as biphasic, and flat ALT patterns seemed to be significantly more frequent in protracted courses than in typical courses.

In comparison with an other study [8], monthly recovery rate for HA patients in the present study seems to be slow. This may be however the consequence of the fact that the cited study, included children, too.

Consistently with recent reports, the present study has presented only one case, which had ongoing infection for more than six months [13, 14]. Pathogenesis of this course remains unclear. However, even in this case the infection resolved entirely within one year.

Most cases with HB usually recovered within three months, while some cases required more than three months to recover.

Monthly recovery rate of HB in the present study was similar to that published by others [8]. Chronic infection was observed in about 6% of our HB patients.

Factors of sex, age, severity of acute phase, existence of underlying disease (*Diabetes mellitus*), and pattern of ALT elevation were found to be associated with chronicity rate. Male gender, older age, mild acute phase, diabetes mellitus, and flat ALT pattern may predict the transition to chronicity.

However, the chronicity rate of HB in the present study is low in comparison with earlier results [15, 16]. This may be related with superinfection with Delta agent or other viruses before the development of the serological tests. On the other hand, chronicity rate of HB seems to be high, in comparison with other studies [4, 6, 17, 18]. This may be attributed to the lower mean age of patients. Other factors also may play a role in this difference, as HLA phenotype has been reported to possess influence on the chronicity rate of HB [19–21].

It is difficult in hepatitis C to decide whether the normalization of liver function tests represents a true recovery, or only a transient remission.

Few cases in the present study recovered within three months. This finding and the recovery rate were consistent with similar well-known data of literature [8, 21–25].

In consistence with other studies [3, 24, 26, 27], factors of sex, age, and severity of acute phase had no effect on the chronicity rate of Hepatitis C in the present study, except patterns of ALT elevation. Flat ALT pattern was significantly more frequent in the ongoing infection than in the resolving infection.

The chronicity rate of Hepatitis C seems to be higher compared with earlier other NANB hepatitis studies [8, 9, 21–23]. This discrepancy may be related to involved other viral hepatitides in early studies which have low chronicity rate. In the same time, the last finding corresponds with the results of recent studies [3, 5, 25, 28–30].

Factors of sex, age, and severity of acute phase had no effect on the chronicity rate of NON A-C hepatitis, except that flat ALT pattern may play a predicting role to the transition to the chronicity state.

The chronicity rate of NON A-C hepatitis is low if compared with chronicity rate of Hepatitis C in the present study. This finding confirms other data reported earlier [3, 5, 25, 31, 32].

The higher frequency of readmission of symptomatic patients of Hepatitis C that of other types of viral hepatitis during the follow-up, can be explained by the fluctuating course of this infection.

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Abbreviations: **HA**: Hepatitis A, **Anti-HAV IgM**: Antibody against A Hepatitis A virus of IgM type, **HB**: Hepatitis B, **HBsAg**: Hepatitis B surface antigen, **Anti-HBs**: Antibody against hepatitis B surface antigen, **Anti-HBcAg IgM**: Antibody against hepatitis B core antigen of IgM type, **NANBH**: Non-A, non-B hepatitis, **Anti-HCV**: Antibody against hepatitis C virus, **HC**: Non-A, non–B hepatitis which is positive to HCV serological test, **NON A-C H**: which is negative to anti-HAV, anti-HBc-Ag and anti-HCV ELISA, **ELISA**: Enzyme linked immunosorbent assay, **ALT**: Alanine aminotransferase, **S.T.B**: Serum total bilirubin.

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EFFECT OF TRACE ELEMENT COMBINATION ON THE IMMUNE RESPONSE OF RATS TREATED WITH CYTOSTATIC DRUG

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The effect of trace element combination, Béres Drop Plus (BDP) on the immune response of rats following single treatment with cytostatic drug (5-fluorouracil, 5-FU) was tested. Animals were treated with 5-FU and SRBC simultaneously, but separately. Rats were pretreated with BDP for 21 days. The body and spleen weight, furthermore the number of spleen cells and antibody producing cells were determined. The antibody titres in blood serum were also measured. The immune system of rats, 5 days following the 5-FU treatment, showed considerable regeneration. Pretreatment of rats with BDP had a beneficial effect on all parameters investigated.

In humans and experimental animals generalized malnutrition and also the deficiency in defined nutrients can influence the immune functions [1–5]. Antibody producing cell response decreased, susceptibility to microorganisms increased in copper deficiency [1, 5]. Furthermore copper deficiency induced splenomegaly and anemia and altered the lipid and protein composition of murin lymphocyte plasma membranes [1, 5, 6]. Zinc deficiency induces dysfunction of lymphocytes in humans [7]. Zinc supplementation influences the ratio of different lymphocyte subpopulations, normalize the CD_4/CD_8 ratio [8, 9]. Copper and zinc influence differently the interleukin production, zinc stimulated the Il-1 secretion and did not influence the Il-6 production whereas, at the same culture condition, copper inhibited the Il-1β and Il-6 secretion [10]. In experimental animals manganese deficient diet decreased antibody production [5].

Trace element combination, so-called Béres Drop Plus (BDP) seems to be useful in supplementation of trace element deficiency. BDP enhances the reticuloendothelial function of irradiated rats [11] and Il-6 production in human monocytes [12]. In earlier experiments [13] it was found that in rats, with intact immune system, all used doses of BDP could enhance the immune response. Especially the moderate high doses were effective. In rats, with sublethally irradiated immune system, the immunomodulating dose

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became narrow, only the dose of 250 µl/kg could enhance the immune response more considerably. These earlier results rise the question, whether BDP is able to enhance the immune response following any kind of immunosuppression. Extremely important is whether the immunosuppressive effect of cytostatica – used in human therapy – could be compensated by the use of BDP. The aim of the present work is, to answer this question under experimental circumstances.

Materials and methods

Animals. In the experiments female Wistar SOTE/NET (SNET/Br) rats were used. Animals (6-6 rats /groups) were kept in plastic cages of 30×40 cm basic area on softwood shavings. Animals were feed with rat-chow (Farmer Prompt KFT/BIOPLAN BT, Zagyvaszántó-Budapest) and water ad libitum.

Trace element treatment. The trace element preparation BDP (Béres Inc., Budapest, Hungary) contains the following inorganic components in coordinative binding to organic molecules in mg/60 drops (18 drops=1 ml): Fe (6.7), Zn (3.79), Na (2.14), Mg (1.35), Mn (1.02), K (0.924), Cu (0.848), Mo (0.634), V (0.406), Ni (0.362), B (0.35), F (0.301), Cl (0.099), Co (0.083). Organic components (mg/60 drops): glicerol (20.0), L-(+)ascorbic acid (10.0), EDTA (7.85), glicin (7.67), L-(+)tauric acid (5.21), succinic acid (1.67), 2,4,5,7-tetraiodofluorescein (0.0159), dissolved in water.

Rats were treated with 250 µl/kg BDP for 26 days. For use, BDP was diluted by distilled water. The appropriate dose was given daily once by stomac tube.

Other treatments. Animals were immunized on the 21th day of the BDP treatment with 4×10^8 sheep red blood cells (SRBC) intraperitoneally. Control animals were treated with SRBC only. As cytostaticum 100 mg/kg 5-fluorouracil (5-FU, Roche) was used, which on the other side of the peritoneum was given, on the 21th day of the BDP treatment, simultaneously with the immunization.

Determination of antibody production. Animals were bled on the 26th day of BDP treatment, and on the 5th day following 5-FU treatment and immunization.

Spleen cell suspensions were prepared from each animal in TC 199 medium. From the spleen cell suspension the number of antibody producing cells were determined by the plaque method according to Jerne et al. [14]. Briefly 0.1 ml of spleen cell suspension and 0.1 ml of 20 percent suspension of SRBC were mixed with agarose in TC 199 tissue culture medium at 49 °C, and it was placed on a performed agar plate. Following incubation at 37 °C for one hour without complement, and an other hour with complement, plates were incubated on room temperature for one hour. This incubation was followed to the next morning at 4 °C, when the number of PFC-s on the plates were determined. By this method the number of 19 S antibody producing cells were counted.

Serum samples were stored at –20 °C, and the haemagglutinin and haemolysin titres were determined by micromethod, according to Takátsy [15]. Briefly, the serum samples were serially diluted in microtiter plates and with equal volume of SRBC suspension and with or without complement incubated throughout one hour at 37 °C.

Statistical analysis. Experiments were repeated twice, and mean value ± SE and spleen index (spleen weight mg / body weight g) were calculated from the results of 12 rats in each group. The mathematical significance of the experimental results were calculated with ANOVA-test from the results of the following groups:

1. Control, immunized with SRBC
2. 5-FU treated + SRBC immunized
3. BDP pretreated, 5-FU treated + SRBC immunized

Results

Table I shows that in the BDP-pretreated group the decrease of body weight was less pronounced than that of the 5-FU-pretreated group. In the 5-FU treated rats a 15% depression of spleen weight could be seen, in the BDP + 5-FU treated group the value was about at the control level. The spleen index (spleen weight mg / body weight g) in the 5-FU treated animals was about as high as that of controls (Table I).

Table I

The effect of BDP and 5-FU treatment on the body weight, spleen weight and on the spleen index of SRBC immunized rats

Group	n	Body weight±SE, g	Spleen weight±SE, mg	mg/g ± SE
Control	12	284.58±7.67	669.10±28.16	2.35±0.07
5-FU	12	245.42±6.17	563.50±24.45	2.30±0.09
BDP+5-FU	12	262.92±5.09	633.68±24.12	2.41±0.90
P (control-5-FU)		0.01	0.05	–
P (control-BDP+5-FU)		0.1	–	–
P (5-FU-BDP+5-FU)		0.1	0.1	–

Rats were treated with BDP p.o., on the 21th day of the treatment they were treated with 5-FU and immunized with SRBC i.p. Parameters were determined on the 26th day of the BDP-, 5th day of the 5-FU treatment

Table II shows that 5-FU treatment reduced the number of spleen cells significantly. Table II also shows that this reduction was partially inhibited if the animals were pretreated with BDP.

For the evaluation of the number of antibody producing cells, following 5-FU treatment, the 5th day was not quite optimal, because at that time the number of antibody producing cells – as compared with that of the control – was elevated considerably.

When the results of BDP + 5-FU treatment to those of 5-FU treatment was compared, it could be seen that the BDP treatment significantly elevated the relative number of antibody producing cells (Fig. 1). The whole number of antibody producing cells of BDP + 5-FU treated rats was significantly elevated ($P<0.01$) as compared with the control and with the 5-FU treated animals. The density of antibody producing cells in the spleen was higher following 5-FU treatment than that in the control animals. In the BDP + 5-FU treated rats this elevation proved to be even more pronounced (Table III).

Table II

The effect of BDP and 5-FU treatment on the number and density of spleen cells in SRBC immunized rats

Group	n	10^7 spleen cell/ spleen±SE	10^7 spleen cell/ spleen±SE
Control	12	23.15±1.89	34.46±2.32
5-FU	12	16.69±1.23	29.98±2.29
BDP+ 5-FU	12	20.98±1.90	33.10±2.54
P (control-5-FU)		0.01	–
P (control-BDP+ 5-FU)		–	–
P (5-FU-BDP+ 5-FU)		0.1	–

For details see Table I

Table III

The effect of BDP and 5-FU treatment on the whole number and density of antibody producing cells (PFC) in the spleen of SRBC immunized rats

Group	n	PFC/spleen±SE	PFC/g spleen±SE
Control	12	104909±22247	155348±30162
5-FU	12	152281±43366	259610±69486
BDP+ 5-FU	12	272170±43590	437894±70132
P (control-5-FU)		–	–
P (control-BDP+ 5-FU)		0.01	0.01
P (5-FU-BDP+ 5-FU)		0.01	0.01

For details see Table I

The haemagglutinin titre was not influenced by 5-FU treatment, but in the BDP +5-FU treated animals an about 80% elevation could be observed, compared to the control or 5-FU treated animals (Table IV). The haemolysin titer was minimally elevated in the 5-FU treated animals, and BDP pretreatment induced a further elevation (Table IV).

Table IV

The effect of BDP and 5-FU treatment on the haemagglutinin and haemolysin titre in sera of SRBC immunized rats

Group	n	Haemagglutinin 2^{-n}±SE	Haemolysin 2^{-n}±SE
Control	12	6.06±0.40	9.52±0.52
5-FU	12	6.54±0.18	9.79±0.59
BDP+ 5-FU	12	7.42±0.42	10.27±0.66
P (control-5-FU)		–	–
P (control-BDP+ 5-FU)		0.05	–
P (5-FU-BDP+ 5-FU)		0.1	–

For details see Table I

Discussion

The immune system can be deeply destroyed by cytostatic drugs or irradiation. The irradiation-induced long-lasting and deep depression of immune functions can be only moderately influenced by treatments following irradiation, for example with endotoxin treatment [16].

It is known that the plasma zinc level is decreased in metastatic breast cancer bearing patients and also in the III or IV stage of cervical cancer [17, 18]. The Cu/Zn ratio of tumor bearing patients is increased significantly and may be used for monitoring the gynecological tumors [19]. It is furthermore well known that trace element deprivations depress the immune function [1–5]. This depression can be corrected in humans and animals by appropriate supplementation [20, 21]. Trace element supplementation can furthermore moderate the toxicity of heavy metals [22, 23].

In our earlier experiments the effect of trace element combination in normal and irradiated rats was investigated. In rats with intact immune system all used doses of BDP induced elevation of antibody production, but in irradiated rats the treatment with trace element combination was less effective. Only 250 µl/kg dose of BDP could elevate the immune response of animals [13].

We supposed that trace element combination possesses the ability to enhance the immune response following impairment. To prove this supposition the combined effect of BDP and cytostatic drug was tested. These experiments were important also from therapeutic view, because the immune system of tumor bearing persons suffer from the side effects of cytostatic drugs.

In the present experiments 100 mg/kg dose of 5-FU was used to destroy the immune system, simultaneously with the immunization. The used dose was higher, than

the dose used in murine [24, 25] experiments. In the human therapy 5-FU is used usually continuously.

To influence the effect of 5-FU a dose of 250 µl/kg of BDP was used, because in irradiated rats, this dose had a moderate immunoenhancing effect [13].

From the results it can be seen that BDP treatment has a beneficial effect on the immune response of rats treated with cytostatic drug. This effect is more pronounced than the effect of BDP in irradiated animals [13].

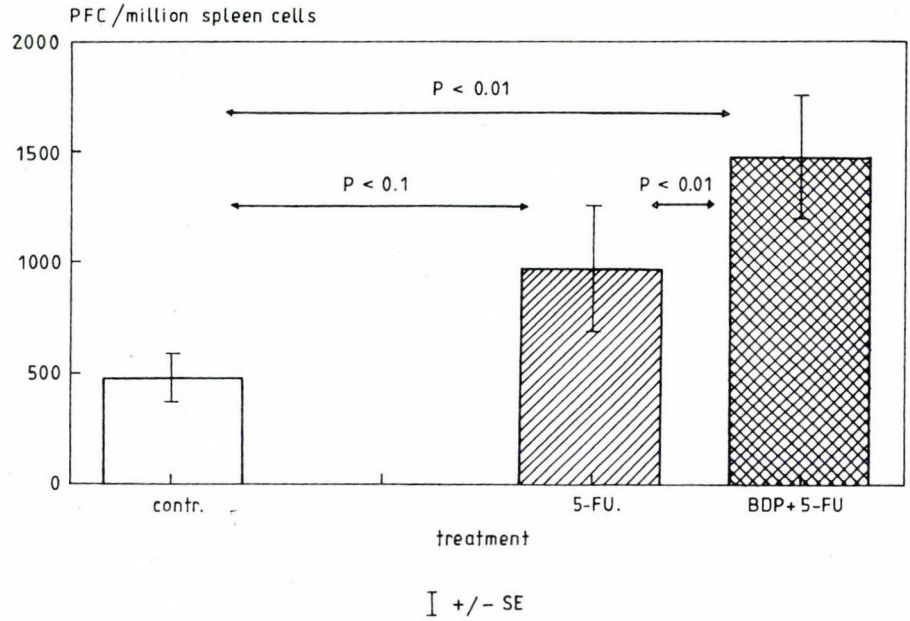

Fig. 1. The effect of trace element combination (BDP) and 5-FU treatment on the relative number of antibody producing cells in the spleen of rats.
Rats were treated with BDP p.o. permanently. On the 21th day of the treatment they were treated with 5-FU and immunized with SRBC i.p. separately. Control group was treated with SRBC only. Parameters were determined on the 26th day of the BDP-, 5th day of the 5-FU treatment and on the 5th day of the immune response

It can bee seen furthermore that BDP treatment influences the immune response differently following sublethal irradiation and cytostatic treatment. The base of this difference lay on the different effect of the destroying treatments. Following sublethal irradiation the spontan regeneration is only moderate, but 5 days following 100 mg/kg dose of 5-FU the regeneration is considerable. The moderate regeneration is coupled with moderate effect of BDP. A considerable regeneration is coupled with expressed effect of BDP. We suppose that certain regeneration of the immune system is necessary to the

beneficial effect of BDP. If the spontaneous regeneration achieves a level, the BDP treatment can induce further elevation of the productivity of immune system.

It follows that in patients, treated not only with irradiation but especially with cytostatic drug, BDP treatment can help the regeneration of the immune system. On the base of the experimental results we suppose that BDP treatment can have a beneficial effect on the immune response in patients treated with cytostatic drugs.

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EFFECT OF DIFFERENT CARBON SOURCES ON THE PRODUCTION OF AMYLASE BY *BACILLUS* SP. MD 124

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An extracellular, thermostable salt tolerant amylase has been obtained from *Bacillus* sp. MD 124 which was previously isolated from municipal garbage. Among the carbon sources used for amylase production, rhamnose, starch, glucose, lactose, galactose, maltose and sucrose favoured enzyme production whereas sorbose suppressed the enzyme production. Maximum amount of enzyme (15 unit/ml culture broth) was produced after 24 h of incubation at 45 °C in the medium containing 0.5% starch, at pH 6.5. The effect of temperature, pH as well as effect of metal ions on enzyme activity was also studied.

Thermostable amylases of microbiol origin are widely used in textile, food, paper and pharmaceutical industries. Several *Bacillus* species are potent producer of this enzyme, but few of them capable of secreting amylases active at high temperature [1–3].

The nature and amount of carbon source in the culture medium is an important factor for growth and production of extracellular amylase in bacteria [4]. Most of the cases starch considered an inducer for amylase production [5, 6]. Tigue et al. [7] mentioned that soluble starch was the most effective among the carbon sources tested far amylase production by the three different *Bacillus* spp. They also mentioned enzyme activity present with all carbon sources except glucose.

The present communication deals with the production of amylase by *Bacillus* sp. MD 124 in presence of different carbon sources. Some properties of the enzyme were also taken into consideration. It differs from others reported in the literature in a number of important aspects.

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Materials and methods

Bacterial strain and culture conditions. Previously isolated *Bacillus* sp. MD 124 [8] was maintained in nutrient starch agar slants. Inoculum was prepared by transferring several loopfuls from a fresh culture slants to 30 ml nutrient starch broth and incubated at 45 °C on a rotary shaker (200 rpm) for 14 h. Then the culture fluid was transferred aseptically to sterilized centrifuge tubes and spinned at 10,000 × *g* for 15 mins. The cell mass deposited in the centrifuge tube was washed with sterile distilled water twice and resuspended in 30 ml sterile water. Next to that 0.5 ml suspension was transferred to 250 ml Erlenmeyer flasks containing 30 ml of enriched medium composed of g/L; Starch, 5; KH_2PO_4, 3; $MgSO_4$, $7H_2O$, 0.5; KCl, 1; $CaCl_2$, $2H_2O$, 0.02; peptone, 10; beef extract. 5; cystin, 1; isoleucine, 1; riboflavin, 0.02; biotin, 0.02; pyridoxine, 0.05; and pH was adjusted at 6.5. Then the flasks were incubated in a rotary shaker at 45 °C for 28 h. To study the effect of different carbon sources on amylase production in fermentation broth various carbon sources at optimum concentration (0.5%) were substituted for soluble starch in the basal medium except glucose and sucrose (0.1%).

Partial purification of amylase. After 28h cultivation, the fermented broth was centrifuged to remove bacterial cells and was treated with 80% ammonium sulfate saturation and allowed to stand for overnight. The precipitate formed was collected by centrifugation (15000 × *g*, 20 min), dissolved in phosphate buffer (10 mM, pH 6.5) and dialysed against the same buffer for 24h at 4 °C.

DEAE cellulose column chromatography. The dialysate solution was put on a DEAE-cellulose column (3×10 cm) preequilibrated with 10 mM phosphate buffer, pH 6.5 and eluted with the same buffer. The flow through enzyme fractions were pooled. This partially purified enzyme solution was used to study the enzyme properties.

Assay of amylase. Saccharolytic amylase activity was assayed following the method of Bernfeld [9]. The reaction mixture consisted of 0.5 ml of 1% starch solution dissolved in 10 mM phasphate buffer (pH 6) and 0.1 ml of enzyme solution. After incubation at 90 °C for 5 min, reaction was stopped by the addition of 1 ml of 3,5-dinitrosalicylate reagent. Absorbance was measured at 540 nm. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of reducing sugar as glucose standard per min under the assay condition specified.

Growth. The growth of the organism was estimated on the basis of changes of optical density at 620 nm using a Klett summerson colorimeter.

Results and discussion

Enzyme production in relation to growth of the organism has been represented in Fig. 1. It has been found that enzyme production is directly proportional to the growth of the organism and both have reached their maxima at 24 h. Previously parallel growth and enzyme production have been observed by Coleman and Elliott [10] and Welker and Campbell [11] in *Bacillus subtilis* and *Bacillus stearothermophilus*, respectively.

The pH of the broth decreased sharply from 6.5 to 5.5 during the first h of growth after which it increased until it reached 8.7 at the end of the decline phase.

Growth of the *Bacillus* sp. MD 124 in presence of various carbon sources has been represented in Fig. 2. The maximum growth of the organism has been obtained at 16 h in all the investigated carbon sources except the media with galactose, starch and rhamnose. In presence of above-mentioned three carbon sources, organism showed highest growth at 24 h. Production of enzyme in presence of different carbon sources by the organism has

been studied and represented in Fig. 3. In all the cases maximum production of the enzyme were after 24 h growth i.e. at the decline phase. Our finding may be comparable with the findings of Medda and Chandra [12]. Maximum growth was obtained in case of galactose and starch, may be due to the better utilization of carbon sources. It has been also found that the level of growth resulting from the utilization of various carbohydrates did not effect the formation of amylase.

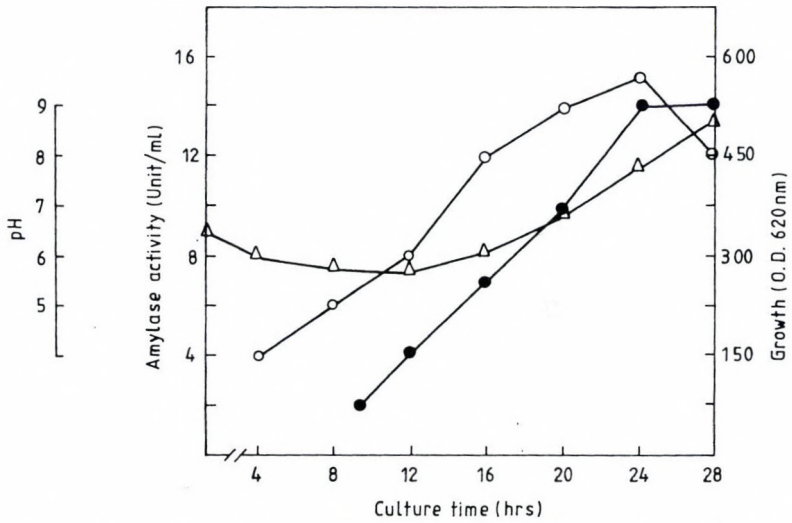

Fig. 1. Time course of cell growth (○), amylase activity (●) and pH (△) by *Bacillus* sp. MD 124.

Depending on the nature of their effect three groups of carbohydrates could be distinguished as poor, moderate and good producers of amylase. Synthesis of amylase was strongly inhibited by sorbose. Lactose, galactose, maltose and sucrose could be considered as moderate sources, whereas rhamnose, starch and glucose were the good carbon sources for enzyme production. The increased order of formation of the enzyme were correlated with rhamnose > starch > glucose > sucrose > maltose > lactose > galactose > sorbose. Of all the carbohydrates, rhamnose was the best carbon sources for enzyme production. Earlier Medda & Chandra [12] also indicated that starch, galactose, rhamnose were the good carbon sources for amylase production in *Bacillus licheniformis.*

It has been also found amylase production was inhibited by glucose and sucrose at higher concentration but good amount of enzyme was produced when same carbon sources were used at extremely low concentration (0.1 or 0.05%) (Table I) whereas repression of amylase synthesis has been noticed in *Endomycopsis* sp. [13] in presence of glucose and sucrose.

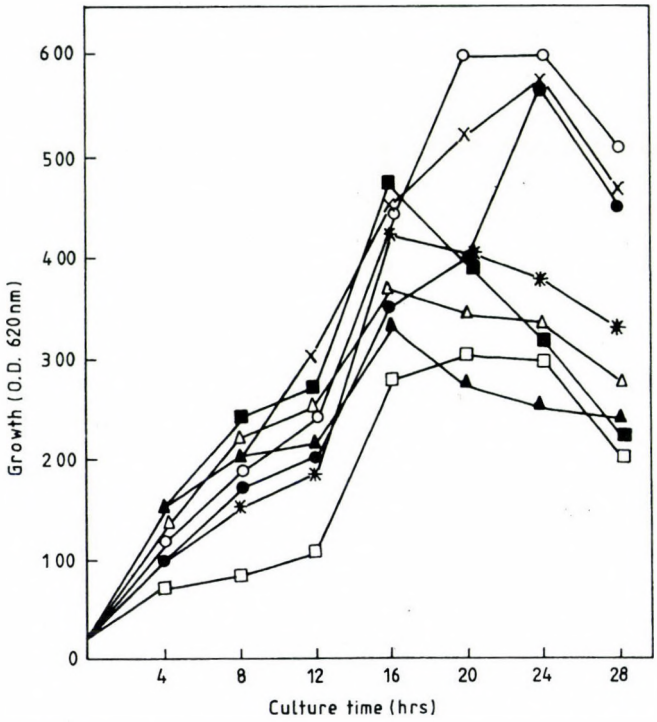

Fig. 2. Effect of various carbon sources on the *Bacillus* sp. MD 124 growth.
(○) 0.5% galactose; (●) 0.5% rhamnose; (△) 0.5% lactose; (▲) 0.1% sucrose; (*) 0.1% glucose; (×) 0.5% starch; (□) 0.5% sorbose; (■) 0.5% maltose

Partial purification of amylase from the culture medium. A summary of the partial purification results is given in Table II. The enzyme was purified about 32-fold over the culture supernatant with 74% recovery in activity.

Optimum temperature of the enzyme activity was 90 °C [8]. The enzyme was stable upto 65 °C for 60 min, even 85% and 65% of the original activity was retained at 75 °C and 80 °C, respectively (Fig. 4). The thermal inactivation data indicate that the enzyme were substrate dependent for the maintenance of thermal stability and enzyme could tolerate high temperature for a longer period in the presence of substrate. The *Bacillus* sp. MD 124 amylase showed steady-state starch hydrolytic activity even at 90 °C for over 1 h in presence of 5% starch slurry (Fig. 5). Similar findings also observed by Bezbaruah et al. [3].

Effects of pH stability of the enzyme have been represented in Fig. 6. The optimum pH was 5.7–6.5 and the amylase was stable in the pH range of 5–10. Most of the *Bacillus* [14] amylases are stable between pH 5–8.

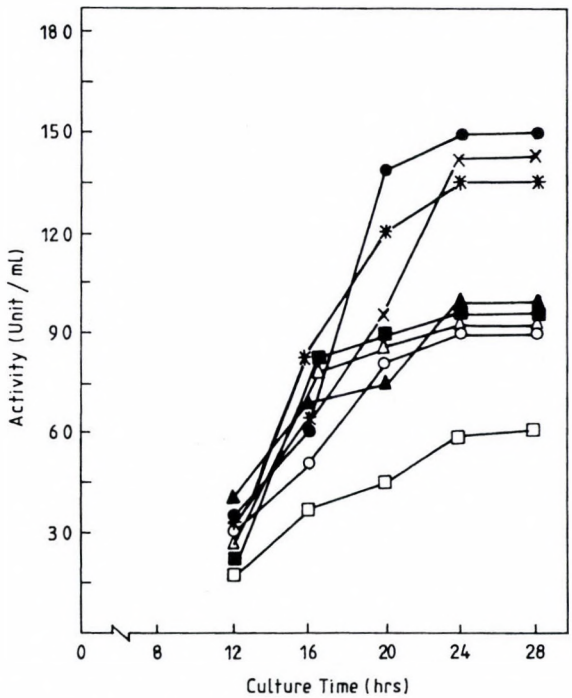

Fig. 3. Extracellular amylase activity of crude enzyme preparation due to the various carbon sources (○) galactose; (△) lactose; (●) rhamnose; (▲) sucrose; (*) glucose, (×) starch; (□) sorbose; (■) maltose

Table I

Effect of glucose and sucrose on the formation of amylase by Bacillus *sp. MD 124*

Concentration % (w/v)	Cell mass growth (OD at 620nm)	Saccharolytic activity (units/ml)
Glucose		
0.05	3.4	13
0.1	3.5	13
0.3	3.8	8
0.5	4.0	4
1.0	4.0	2
Sucrose		
0.05	2.0	10
0.1	2.5	10
0.3	2.7	6
0.5	3.0	3
1.0	3.2	2

Table II

Summary of partially purification of amylase from Bacillus *sp. MD 124*

Procedure	Volume (ml)	Total activity (units)	Total protein (mg)	Sp. activity (units/mg)	Yield (%)	Purification (fold)
Crude extract	194	3724.8	958.3	3.88	100	–
Salting out with 40–80% $(NH_4)_2SO_4$	9	3200	42.56	56.18	85.91	14.99
Chromatography on DEAE cellulase	9	2772	22.5	123.2	74.4	31.75

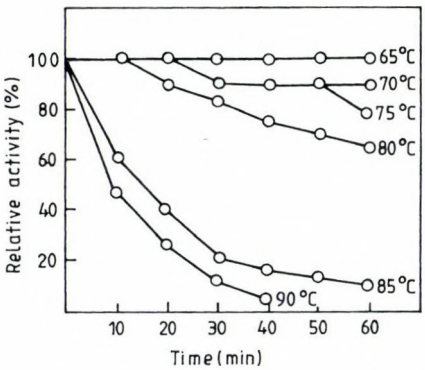

Fig. 4. Effect of temperature on stability of the amylase. For thermal stability, the enzyme solution was held at different temperature for 60 min in phosphate buffer (pH 6.10 mM) and then cooled immediately in ice and the residual activity was measured

Effect of various metal ions on the amylase activity have been shown in Table III. Hg^{2+} was the only metal causing total inhibition, Zn^{2+}, Pb^{2+}, Cu^{2+}, Fe^{2+} completely inhibited enzyme activity at higher concentration tested while Mn^{2+}, Pb^{2+}, Ca^{2+}, Mg^{2+} inhibited enzyme activity slightly in all concentration. Ni^{2+}, Ba^{2+}, Sn^{2+}, Ag^{+} enhanced enzyme activity at lower concentration. Whereas K^{+} enhanced at higher concentration.

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Table III

Effects of metals ion on amylase activity

Ions	Concentration (mg/ml)	Relative activity (%)
Control		100
Ba^{2+}	0.01	87.5
	0.001	100
	0.0001	110
Ni^{2+}	0.01	20
	0.001	90
	0.0001	108
Ag^{+}	0.01	12
	0.001	110
	0.0001	112
Sn^{2+}	0.01	25
	0.001	108
	0.0001	108
K^{+}	0.01	105
	0.001	95
	0.0001	95
Zn^{2+}	0.01	0
	0.001	83
	0.0001	90
Pb^{2+}	0.01	0
	0.001	80
	0.0001	96
Cu^{2+}	0.01	0
	0.001	43
	0.0001	57
Ca^{2+}	0.01	90
	0.001	85
	0.0001	85
Mg^{2+}	0.01	90
	0.001	88
	0.0001	98
Mn^{2+}	0.01	80
	0.001	86
	0.0001	90
Fe^{2+}	0.01	0
	0.001	76
	0.0001	81

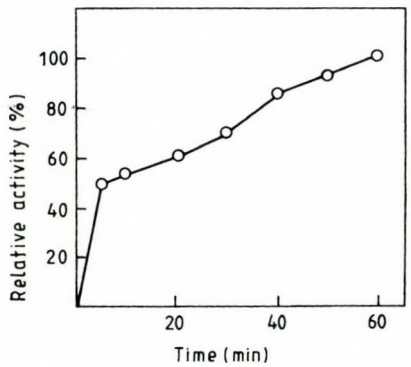

Fig. 5. Effect of temperature on the enzyme activity in presence of substrate (5% starch) over a 60 min period

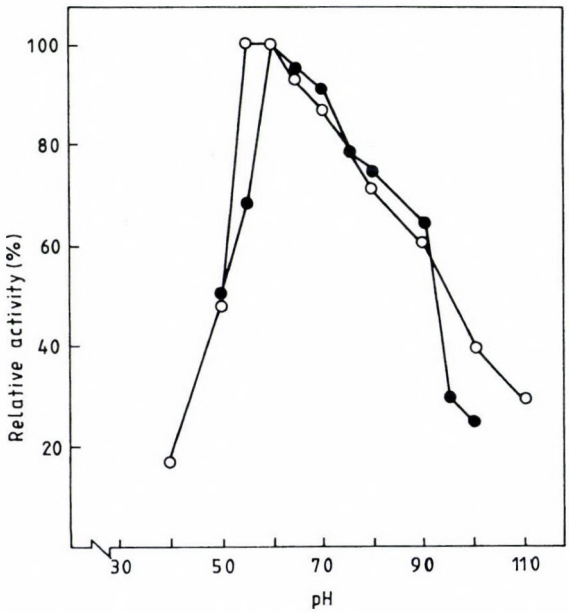

Fig. 6. Effect of pH on amylase activity (○) and stability (●). For optimum pH, amylase activity was measured using desired buffer (10 mM sodium citrate, sodium phosphate and carbonate bicarbonate) at 90 °C for 5 min. For pH stability the enzyme solutions at a desired pH were kept at 4 °C for 24 h and then used for activity measurement

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PARTIAL INHIBITION OF AMPLIFICATIONS BY PRIMERS OF EHEC GENES

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Diagnostic value of multiplex polymerase chain reaction (PCR) was examined by using three primer pairs, specific for the common conserved region of *stx1* and *stx2*, *eae* and an enterohaemolysin A gene (ehxA). The sensitivity in respect of each amplicon decreased with three exponents comparing to the individual PCR reactions. These PCR reactions were partially inhibited by the presence of certain additional primers. This inhibitory effect was template-concentration dependent, and was partially balanced by usage of increased amount of dNTP. Taq DNA polymerase in a range of 0.3–1.25 U/reaction did not influence the inhibition. The same inhibiton was detected if the annealing temperature was changed from 48 °C to 57 °C. Pairs of EHEC primers inhibited a *Salmonella enteritidis* virulence-plasmid specific gene amplification, as well. Theoretical inhibiting effects were predicted by Primer Premier software but our observations can be sufficiently explained neither by the competitions between the specific and aspecific amplifications nor by the inhibition caused by dimerization of primers.

In the nineties the multiplex PCR methods were quite widespread being quicker and more economic compared to the single amplifications. Furthermore, the published procedures were sufficiently sensitive to detect the target genes without preparation of pure cultures, e.g. the verotoxin genes (*stx1* and *stx2*) were demonstrated by two primer pairs in the presence of 1 cfu (by Brian et al.) [1]. The sequences of two *Salmonella* genes were amplified with a sensitivity limit of 10 cfu (by Mahon et al.) [2]. Way and coworkers [3] could detect three *Salmonella* genes in the presence of minimum 100–1000 cfu. However Weaver and Rowe [4] demonstrated, that a high concentration of target-free bacteria might decrease the sensitivity of multiplex PCR. Our purpose was to evaluate the multiplex PCR described by Fratamico et al. [5]. Three genes of the EHEC strains were detected by these authors: a common, conservative sequence of the verotoxin genes (*stx1-stx2*), the variant of *eae* gene characteristic to serotypes O157:H7 and O55:H7 coding intimin and a 60 MDa plasmid sequence which was probably localized in an

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enterohaemolysin gene. The sensitivity of PCR was equally very high in respect of this latter gene with single or multiplex amplification.

Very definite differences were found, however, between the sensitivities obtained with multiplex PCR and with separate amplifications of single genes. Therefore we examined, whether the single amplifications could be inhibited by the primers of the other two genes obtaining inhibitions depending on the system to be inhibited, on the quality of the third primer and on the template concentration. The inhibition could be elicited with EHEC primer pairs also in *Salmonella* system not containing the adequate targets. As a working hypothesis it was supposed that the cause of the inhibitions was the competitions for the dNTP precursors and/or Taq DNA polymerase among specific and aspecific amplifications and primer dimerizations. Our results can be only partly explained with this hypothesis suggesting a more complex phenomenon.

Table I

EHEC strains used in this study

Strain	Serotype	Origin	EHEC genes			
			stx-1	*stx-2*	*eae*	*enthly*
c276/95*	O157:H7	Stool, HC	+	+	+	+
c33/97	O96:HNt	Stool, diarrhoea	+	-	-	+
c90/97	O157:H7	Stool, HC	-	+	+	+
c130/97	O157:H7	Raw milk	+	+	+	+

* generally used strain
HC: hemorrhagic colitis

Methods

Bacterial strains. The experiments were performed with EHEC strains isolated in 1995–97 (Table I) and with *S. enteritidis* strain S24 (PT 1b), isolated in 1995. All of these strains originated from clinical samples.

Preparation of templates. Generally, crude lysates of cells grown overnight in nutrient agar at 37 °C served as template. 2–4x10^9 cfu/ml were suspended in a solution containing Triton X-100 0.5%, Tris.HCl (pH=8) 20 mM and EDTA 2 mM, and boiled for 10 min. For genomic DNA preparation the method of Versalovic et al. [6], and for plasmid preparation the method described by Birnboim and Doly [7] was used.

PCR amplifications. The reaction mixture (50 µl) contained 5 µl 10× Promega buffer (100 mM Tris.HCl pH=9, 500 mM KCl, 1% Triton X-100), 1.5 mM $MgCl_2$, dNTP-s 200 µM each, 0.5 U Taq

DNA polymerase (Promega), primers (Table II) generally 25 pmol each, and the supernatants of the boiled cell suspensions in different dilutions. The primers determined the amplicons of 3 EHEC genes (*stx*, *eae*A and enterohaemolysin*) with 227/224, 1087 and 166 bp, resp., and a 620 bp amplicon produced by a *Salmonella* virulence plasmid. The amplification was made in thermocycler „Progene" (Techne, Cambridge, UK) with the following cycling conditions: in case of EHEC strains an initial denaturation at 94 °C for 5 min, followed by 35 cycles, with 1 cycle consisting of 1 min at 94 °C, 3 min at 48 °C and 4 min at 72 °C. In case of the *Salmonella* strain the same initial denaturation was followed by 40 cycles, with 1 cycle consisting of 60 s at 94 °C, 60 s at 54 °C and 90 s at 72 °C with a final elongation at 72 °C for 10 min (Mahon et al. [2]). For hot start AmpliWax was used according to the manufacturer's instructions, giving Taq DNA polymerase and template above the wax.

Detection of PCR products. The amplicons were generally examined with agarose gel electrophoresis using 1.6% agarose (Sigma type II) in TAE buffer containing 1 μg/ml ethidium bromide. Λ phage digested with *HindIII* enzyme was applied as mwt marker (23.1, 9.4, 6.5, 4.3, 2.3, 2.0, 0.56 kbp). The inhibition effects were determined comparing the bands to a dilution series in twofold steps of the control reaction mixture. PAGE was made with Laemmli's method (10) applying 10% of separating gel, 10 μl was used from the reaction mixture and stained with 1 μg/ml ethidium bromide. For the detection of amplicons produced less than the visible limit of the agarose gel electrophoresis, dot blots were performed with 15 μl of PCR reaction mixtures according to Sambrook et al. [11]. The hybridization was carried out at 65 °C for 16 hours. The probes were the amplicons of the EHEC genes isolated from the agarose gel with ultra-free MC filter (Millipore) according to the manufacturer's instructions using [α-^{32}P] dCTP labelled with random priming method.

Table II

Primers used in this study

Primer	Sequence (5'-3')	Target	Amplicon (bp)	Reference
MK1	TTTACGATAGACTTCTCGAC	*stx-1* and *stx-2*	227/224	Karch and Meyer [8]
MK2	CACATATAAATTATTTCGCTC			
AE19	CAGGTCGTCGTGTCTGCTAAA	*eae*	1087	Gannon et al. [9]
AE20	TCAGCGTGGTTGGATCAACCT			
MFS1F	ACGATGTGGTTTATTCTGGA	*ehx*A	166	Fratamico et al. [5]
MFS1R	CTTCACGTCACCATACATAT			
S1	GCCGTACACGAGCTTATAGA	a gene of the 38 MDa virulence plasmid	620	Mahon et al. [2]
S2	AAAGTGATGCCTTCTGCATC			

* abbreviations: tox, int and enthly, resp.

Results

The PCR results of four EHEC strains are demonstrated in Fig. 1. The single amplifications of tox, int and enthly amplicons (lanes 1–3) were compared with the multiplex method (lanes 4–5) using 4×10^7 cfu/50 µl template concentration. The quantity of primers in case of multiplex PCR was either 25–25 pmol (lane 4) as in the single PCR or 8.3 pmol each (lane 5). The effect of the multiplex method varied in the different strains. Strain c276/95 produced all three amplicons, with a little decrease in the *int* band. In case of strain 90/97 the band intensity weakened, while with strain 130/97 the appearance of all the three amplicons were fully inhibited. The production of *tox* amplicon in the *int*$^-$ strain c33/97 was more definitely inhibited and the inhibition was smaller at lower primer concentrations.

The minimal template concentrations necessary to detect amplicons in single and multiplex PCR were compared using EHEC strain c276/95 and 25 pmol of specific primers. Figure 2 shows that this value was 2.4×10^3 cfu/50 µl in single PCR (lanes 3 of A-C panels), while none of the amplicons could be demonstrated under 2.4×10^6 cfu/50 µl (lane 5 of panel D) in multiplex PCR.

The appearance of *tox* amplicon in the agarose gel could be totally inhibited by 12.5 pmol *int* primer AE19, i.e. with 25% of the standard primer quantity used for amplification (Fig. 3, lane 3). There was no inhibition using *int* primer AE20 (lanes 4–5). The 1:1 mixture of AE19 and AE20 did not give synergistic effect and *int* amplicon did not appear (lanes 6–7).

The inhibition was not absolute, as in dot blot experiments positive results were obtained applying the adequate amplicons as probes (data not shown). The appearance of aspecific amplicons either in agarose gel electrophoresis or in PAGE was not observed (data not shown).

Applying hot start to exclude the connection of Taq DNA polymerase and the primers before the first melting of the template, the extent of the inhibition was unchanged and the change in the concentration of Taq DNA polymerase between 0.3–1.25 U/50 µl was indifferent. A threefold increase of dNTP concentration diminished the inhibition of *tox* amplification induced by primer AE19 (Fig. 4). This amplification was inhibited by more than 300 µM dNTP (data not shown). There was an opposite relation between template concentration and inhibition: the highest inhibition was found near the minimal template concentration (Fig. 4).

The results of semiquantitative inhibition experiments are summarized in Table III. The amplification of *tox* and *int* amplicons could be inhibited by one or another primer in a different degree. A very strong inhibition was caused by primer AE19 to *tox*, and by primer MK2 to int amplification: there was some effect even with 5 pmol primer. The synthesis of *enthly* amplicon production was also inhibited significantly by primer AE19. The *tox* amplification could not be inhibited by the *enthly* primers and vice versa.

The increase of annealing temperature from 48 °C to 57 °C did not abolish the inhibition (data not shown).

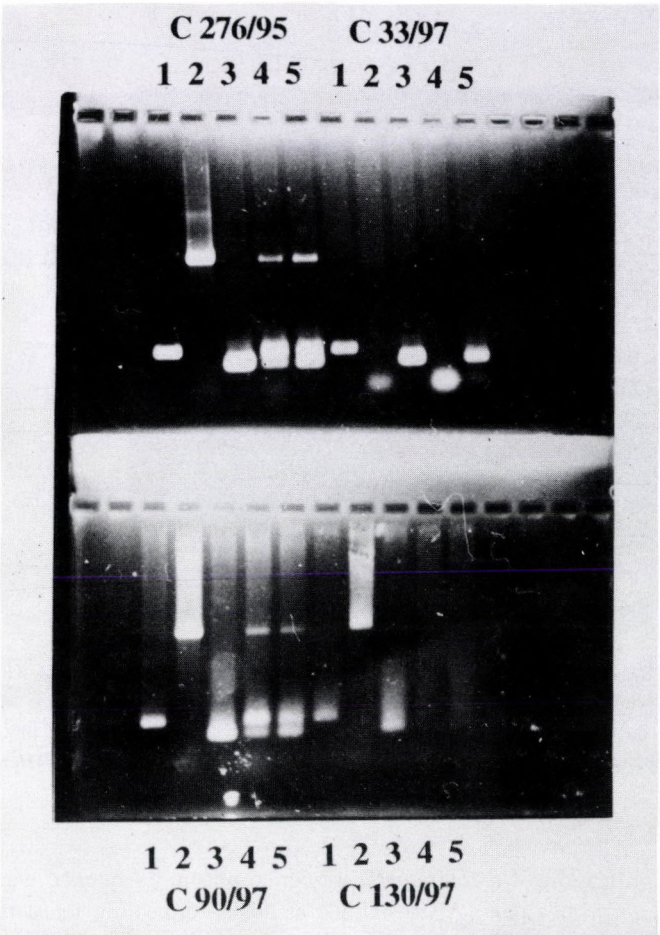

Fig. 1. Detection of EHEC genes in pure cultures with single and multiplex PCR. **1–3**: single amplifications in the presence of tox (1), int (2) and enthly (3) pairs of primers, 25+25 pmol each. **4–5**: multiplex PCR in the presence of 25+25 pmol (4) or 8.3+8.3 pmol (5) *tox, int* and *enthly* pairs of primers. Template conc. 4×10^7 cfu/50 µl

In the experiment presented in Table IV the effects of two pairs of primers used equally in 25+25 pmol concentration were studied. In case of *int+tox* combination the detectability of both products were totally inhibited, while in *enthly+int* relation a partial inhibition was found. The amplification of mainly the *enthly* product was inhibited in case of *tox+enthly* combination. There was no inhibition if only one pair was applied in a double concentration.

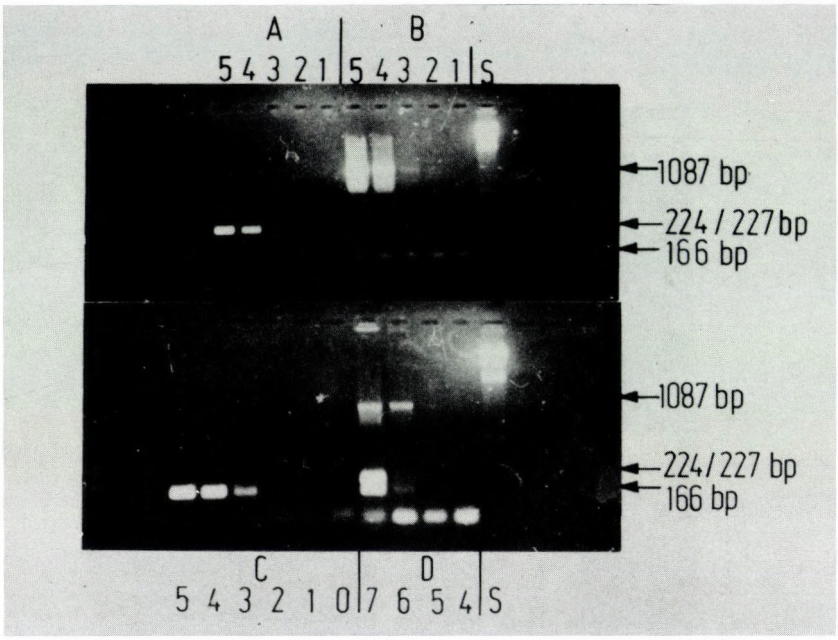

Fig. 2. Sensitivity of detection of EHEC genes in single and multiplex PCR. A–C: single amplifications in the presence of *tox* (A), *int* (B) and *enthly* (C) pairs of primers. Multiplex PCR in the presence of all the three pairs of primers (D). Conc. of primers: 25 pmol each. Template: c276/95, 1–7: template conc.-s (2.4×10^n cfu/50 µl). S: standard (l*HindIII*)

The amplification of a *Salmonella* virulence plasmid sequence was decreased by 5–50 pmol of *int* primer pair (AE19+AE20) at 54 °C annealing temperature, being in correlation with the template concentration (Fig. 5). Similar inhibitions could be induced with the other two EHEC primer pairs. In contrast with the observation found in the EHEC system, the inhibitory effects of primer pairs strongly exceeded those obtained with single primers (Fig. 6).

The inhibition of specific amplifications observed in the presence of a third primer could be reproduced if genomic DNA (EHEC systems) or plasmid DNA (*Salmonella* system) were used as templates (data not shown).

Discussion

We have not seen any systematic studies comparing PCR with multiplex PCR, and the inhibition of amplifications by additional primers was generally not mentioned. Frankel et al. [12] described significant inhibitions using multiplex method with ETEC ST, LT and *Shigella ial* primer-pairs but these results were not explained. Fratamico et al.

[5] carried out a comparison study using enthly-specific primers; PCR and multiplex PCR both resulted in a sensitivity of 1.2 cfu/100 µl.

In our present work using Fratamico's method it was found that the sensitivities decreased by three exponents in respect of all the three genes by multiplex method (Fig. 2). This means that in practice the simultaneous detection of the three genes may not be reliable even using pure, concentrated cultures since false negative results may be obtained in case of certain strains (Fig. 1).

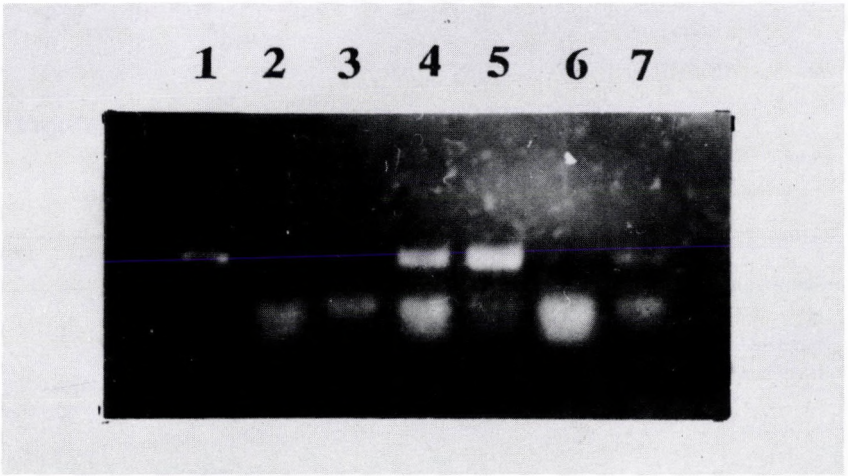

Fig. 3. Inhibition of tox amplification with int primers. 1: amplification in the presence of *tox* primers 25 pmol each. 2–7: supplementation with 50 (2) or 12.5 pmol (3) of primer AE19, with 50 (4) or 12.5 pmol (5) of primer AE20, with 25+25 (6) or 6.25+6.25 pmol (7) of primers AE19 and AE20. Template: c276/95, 2.4×10^4 cfu/50 µl

The inhibition experiments carried out with strains *E. coli* c276/95 and *S. enteritidis* S24 proved that the amplification might be more or less inhibited by a third primer (Fig. 3, Table III) or an additional primer pair (Table IV). There are two possible explanations for this phenomenon, not excluding each other. Firstly, the competition for the Taq DNA polymerase enzyme and/or for the dNTP precursors is indirectly responsible for the inhibition. Secondly, a direct inhibition of the amplification is also possible. Concerning the indirect hypothesis, almost certainly the competition for the Taq DNA polymerase can be excluded since decreased amount of the enzyme did not increase the level of inhibition. Competition for dNTP could be more likely, because the degree of inhibition was decreased by a higher dNTP concentration (Fig. 4) and the sensitivity of the multiplex PCR was somewhat higher (results not shown).

Table III

Inhibition of the amplification by the primers of the other two genes in E. coli 276/95

The inhibited amplifications			Inhibiting primers					
Amplicon	Primer		*Tox*		*Int*		*enthly*	
	(25+25pmol)	pmol	MK1	MK2	AE19	AE20	MFS1F	MFS1R
Tox	MK1+MK2	50			100	25	0	0
		12.5	**nt**	**nt**	100	0	0	0
		5			75	0	0	0
Int	AE19+AE20	50	50–75	100			0	94
		12.5	0	100	**nt**	**nt**	0	88
		5	0	100			0	100
enthly	MFS1F+	50	0	0	88	0–50		
	MFS1R	12.5	0	0	50	0	**nt**	**nt**
		5	0	0	0	0		

Numbers: inhibition (%), on the basis of the intensity of amplicon bands
100 %: there was no visible band
Template concentration: 3×10^4 cfu/50 µl
nt: not tested

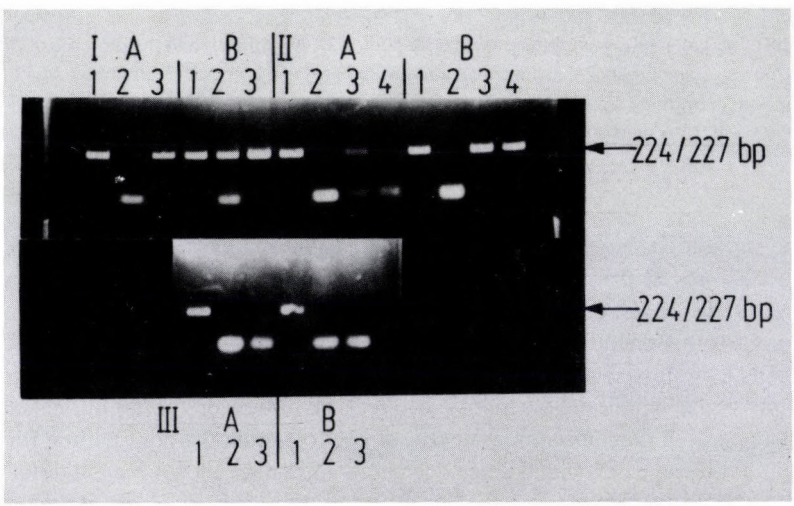

Fig. 4. The effect of dNTP and template concentrations on the inhibition of *tox* amplification caused by primer AE19. 1: amplification in the presence of *tox* primers 25 pmol each. 2–4: supplementation with 50 (2), 12.5 (3) and 5 pmol (4) primer AE19. Template: c276/95. Template conc.-s: 2.4×10^6 (I), 2.4×10^5 (II) and 3.4×10^4 (III) cfu/50 µl. dNTP conc.-s: 100 (A) and 300 (B) µM each

Table IV

Amplification in the presence of two pairs of primers in E. coli 276/95

Primer pair „a" Primer pair „b"	MK1+MK2	AE19+AE20	MFSF1+MFS1R
MK1 +MK2 *(Tox)*	*Tox**: 0		
AE19+AE20 *(Int)*	*Tox*: 100 *Int*: 100	*Int**: 0	
MFS1F + MFS1R *(enthly)*	*Tox*:50 *enthly*: 75	*Int*: 6 *enthly*: 25	*enthly**: 0

Numbers: inhibition (%), on the basis of the intensity of amplicon bands
100 %: there was no visible band
Template concentration: 3×10^4 cfu/50 µl
Concentration of primers: 25 pmol each
* 50+50 pmol

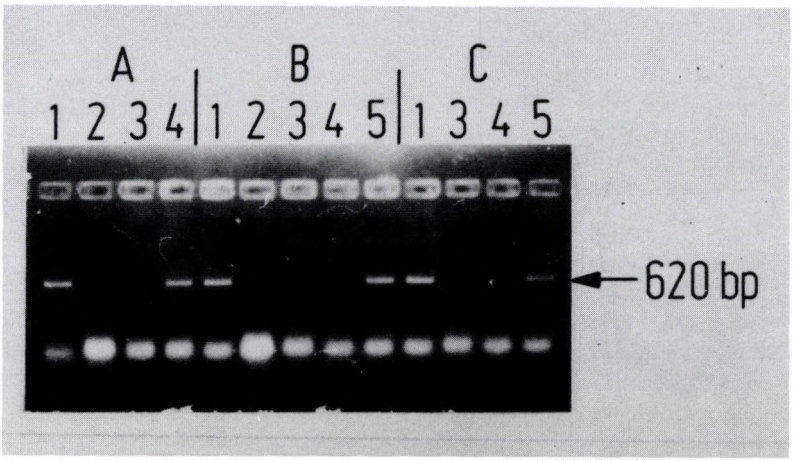

Fig. 5. Inhibiting effect of the *int* pair of primers on the amplification of a *S. enteritidis* virulence plasmid gene. 1: amplification in the presence of S1 and S2 primers 25 pmol each. 2–5: supplementation with 25 (2), 6.25 (3), 2.5 (4) and 0.6 (5) pmol of AE19 and AE20 primers. Template: S24. Template conc.-s: 6×10^3 (A), 2.4×10^3 (B) and 1.2×10^3 (C) cfu/50 µl

The competition between specific amplifications could be excluded, when the inhibition was induced by single primers or there was no adequate target for the additional primer pairs (EHEC primers in *Salmonella* system). The reciprocal inhibition, observed in

the presence of *tox-int* primer pairs in EHEC strains (Table IV) could not be explained in this way, either.

Neither the higher annealing temperature, nor hot start, which were unfavourable for the aspecific amplifications (Chou et al. [13]), reduced the inhibition. The dilution of the template enhanced the degree of inhibition although the ratio of specific and aspecific targets was not changed. Both of these observations contradict to the possible competitive effect of aspecific amplifications.

Theoretically, the amplifications may cause concurrency by starting the hairpin-like annealing of primers with themselves. However, the primer AE19, which induced a very strong inhibition added to the tox system, did not form hairpin-like structures according to Primer Premier software.

Table V

Categories of primer dimers on the basis of their expectable inhibiting effects

	MK1	MK2	AE19	AE20	MFS1F	MFS1R
MK1	–6.8[2]	(–4.9)	–6.5[2]	No	No	–2.9[4]
MK2		–5.4[3]	No	–8.3[1]	(–6.3)	No
AE19			No	No	(–6.5)	(–6.9)
					–4.9[4]	
AE20				–8.3[1]	–6.4[2]	(–6.9)
MFS1F					No	(–6.4)
						–4.8[4]
MFS1R						–3.9[4]

Analysis made on the basis of Primer Premier software
Numbers: Δ G values

Categories:
1. ΔG is between –9.4 and –5.5; at the 3' ends of primers there are minimum 4 bps among 6 or 5 continual bps anywhere.
This type has strong inhibiting capacity
2. ΔG is between –9.4 and –5.5 kcal/mol but the above criterions of basis pairing are not fulfilled
3. ΔG is greater than –5.5 kcal/mol but one of the above criterions is fulfilled.
Categories 2–3 have weak inhibiting capacities
4. ΔG is greater than –5.5 kcal/mol. There is no inhibiting capacity
The condition of continuable dimerization is the pairing at the last or one but last basis of 3' ends. In case of dimers signed with parentheses this condition was not fulfilled

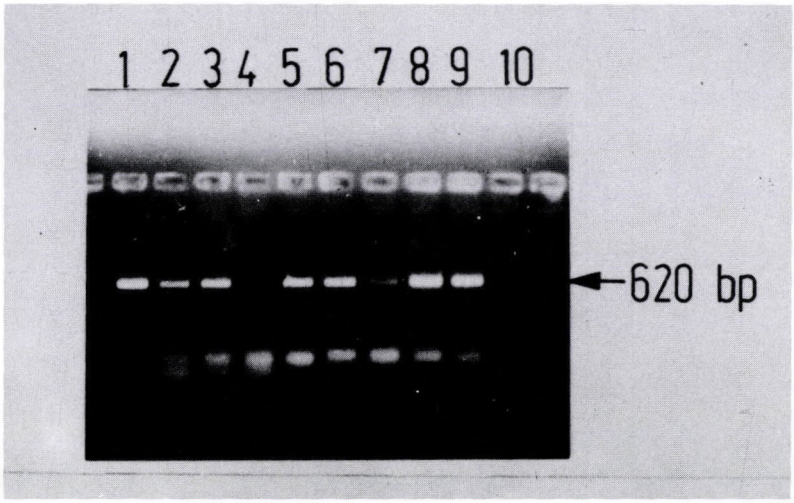

Fig. 6. Inhibiting effect of the primers of EHEC genes on the amplification of a *S. enteritidis* virulence plasmid gene. 1: amplification in the presence of S1 and S2 primers 25 pmol each. 2–10: supplementation with AE19 (2), AE20 (3), AE19 and AE20 (4), MFS1F (5), MFS1R (6), MFS1F and MFS1R (7), MK1 (8), MK2 (9), MK1 and MK2 (10) primers. Conc.-s of single primers: 50 pmol, those of pairs: 25+25 pmol. Template: S24, 5×10^3 cfu/50 µl

The formation of primer dimers are disadvantageous for specific amplifications, this must be avoided when planning the primer pairs (Meng et al. [14]) by excluding the complementarity between their 3' ends. Amplification can be started by the annealing of the 3' ends; the Δ G value of dimerization depends on the number, the quality and position of the basis pairs, which are expressed in the Δ G value. Table V shows the dimerization capacity of all combinations of the used primers classifying them on the basis of their possible inhibitory effect claimed by the software Primer Premier. Intensive inhibitory effect can be expected, if the Δ G of the most stable domain of 5' overhang dimer is <9.4 kcal/mol (such value did not occur here) or Δ G is between –9.4 and 5.5 kcal/ml and basis pairing develop at their 3' ends at least 4 out of 6 basis, or 5 there are continuos pairing somewhere (Table V, category 1). The only combinations which fell into this category were AE19-AE20 and AE20-MK2. Smaller inhibitions are only permitted by the partial fulfilling of the above requirements. (Table V, categories 2–3).

As it can be seen in Table VI, there was no correlation between the calculated stability of dimers and the actually observed inhibitions, primer AE20 did not induce any inhibition in either combinations. The presence of primer AE19 was the condition for a considerable effect, but this fact was not explained by the possibilities for dimerization. The hypothesized inhibitory role of dimerization was in contrast with the observation, that the inhibition did not decrease in case of hot start and in *Salmonella* system higher inhibitions were caused by EHEC primer-pairs than by single primers, but in EHEC system this effect was not observed.

Table VI

Correlation between the inhibitions found and the theoretical inhibiting effects of primer dimers

Inhibited amplification		Additional	Inhibition	Possible inhibiting dimers	
Amplicons	Primers*	primers**	%	Homo-dimers	Hetero-dimers
Tox	MK1-MK2	AE19	100	MK1-MK1[2], MK2-MK2[3]	MK1-AE19[2]
		AE20	25	MK1-MK1[2], MK2-MK2[3], AE20-AE20[1]	MK2-AE20[1]
		MFS1F	0	MK1-MK1[2], MK2-MK2[3]	–
		MFS1R	0	MK1-MK1[2], MK2-MK2[3]	–
Int	AE19-AE20	MK1	50–75	AE20-AE20[1], MK1-MK1[2]	AE19-MK1[2]
		MK2	100	AE20-AE20[1], MK2-MK2[3]	AE20-MK2[1]
		MFS1F	0	AE20-AE20[1]	AE20-MFS1F[2]
		MFS1R	94	AE20-AE20[1]	–
enthly	MFS1F-MFS1R	AE19	88	–	–
		AE20	0–50	AE20-AE20	AE20-MFS1F[2]
		MK1	0	MK1-MK1[2]	–
		MK2	0	MK2-MK2	–

* 25+25 pmol

** 50 pmol

[1,2,3]: categories of the inhibiting effect of dimers on the basis of Primer Premier software (explanation see in Table 5).

Our results suggest that the additional oligonucleotides may exert also some direct inhibition. For this effect more possibilities are known, e.g. a primer bound aspecifically between two specific targets may inhibit the appearance of the original amplicon synthetizing a shorter product (Lewis et al. [15]). Annealing may happen between the newly formed DNA chain and the additional oligonucleotide and this may result in an abortive replication (Guieysse et al. [16]). Products of these processes may remain under the visible level. The Taq DNA polymerase function was hindered if it was bound to a mismatch basis pair formed at the 3' end of the primer (Kwok et al. [17]). Last, but not least, an allosteric change of the enzyme induced by the additional primer cannot be excluded.

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FAST METHOD TO REMOVE UV ABSORBING AGAROSE GEL CONTAMINATION FROM DNA SAMPLES

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Agarose gel electrophoresis and subsequent purification of DNA bands from the agarose gel is a widely used molecular biological method. There are different methods to achieve this goal, however they have different advantages and disadvantages. One major problem is the presence of different contaminants in the final sample. We developed a method which is effective in removal of the agarose contaminants.

Different methods are used to purify the DNA from agarose gel after agarose gel electrophoresis including electroelution [1], different chromatographic matrix based methods [2] and direct physical methods collapsing the gel structure and separate the trapped buffer carrying the DNA [3]. The latter approach is particularly fascinating because of its speed, simplicity and the possibility of parallel processing of more samples; one can obtain the DNA sample with an average 80% yield in two minutes using a Wizard minicolumn centrifuged for one minute at 14000 g. The sample is ready for further processing including ligation, restriction digestion and others. But the method has a major drawback: the buffer also carries some UV absorbing material along the DNA which can be removed by ethanol precipitation but the ethanol precipitation is time consuming eliminating the advantage of the speedy preparation of the DNA sample and it is dependent on the exact details of the method and in some cases it cannot be complete (personal observations). The other way to overcome this difficulty is to measure the DNA content of the sample by fluorimetry, but the fluorimeter is not available in every molecular laboratory. Moreover, although the agarose gel contamination does not influence most enzymatic reactions but making the sample chemically more complex limits the free application of the DNA and also adds some uncertainty, because of different agarose brands contain different levels of contaminants (personal observation). Recently the spincolumn technique was introduced for buffer exchange, for presequencing clean-up of DNA samples. Using the Pharmacia minicolumn based DNA

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preparation technique we tried to remove the low molecular weight agarose gel contamination from the DNA samples by the spincolumn method with gelfiltration of the sample through Sephacryl S-400 HR matrix.

Materials and methods

To prepare the DNA sample about 100 µl excised agarose plug was placed in an empty Pharmacia minicolumn and centrifuged at 14000 *g* for two minutes resulted in about 80 µl DNA sample. A second minicolumn was filled with 500 µl Sephacryl S-400 HR matrix, centrifuged for one minute at 735 g in an Eppendorf tube to remove the storage buffer and subsequently washed with 400 µl MilliQ water two times. The prepared DNA sample (80 µl) was applied in the center of the gel and centrifuged for one minute and the eluent was collected with an almost 100% volume yield. The purity of the sample was measured by spectrophotometry at 260 nm, 280 nm and 320 nm in a 50 µl microcell. Fluorimetry is done at 1 µg/ml Hoechst dye concentration in 1 M NaCl.

Results

The described procedure was carried out and the data obtained are summarized on Table I. The DNA containing sample was free from contaminating UV absorbing material judged by processing a DNA sample alone and in agarose and measured spectrophotometrically. The A260/A280 ratios were very close to 1.8 indicating pure DNA sample. An empty agarose plug was also processed to measure the effectiveness of the contaminant removal (Table I). Comparing the spincolumn method with the ethanol precipitation we could get the same A260/Hoechst ratio indicating parallel readings with the two methods (Table II). Considering that no UV absorbing agarose contaminants were left after spincolumn clean-up (Table I) and the Hoechst dye DNA specificity it is proved that all the UV absorbing material of the sample is DNA.

Discussion

During the experiments we compared different agarose brands. We have found differences but these differences do not show any correlation with the published chemical characteristics of the agarose brands. Some agarose brands have higher 320 nm absorbance than the others and this contamination can be copurifying with the DNA during spincolumn clean-up step. This is absent when an empty agarose plug is processed and influences only the 320 nm absorbance value, so does not cause misreading at 260 or 280 nm (data not shown). Considering that even the unprocessed sample is suitable to perform the subsequent enzymatic step, this contamination should be no problem. The yield varies with the fragment size and DNA terciary structure but it is always better than 50% and even 90% can be achieved. However the overall yield mainly depends on the collapsing step and not on the Sephacryl S-400 HR step (data not shown). Purifying

smaller plasmid superhelical forms from agarose gel can result in lower yields, because of their small Stokes radius they can be trapped partly in the matrix.

Table I

Purity of the samples measured by spectrophotometry

Sample	A260	A280	A260/280	mg/ml
DNS+agarose+cleanup	0.041	0.022	1.864	2.05
DNS+cleanup	0.046	0.026	1.770	2.29
Agarose (w/o DNA)	0.050	0.047	-	-
agarose+cleanup (w/o DNA)	0.006	0.004	-	-

Agarose means the eluent produced by the centrifugal collapsing method processing an empty agarose plug. Cleanup means the removal of contaminants by Sephacryl S 400 HR column. CsCl gradient purified pUC18 superhelical plasmid preparation has been used as DNA. The experiments have been carried out at least three times, typical results have been shown

Table II

Comparison of the ethanol precipitation and the Sephacryl S 400 HR column method

Sample	A260	Hoechts	A260 / Hoechst	%
DNA+agarose+EtOH	0.063	1.051	0.0599	100
DNA+agarose+cleanup	0.052	0.863	0.0602	99.5

'EtOH' means ethanol precipitation. The spincolumn clean-up has lower yield than the ethanol precipitation, this caused lower readings. Other abbreviations see at Table I.

Summarizing: the method is fast and effective to remove the agarose gel contamination from DNA samples prepared from collapsed agarose gels making possible to measure the DNA content of the sample by spectrophotometry. The further manipulation of DNA often necessitates to determine the concentration of the DNA in the sample and with the described technique it is now possible to use the spectrophotometry for this purpose. Additional advantage of the method is that buffer exchange can be carried out at the same time. Pure DNA sample ready for spectrophotometric quantitation can be prepared in less than 5 minutes, which make this method one of he fastest DNA preparation available today.

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EFFECT OF ANTILYMPHOCYTE SERUM ON BACTERIAL TRANSLOCATION IN MICE

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Following intraperitoneally applied treatment with antilymphocyte serum (ALS) of immunosuppressive effect no bacterial translocation (BT) was observed in mice. The ALS treatment applied in combination with other immunosuppressive agents such as lymphotropic cytostatics as dianhydrogalactitol or chlorpromazine did not increase the mice's drug sensitivity to the used agents. According to our results, ALS can be suitable for combined application with other immunosuppressive agents as it can increase immunosuppression without side-effects such as those induced by bacterial translocation.

It is known from literature that in certain immunosuppressive conditions bacteria of the normal intestinal flora may permeate the intestinal wall and can be translocated into the otherwise sterile inner organs [1]. In our earlier experiments, following cytostatic dianhydrodulcitol (DAD) or chlorpromazine (CPZ) treatments bacterial translocations (BT) and simultaneously enhanced sensitivity to the applied agents as compared to germ-free mice were observed in conventional animals [2, 3]. It is also known from published data and learned from our experiments, too that both agents have definite adverse effects on the intestinal wall [2] and presumably the BT appearance is a consequence of the intestinal wall injuries [4].

In the present study we looked for immunosuppressive agent or agents without any effect on the intestinal mucosa. First, antilymphocyte serum (ALS) was examined. We tried to find an answer as to whether

– BT can be identified in immunosuppressive state following ALS treatment,

– ALS treatment can induce changes in drug sensitivity when used in combination with other immunosuppressive agents.

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Materials and methods

Experimental animals. Six to eight-week-old young adult conventional CD-1 mice (Charles River Hungary Kft) of both sexes were used.

Antilymphocyte serum treatment. Antilymphocyte serum was prepared by the method of Gray et al. [5] then stored in 5 ml doses frozen until its use. Right before use the ALS was *ana partes* diluted with phosphate-buffered saline (PBS) and 0.5 ml was intraperitoneally (i.p.) applied once to each mouse.

Dianhydrogalactitol treatment. Dianhydrogalactitol (DAG) which is epoxide of dibromdulcitol (DBD) and stereoisomer of dianhydrodulcitol (DAD) is a lymphotropic cytostatic agent of the alkylating group (Chinoin Pharmaceutical Works Ltd., Budapest). The substances were dissolved in sterile distilled water and used within half an hour. Dilution was made according to 0.01 ml/g body weight. The mice were inoculated i.p. on one occasion.

Chlorpromazine treatment. A 2.5% aqueous solution of chlorpromazine hydrochloricum (EGIS, Budapest) was used. The required dilution was prepared with PBS, immediately before use. The mice were inoculated i. p. once.

Control groups' treatment. Control mice (Group C) were inoculated by sterile PBS or normal rabbit serum in a volume corresponding to that of DAG, CPZ and ALS.

Examination of bacterial translocation. Preparation of cell suspension: After the mice were killed, two mesenteric lymph nodes, the spleen and the liver were removed with sterile instruments. Cell suspension was prepared in 0.5 ml PBS individually from the removed lymph nodes as well as from the fragments of 100 mg of spleen and liver, respectively.

Isolation of bacteria. The whole quantity of each of the cell suspensions was inoculated into serum bouillon and incubated at 37 °C. Subcultures were made of the serum bouillon on blood agar medium at 24 h intervals and they were aerobically incubated. Isolations were considered negative when no bacterial growth was observed on the blood agar media after 48 h incubation. (Inoculations were repeated daily on five successive days).

Lymphocyte count. It was determined in caudal vein samples under standardised conditions.

Statistical evaluation. It was carried out by EXCEL and a significance level of $p=0.05$ was accepted.

Experiments and results

In our earlier experiments the immunosuppressive effect of this ALS substance had been proved both *in vivo* and *in vitro* [6] and now it was proved *in vivo* experiment. Twelve-twelve young adult mice were treated by ALS or normal rabbit serum and then lymphocyte count was determined at 6 and 24 h after i.p. treatment (ALS/6, ALS/24, C/6 and C/24 groups). The results are shown in Table I.

Table I

Lymphocyte count (ly) averages in 6 h and 24 h after ALS treatment

Groups	Ly averages	Standard deviation	p value
ALS/6	370.0	201.7	
			< 0.001
C/6	2934.0	759.8	
ALS/24	1371.4	667.6	
			< 0.001
C/24	3180.0	383.4	

Table I shows that similarly to our earlier results – lymphopenia was resulted after the use of this ALS, and we considered it as indication of immunosuppression.

Two series of experiments were performed.

Experiment I. Groups of 18-18 mice were treated with ALS or PBS as a control. The heaviest immunosuppression induced by ALS can be experienced on the first days after treatment, therefore we carried out daily isolations on the first, the second and third days, killing 6 mice both from the ALS treated (ALS) and control (C) groups, to study BT. We could not demonstrate BT during the three bacterial isolation periods, either in the ALS or in the C groups (Table II).

Table II

Frequency of bacterial translocation in mice or in their organs in Experiment I

Groups of mice	i.p. treatment	Examination after treatment on	Translocation frequency* in	
			mice	organs
ALS	0.5 ml ALS	1st day	0/6	0/18
		2nd day	0/6	0/18
		3rd day	0/6	0/18
C	0.5 ml PBS	1st day	0/6	0/18
		2nd day	0/6	0/18
		3rd day	0/6	0/18

* Number of positive mice or organs / Number of examined mice or organs

Further, we applied ALS treatment in combination with immunosuppressive agents and examined whether ALS treatment can change the drug sensitivity. On the day after ALS treatment we inoculated mice's groups with subtoxic doses of DAG or CPZ, namely with 30 mg/kg DAG or 75 mg/kg CPZ (CPZ LD50: 100 mg/kg, DAG LD50: 35 mg/kg).

Table III shows the groups established and the number of mice in each group as well as the treatments applied and mortality.

Table III

Groups of mice, number of mice as well as the applied treatments and mortality rate

Groups of mice	No. of mice	i.p. treatments				Mortality rate
		ALS (ml)	DAG (mg/kg)	CPZ (mg/kg)	PBS (ml)	
ALS	20	0.5	–	–	–	0/20
DAG	20	–	30	–	–	2/20
ALS–DAG	20	0.5	30	–	–	2/20
CPZ	20	–	–	75	–	7/20
ALS–CPZ	20	0.5	–	75	–	7/20
C	20	–	–	–	0.5	0/20

From Table III it can be seen that ALS pretreatment that caused no mortality by itself (ALS group), did not affect the mortality rates caused by DAG or CPZ (ALS-DAG, or ALS-CPZ groups) and consequently did not lead to enhanced drug sensitivity.

Discussion

The immunosuppressive effect of DAD has been proved in our previous investigations [7]. BT caused by DAD and DAG has been demonstrated as well after a single large dose or chronic treatment [4]. The BT occurrence is due to the cytostatic effect of the agent and connected at the same time with its adverse effect on the intestinal mucosa [2]. It is known from literature and our results, too that CPZ has an immunosuppressive effect and further induces intestinal atony as well as consecutive increased permeability [3]. Stress is well known of immunosuppression that enhances permeability of the intestinal wall and induces consecutive BT [9]. Because of stress, the DAD LD50 value has definitely decreased in conventional mice [10], and they have become more sensitive to the examined agent. Similar drug sensitivity developed when we used two agents (DAG, CPZ) in combination [8].

In our present experiments we investigated the effect of ALS on BT. ALS has no adverse effect on the intestinal mucosa. This may explain our results that no BT and

consecutive endotoxic effect were experienced as well as ALS does not increase the adverse effect on the intestinal wall of the agents – DAG or CPZ in our case – used in combination, i.e. no increased drug sensitivity develops.

In previous experiments we have demonstrated that mice with injured lymphoid system show an increased sensibility to further interventions in injury of the lymphoid system and effects of various interventions on the lymphoid system as for example the adverse effects on the mucous membrane of intestines of the combined use of cytostatics are summed up [11], and lead to BT occurrence with consecutive endotoxic effect [12].

From the present results one can conclude that ALS seems to be suitable for combined use with immunosuppressive agents because immunosuppression can be increased without adverse side-effects and thus without increasing BT.

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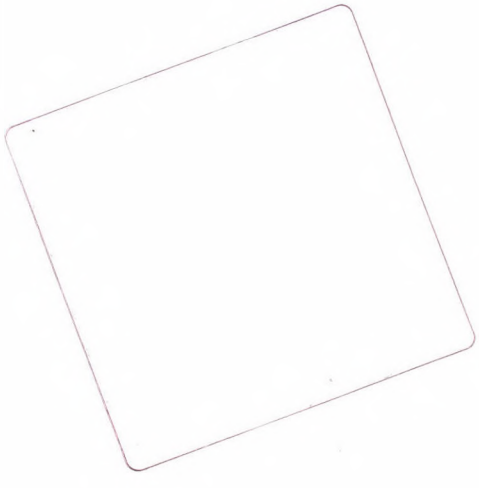

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FIFTIETH ANNIVERSARY OF THE INSTITUTE OF MICROBIOLOGY, SEMMELWEIS MEDICAL UNIVERSITY, BUDAPEST 1948–1998*

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In 1998 the Acta Microbiologica and Immunologica Hungarica (AMIH) commemorates the 50th anniversary of the Institute of Microbiology of Budapest, later Semmelweis Medical University (Budapest, Hungary) with a special issue. All members as well as their collaborators taking part in the research work of the Institute in the past and present were requested for publications to establish the success of this issue. We feel highly honoured by other collegues, too, who also have sent papers to this special issue.

In history 50 years represent a short period, but in life of a University Institute it is long enough for looking back to the past, to the multilateral activity, to the results achieved, among them the scientific results. The more timely this retrospection is as this period is characterized by a rapid world-wide progress of biological sciences. In consequence of this fact it seems to be highly opportunate to publish comprehensive summaries of some of the more important subjects in the scientific research work in the past or in the present of any members, coworkers or collaborators of the Institute of Microbiology, based on their previous literary activity.

During the past 50 years the Institute of Microbiology exerted comprehensive scientific activity in different fields of microbiology. The research work involved subjects of virology, immunology, bacteriology and parasitology. Virological research became, however, the chief tendency. Within this field a wide-range, comprehensive and more and more expanded study of adenoviruses was performed in the Institute in the last 40 years.

In the first years after the foundation of the Institute (1948–1950) the principal trend of the research work was directed, with the leadership of professor Ferenc Faragó (1948–1950 director of the Institute), to active and passive immunization and to elaboration of combined vaccines. This research work resulted in the preparation of tetanus toxoid for mass immunization, as well as of pertussis hyperimmune serum.

The first half of the '50s was dominated by the bacteriological research work. In this period the special line of scientific work became the leptospira research, leaded by professor Zoltán Alföldy (1950–1974 director of the Institute). In these years the regularities of leptospiroses in Hungary, the etiological role of leptospirae in meningitis serosa epidemics as well as the sources and other characteristics of leptospira infections were elucidated. In years 1951–1955 the scientific interest of aspirants for Candidate's

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degree (PhD) working in the Institute was drown towards basic general biological problems of this period, which they approached by bacteriological methods. The transformation of filterable forms to cellular forms of bacteria was studied. Possibility of humoral immune response induction by conditioned reflex and the effect of experimental tiring on the resistance of animals to infection was also investigated. These studies resulted in valuable PhD theses. After this period, from year 1956, adenovirus research in addition to other valuable immunological, bacteriological and parasitological research fields became the chief special scientific line of the Institute, leaded by Professor István Nász (member of the staff since 1948, the foundation year of the Institute).

Several results of the multilateral research work of the Institute have been appreciated on international and national level, some of the scientific findings are cited in handbooks, too. In the following papers only the results of certain selected topics are shown in chief lines – without any demand of completeness – in the form of summarizing articles based only on selected publications of the members and collaborators of the Institute. Detailed results of these topics can be found in the bibliographical data given at the end of the papers.

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Editor in chief of AMIH
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OUTLINES OF THE 42 YEARS ADENOVIRUS RESEARCH IN THE INSTITUTE OF MICROBIOLOGY, SEMMELWEIS MEDICAL UNIVERSITY*

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Adenoviruses, as it is well known, were discovered, due to the development of tissue culturing techniques, in year 1953, and the first reports dealing with them – except their description in 1953 – were published in the scientific literature in years 1954–1955. Thus, the adenovirus research group came into existence relatively early, in 1956, by the leadership of István Nász with coworkers Anna Lengyel and Margit Tóth. This group was the first to isolate adenoviruses in Hungary from tissue cultures of excised tonsils [1]. In the following years Pál Dán, Gizella Kulcsár and later Ida Cserba joined the group, which performed wide-range investigations to approach the adenovirus problem from different sides including aspects of general virology as well as of the demands of Hungarian health care. In the course of their work they endeavoured to perform besides basic research of theoretical significance – studies concerning the practice, problems arising directly from the pretensions of public health.

The triple significance of adenoviruses 45 years after their discovery

The significance of adenoviruses in medical sciences is mainly determined by their pathogenic effect. It can be supposed that among the viruses adenoviruses can produce the most various clinical pictures. In general respiratory, ophthalmological, gastrointestinal and urogenital infections are caused by them [2, 3, 5, 9, 10]. But they are able to produce several other diseases and infections of internal organs in almost all age-groups of the population. In the course of recognition and investigation or their role in latent infections

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and in malignant tumorigenesis it became evident that the pathomechanism is intricate and even today not elucidated in every respect. During the last ten-fifteen years it was recognized that in immunodeficient or immunosuppressed people, in organ transplant recipients and in AIDS patients they can produce very serious infections with high lethality. Data concerning the special relation and interaction between AIDS and adenovirus infection were also revealed which are to be explained and elucidated in the future. Adenovirus somehow aggravates and accelerates the course of the disease. Several new, formerly unknown serotypes and intermediate antigenic types of adenoviruses could be isolated from AIDS patients.

There may be an apparent contradiction between the pathogenicity of adenoviruses on the one side and two very hopeful possibilities on the other side, namely the use of adenoviruses in the treatment of different diseases and in the prevention of infectious diseases. A wide-range investigation is carried out worldwide of both possibilities, based on the fact that foreign genes can be built in the DNA of adenoviruses and the emerging recombinant adenoviruses can invade the different cells of organism as vectors, introduce the required genes, and there they are expressed and exert the intended effect. Recombinant adenoviruses were applied in a number of experiments for curative and preventive purposes [11, 12]. Adenovirus vectors are used woldwide in experimental gene therapy in hereditary gene deficiency and other complex diseases, in immune therapy and molecular therapy of different tumours. The combination of recombinant adenovirus technique and drug treatment ("pro-drug" system) seems to be promising as well as the specific destroying of tumour cells by genetically modified, non-recombinant adenoviruses. And if the expressed product of the inserted gene corresponds to a certain protective antigen of a microbe causing an infectious disease, than the organism produces antibodies after the expression, thus a significant part of infectious diseases would be prevented with such a "recombinant vaccine". The experimental administration of recombinant vaccines is also frequently used for the prevention of different infections, mostly of viral origin. To improve the results in the experiments, a general tendency is to construct so-called second generation recombinant adenovirus vectors. In this respect there is a double aim i.e. to prepare "targeted" vector, which invades only the intended cells by the modification of the cell-tropism of the virus, and to avoid the undesired effect of the host cell's immune response, which is directed against the antigen determinants (epitopes) of the recombinant vector and the vector-infected cells, thus diminishing the efficacy of the treatment.

The adenovirus research projects in the Institute

In the first ten years the adenovirus research group performed studies concerning primarily the manifold interrelationships between viruses and cells, thus the cytopathic effect, the haemagglutinating activity, the pathogenicity, the humoral aspects of immunology, serological surveillances and methods for promoting laboratory diagnostics. Besides continuing the studies on these fields, parallelly with the international research trends, the bulk of the studies were shifted over to fields of cellular immunity,

pathomechanism and structural and chemical properties of viral proteins. Studies on the antigenic structure of viral proteins and viral DNA [6–8, 10] were followed which have a perspective significance in respect of experiments concerning DNA manipulation, recombination and so in gene therapy and recombinant vaccination [11, 12]. During this time a number of new participants joined to the virus research group of the Institute in addition to coworkers mentioned earlier: Péter Medveczky, József Horváth, Éva Ádám, György Berencsi, Péter Geck, József Ongrádi, Éva Szomolányi and others.

In the last fifteen years the adenovirus research was focused essentially on three main fields: 1. Studies on the etiological and pathological role of adenoviruses on the level of different cells and of the organism, including the elucidation of pathomechanism and promotion of laboratory diagnostics. 2. Complex examination of the different structural proteins – penton, fibre and hexon – constructing the protein shell, the capsid of adenoviruses, determination of their physical and chemical characteristics, their structural organization, their biological functions and their epitope structure with special regard to the hexon. 3. Separation and comparative examination of adenovirus DNA, digestion with different restriction enzymes and determination of its restriction and physical map, cloning of its fragments in bacteria and provoke their expression. The latter two fields concern mainly to the study of molecular virology of adenoviruses.

*

Due to good international relationships fellowships were realized in many different institutions and countries (London, U. K.; Heidelberg, Homburg/ Saar and Berlin (Buch), G.F.R.; Moscow, and Kiew, UdSSR; Uppsala and Stockholm, Sweden; Sherbrook, Canada; Glasgow, Scotland; Lyon, France; Sofia, Bulgaria; Worcester, New York; Philadelphia and Tampa, USA etc.). Several research fellows were educated at the Szeged Biological Center of the Hungarian Academy of Sciences, and several graduate and postgraduate students spent years with the team. Several of them entered the team following their graduate studies.

The scientific achievements of the team were high relative to the available funding per person. At present several members of the group are working at a leading post in other research institutions in Hungary and all over the world and the members of their mother institute, their former coworkers, are delighted with their achievements gained in the field of virus research.

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INTERRELATIONSHIP OF VIRUSES AND CELLS. THE CYTOPATHIC PROPERTY OF ADENOVIRUSES*

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The application of *in vitro* cell cultures in virus research has a great importance in studies on the interaction between virus and cell. A great number of the viruses causes characteristic degeneration in the infected cells, well visible in the light-microscope, and due to their cytopathic effect the existence of viruses became directly detectable. Based on characteristic changes even the serological type of virus can be determined in some cases. Thus the multilateral examination of the interaction between virus and host cell has immense importance both in virus research and in therapy.

To study the cytopathic effect of adenoviruses on human amniotic and Detroit-6 cells sixty-three strains belonging to types 1 to 18 were examined [1, 2]. The examination of some of these strains was repeated after several years, too. On the basis of characteristics of their cytopathic effect on human amniotic cell cultures the virus strains could be divided in two groups. The difference appeared to be most characteristic between type 3 and type 5 strains, thus the different cytopathic changes were denoted as "type 3" and "type 5 degeneration". Of the sixty-three strains forty caused type 3 and twenty-three type 5 degeneration. In type 3 degeneration the cell culture became network-like and later grape-like groups of cells are formed. In type 5 degeneration the cells are completely separated from each other, the structure of the cell culture becomes loose, the cytoplasm of cells disappears and mostly solitary nuclei can be found coated with cytoplasmic residues [3]. The characteristic cytopathic effect of the strains proved to be a stable feature and could be demonstrated in repeated experiments, in some cases after several years of laboratory maintenance [4]. Most of the strains of identical serotype exhibited identical cytopathic effect, nevertheless different cytopathic effects within strains of the same serological group occurred, too. The degenerative changes caused by

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the viruses involved the nucleus and cytoplasm equally and the differences between the two kinds of degeneration could be demonstrated both in nucleus and cytoplasm. In the course of infection various morphological structures developed in the nucleus resulting in most cases in a so-called "central mass" at the end of the process. Parallelly, the eosinophilic staining of these morphological structures became basophilic. The different nuclear changes were in general positive with Feulgen reaction.

It was demonstrated by immunofluorescent method that the antigenicity of the morphological structures arising in the nuclei due to the virus infection is identical with that of adenoviruses, which suggests that the site of adenovirus multiplication in amniotic cells is the nucleus. It was found that the development of viruses or rather the specific viral antigens begin 7 to 10 hours after the infection [5].

The behaviour of virus strains in human embryonic kidney cell cultures was investigated, too [6]. The virus strains studied were found to have characteristic cytopathic effect and could be readily subcultured in these cell cultures. The degenerative changes caused by type 5 strains in individual human embryonic cells had been investigated in details. Changes were localized primarily to the nuclei of infected cells [7]. The initial change was an eosinophilic granulation, either diffuse or in form of sometimes round-shaped aggregates in the nuclei which became swollen. The granules through several intermediate stages progressed to a basophilic central mass. Later the whole cell became shrunken, the cytoplasm decreased, and finally the rounded cells detached from the glass. In addition to these changes in the degeneration process often were found nuclei containing 20 to 30 eosinophilic corpuscles or 2 to 3 large eosinophilic bodies surrounded by a basophilic ring. Sometimes 2 or 3 rod-shaped, crystal-like body occurred in the nuclei of infected cells.

Cytopathic changes caused by adenoviruses in HeLa, Detroit-6 (adapted to different sera) [8], human embryonic liver and lung, KB, permanent human amniotic, primary human fibroblast, monkey-, rabbit-, pig- and calf-kidney cell cultures were also investigated. In the course of virus isolation experiments with excised tonsils – which led to the first successful demonstration of adenoviruses in Hungary –, data were revealed concerning the cytopathic effect of certain adenovirus types in tonsil and adenoid tissue cultures.

Some of the frequently used tissue cultures mentioned were examined for their susceptibility to adenoviruses. In these experiments human embryonic kidney cell cultures proved to be the most susceptible both in respect of effective virus quantity, and the temporal appearance of the cytopathic effect.

In studies on the susceptibility of Detroit-6 and human amniotic cells adapted to different heterologous sera, it was found that their susceptibility to adenoviruses did not change significantly after adaptation [8].

In the course of experiments on the cytopathic effect of adenoviruses it was observed that under identical conditions the time required for the development of cytopathic effect was influenced to some extent by the age of the cell cultures applied. Comparative experiments were made therefore with 18 different virus strains belonging to the first 16 serotypes and 14 strains belonging to adenovirus type 8 on the adenovirus susceptibility of 5–10 days old (young) and 28–31 days old (old) primary human amniotic cell cultures. Using identical virus doses of these strains for the infection the onset of

cytopathic effect and the complete destruction of the cells manifested earlier in the old cultures than in the young ones. In some cases the virus yield was higher in the old cultures than in the young amniotic cells. According to these experiments, the relative "resistance" of the young primary amniotic cell cultures lasted until about their 3 weeks age [9].

The cytopathic properties of type 8 adenovirus strains were separately investigated, as these strains sharply differ from the other members of adenovirus group in respect of several biological characteristics. In contrast to other serotypes the type 8 strains never produce in the supernate of human amniotic cell cultures higher yields than 100–300 TCID/50.

The correlation between the cytopathic effect and the adsorption time was studied in details, too. Among adsorption times 30, 60, 90, 150 and 300 minutes, in contrast to results of other authors on HeLa cells, in human amniotic cell cultures the 300 minutes adsorption time was optimal. Using identical virus doses both the onset of cytopathic effect and the complete destruction of cells occurred earliest by this adsorption time.

Using the optimal adsorption period and inocula of 1, 2, 4, 8, 20 and 40 TCID/50 three zones could be distinguished in the onset of degeneration and its complete development in primary amniotic cell cultures. With the decrease of virus doses gradually more and more time was required: 1–2 days, 3–4 days and about 7 days for the onset of degeneration and its progression by 25% [10]. This phenomenon refers to the fact that in case of doses mentioned the linear relationship between virus dose and cytopathic effect described for other adenovirus types is not valid for type 8. This is a possible explanation of the difficult and time-consuming isolation of type 8 adenovirus strains.

In human amniotic cell cultures the effect of 5-iodo-2-deoxyuridine on type 8 adenovirus was investigated. In 0.05% solution it did not damage the cells, while it delayed or in some cases inhibited the cytopathic effect and the virus multiplication [11, 12].

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THE HAEMAGGLUTINATION SPECTRA OF ADENOVIRUSES AND FACTORS INFLUENCING THE HAEMAGGLUTINATING ACTIVITY*

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Certain serotypes of adenoviruses possess a direct haemagglutinating ability for erythrocytes of certain mammalian and avian species. The serotypes of human adenoviruses can be divided in three haemagglutination (HA) subgroups on the base of the reaction with rhesus and rat erythrocytes. Types belonging to subgroup I are agglutinating rhesus erythrocytes. The members of subgroup II cause complete HA of rat erythrocytes, while types of subgroup III induce a partial agglutination of rat erythrocytes. Except a few experiments with erythrocytes of some laboratory and domestic animals there was no comprehensive knowledge on the haemagglutinating properties of adenoviruses, though it is obviously of great importance not only for laboratory diagnostics but in immunological and pathogenetical respect, too. This suggested to perform a series of up till now unique experiments to determine the HA spectra of adenoviruses.

HA experiments were made with forty-two virus strains, examined also for their cytopathic effect, belonging to the first 16 serotypes of adenoviruses using the erythrocytes of 97 different mammals and birds from the zoological garden, as well as of some laboratory animals and human erythrocytes of different blood groups.

Erythrocytes of 26 bird species out of 47 were agglutinated by one or more adenovirus strains, the titres varied between 1:16 and 1:128. Erythrocytes of only 7 out of 33 mammals of the zoological garden were agglutinated by one or two strains at low titres. Experiments with erythrocytes of 17 birds and mammals of the zoological garden could not be evaluated from various reasons. Of laboratory animals no HA was found with rabbit erythrocytes, while a few strains agglutinated the erythrocytes of guinea-pigs and white mice at titres of 1:16 to 1:32. Testing 38 rats considerably high titres, 1:128 to

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1:512, but in some cases even 1:16384, were found. In tests with 56 human erythrocytes in general titres of 1:16 to 1:64 were obtained with strains belonging to types 9, 10, 13 and in some cases type 15, without any relevance to blood groups [1].

No species was found having erythrocytes agglutinable with all virus strains tested. Of the 42 strains examined 14 did not haemagglutinate at all and an other considerable group agglutinated only one or very few erythrocyte species at rather low titres. Only 6 strains agglutinated the erythrocytes of more then 5 species. The HA could be always inhibited by specific immune sera [2].

The results indicate that HA with adenoviruses is a specific but complex and in several respects unelucidated phenomenon. Erythrocytes well agglutinable by some strains are agglutinated only in very low titres or not at all by others. Under identical experimental conditions the same erythrocytes are well agglutinated by certain adenovirus types and scarcely or not at all by strains belonging to the same serotype. Furthermore the erythrocytes of different individuals of a given species display different HA titres with an identical virus strain.

Correlation was found between the type of cytopathic effect and HA. Namely adenovirus strains causing type 3 degeneration were much more active in HA, agglutinating the erythrocytes of far more mammals and birds than those causing type 5 degeneration.

The influence of certain factors on the haemagglutinating activity of adenovirus was studied, too, especially the influence of temperature and pH. The experiments were made with adenovirus types 9, 10 and 13, which cause the complete HA of rat erythrocytes. The 145 experimental series were carried out in 6 different pH-s (between 6 and 10) on 5 different temperatures (4 °C, room temperature, 37 °C in incubator, 37 °C and 40 °C in waterbath). With all serotypes 4 °C and room temperature proved to be optimal. In more than 500 titration series in pH range 6.0 to 10.0 HA titres were the lowest at pH 6.0, they grew gradually, reached the maximum at pH 9.0, then their value decreased at pH 10.0 [3]. During storage the sensitivity of erythrocyte suspensions to adenovirus haemagglutinins increased to some extent between pH 7.0 to 9.0. Though the differences observed in these experiments regularly occurred, their practical importance seemed to be doubtful being their order within the limits of error of the methods employed [4].

Further on manifold studies were made for the characterization of viral components playing role in the HA process. Experiments were carried out with components of type 7 "Pinckney" strain, which belongs to subgenus B of human adenoviruses and proved to be oncogenic in animals. By fractionation with anion-exchange chromatography and ultracentrifugation, a soluble, heat- and trypsin-sensitive, type-specific haemagglutinin was separated, which induced the complete agglutination of monkey erythrocytes and became eluted from them at 4 °C. This haemagglutinin corresponded to the soluble component arising from aggregated penton antigens, later described for several other adenovirus types as the dodecon [5].

Dodecon, the soluble complete haemagglutinin was separated from several serotypes of the most numerous subgenus D of human adenoviruses and its properties were examined by different methods. In these experiments types 8, 9, 10, 13, 15, 19 and 23, as well as intermediate types 9/15 and the newly described 9/13 [6] were involved. By

zone-electrophoresis of six strains the soluble haemagglutinin of three, and the non-soluble but also complete haemagglutinin, represented by the virion of four strains could be separated [7]. More detailed investigation of adenovirus type 8, causative agent of epidemic keratoconjunctivitis using combined density gradient centrifugation, zone-centrifugation, anion-exchange chromatography and gel-filtration had shown that the virus possess three soluble haemagglutinins: one complete (dodekon) and two incomplete ones. The latters agglutinated only in the presence of appropriate heterotypic immune sera. One of them proved to be type-specific and trypsin-resistant, this represented the fibre antigen, while the other was trypsin-sensitive and cytotoxic, this corresponded to the penton antigen. Trypsin treatment converted the properties of the latter into that of the fibre antigen [8]. Similar results were obtained in experiments with type 9 and type 19 [9, 10], with the difference that the latter possessed only one incomplete haemagglutinin, which corresponded to the fibre antigen. In experiments with the penton antigen of adenovirus type 8 it was found to induce interferon in chick fibroblast cells in form both of complete and in incomplete haemagglutinin [11].

For better understanding the mechanism of HA cell receptors were examined, too. The acethylcholinesterase activity of erythrocytes was not influenced by viral HA [12]. Adenoviral haemagglutinins could be eluted from the erythrocytes, thus they bind to the neuraminic acid similarly to influenza viruses [9]. Lipid changes of erythrocytes due to adenovirus adsorption, however, proved to be heterogeneous. Lipid changes of human erythrocytes treated with adenovirus serotypes belonging to different subgenera and with type A and B influenza viruses were analysed by thin-layer chromatography. Several qualitative and quantitative changes were observed, which manifested in disappearance of certain compounds or development of new ones, changes of Rf values (migration) and staining characteristics [13]. Some of these changes were restricted to single serotypes, others were induced by two or more types and some characterized all adenovirus types, furthermore lipid changes induced by all adeno and influenza viruses tested were observed, too, though the basis of certain apparently uniform biological phenomena may be significantly deviating on the molecular level [14].

In the culture of type 12 human adenovirus a parvovirus strain with active haemagglutinating activity was found. Based on the adsorption of human erythrocytes combined with other techniques a method was developed for isolation and purification, and a new non-defective parvovirus strain was described [15].

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SEROLOGICAL AND VIRUS ISOLATION EXPERIMENTS ON THE INCIDENCE OF ADENOVIRUSES IN HUNGARY*

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To study the incidence of adenovirus infections in Hungary about 2000 human single or paired sera were examined by complement fixation (CF), neutralization and haemagglutination inhibition (HI) tests.

Six groups of sera were examined by CF test with the results as follows:

i) sera of children convalescent after poliomyelitis (age 0 to 10 years) positive in 48.2%, *ii)* sera of 10 to 20-year-old pulmonary tuberculosis patients positive in 24.5%, *iii)* sera of 18 to 20-years-old healthy persons positive in 14.7%, *iv)* sera of healthy adults over 20-years of age positive in 13.6%, *v)* sera of patients with different non-respiratory diseases positive in 42.4% and *vi)* sera of patients with acute respiratory illness positive in 48.2%. The rate of incidence of positive sera generally decreased over 40 to 50 years of age [1, 2].

In neutralization tests with the first seven serotypes of adenoviruses antibodies to types 1, 2 and 7 were the most frequent and to types 3 and 4 the rarest. The sera contained antibodies to one or two serotypes in 19.5 and 18.2%, respectively. About 9% of sera neutralized 3 to 5 serotypes, while antibodies against all seven types tested were found only in 2.7% [3, 4].

The incidence of HA-inhibiting antibodies was examined in sera of healthy persons and of patients recovered from epidemic keratoconjunctivitis (EKC) to types 8, 9, 10, 11 and 16, and in case of healthy persons to type 13, too [5]. Sera of EKC patients gave positive results in more than 90% with prototype strains 8 and 9, in 68% with type 10 and in 32% with type 11 [6]. The titres of HI antibodies were found to be higher 4–6 months after the infection than one month after it and even 1 year after the disease high titres were detected. Examination of paired sera of EKC patients revealed a heterotypic HI

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reaction [7, 8]. With adenovirus type 16 no antibodies were detectable in sera either of EKC patients or in that of healthy persons. The latter were reacting with types 8, 9, 10 and 13 in 42–47%, while with type 11 in 15%.

In the course of a 6 years long survey of healthy persons and different patient groups using different serological methods the wide spread incidence of adenoviruses in Hungary had been proved [9].

Studying the epidemic of EKC in Budapest during winter 1961–1962 attempts were made to elucidate the questions both of etiology and of laboratory diagnostics in Hungary. Virus isolation experiments were made with conjunctival swabs of 58 patients on Detroit-6- or HeLa cell cultures and 9 virus strains were isolated which proved to be adenoviruses by CF tests. HI and neutralization tests revealed that 7 of the strains belonged to serotype 8 and 2 of them to serotype 6 of human adenoviruses [10]. These results confirmed the observation that typing of adenovirus strains can be accomplished exclusively by HI tests [11].

The etiological role of the isolated type 8 strains in EKC was confirmed by the serological examination of the patients' paired sera. Of patients included in isolation experiments 38 paired serum samples were available for neutralization, CF and HI tests. In 78% of convalescent sera an increase of antibody level to type 8 with CF and neutralization tests was found, but in about 50% of these cases the increase remained under a fourfold rise. With HI test, however, 94% of the samples had shown significant (at least fourfold) rise of antibody level. In these experiments the titres to adenovirus type 8 were in average considerably higher in HI test than in neutralization and CF tests [10].

Sera of 350 healthy persons and 122 KCE patients taken 1 to 12 months after the infection were examined by HI test [6] with one strain of the type 8 adenoviruses isolated from the epidemic. Sera of healthy persons contained in 37%, those of EKC patients in 96% HI antibodies.

By immunofluorescent method the antigenic properties of EKC inclusion bodies corresponded to those of type 8 adenovirus and the inclusion bodies consisted partly or totally of infective virus particles [12].

The results of HI tests, supported by those of neutralization and CF tests, as well as the immunofluorescent experiments indicate that type 8 adenovirus could have been regarded as the causative agent of the EKC epidemic. This was confirmed by the fact that three persons who got accidentally infected with one of the adenovirus type 8 strains isolated from the epidemic developed typical symptoms of EKC [13].

The experiments on EKC demonstrated – supposedly for the first time – that HI antibodies develop is humans as a consequence of adenovirus type 8 infection and that the HI test is a suitable method for diagnostics with several advantages over the neutralization and CF tests [11].

The wide spread incidence of adenoviruses in Hungary was supported by further virus isolation experiments in collaboration with the Clinics of Semmelweis Medical University and other medical institutions on patients suffering from different diseases. For instance in a community of young adults the causative agent of a tonsillo-pharyngitis epidemic was verified as adenovirus type 14 by virus isolation, serological and immunofluorescent methods [14]. This was the first instance in Hungary to prove the etiological role of adenovirus type 14 in an epidemic of acute upper respiratory disease.

On the course of a conjunctivitis outbreak in the II. Clinic of Ophthalmology, Semmelweis Medical University, the, adenovirus type 6 had been proved as etiological agent and the transmission of infection, by a collectively used eye-drop (Naphasolin) was revealed, too [15, 16]. Of specimens of the Clinic of Oral Surgery type 1 adenovirus was observed in the scrapings of recurrent aphthae, this finding was unique in the scientific literature [19]. Several adenovirus strains were successfully isolated from gynaecological cases, appendicitis and different other diseases and the significance of these findings were evaluated [18–21].

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CHARACTERIZATION OF THE MAIN PROTEIN COMPONENTS OF ADENOVIRUS VIRION AND ITS POSSIBLE USE IN LABORATORY DIAGNOSTICS*

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A complex method had been developed for the detailed examination of structural proteins of adenoviruses, for separation, purification and concentration of certain proteins. This included the combination of different ultracentrifugation methods (density gradient and zone centrifugation), repeated anion-exchange chromatography (DEAE Sephadex A-50 and A-25) and gel-filtration (Sephadex G-200) [1]. The molecular weight, isoelectric point, migration velocity in electric field (PAGE), amino acid composition had been determined [2]. A penton-associated endonuclease had been revealed [3].

Electrophoretic methods had shown considerable relative divergence between the hexons of serotypes belonging to different subgenera, which finding was supported by the numeric values of electrophoretic mobility [4]. The diversity of the physico-chemical properties was indicated also by the finding that the hexons of different serotypes were eluted by different concentrations of NaCl from the columns in anion-exchange chromatography experiments.

Comprehensive investigations were carried out to study the antigenic structure of adenoviruses. Of an oncogenic type 7 adenovirus strain the soluble antigen fractions obtained by chromatography and cesium chloride equilibrium centrifugation had been analysed [5]. In chromatography the group-specific A (hexon) antigen was eluted first, followed by the type-specific haemagglutinin and a small amount of type-specific C (fibre) antigen. This order was reverse in comparison with the fractions of type 2 and 5, but identical with that of the similarly oncogenic type 12. The haemagglutinin and the

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infective virus could be separated from each other by ultracentrifugation and electron microscopy.

For investigation of the antigenic properties of purified and concentrated adenovirus proteins with sera of rabbits immunized with purified proteins complement fixation, gel diffusion precipitation, immuno-electrophoresis and a newly developed electrophoretic method, immuno-osmophoresis [4, 6] were applied.

By the immuno-osmophoresis technique the agar-gel precipitation bands produced between antigenic components separated, purified and concentrated by repeated chromatography on DEAE Sephadex A-50 columns and specific immune sera against them had been determined. By these methods adenoviruses can be diagnosed in 15–60 minutes. Based on this technique two methods had been developed which are applicable for examination of antigens migrating in the electric field toward the negative pole if their migration is slower than that of immuno globulin in the given experimental conditions. These are the "two step two way immuno-osmophoresis" and the "two step post migration immuno-osmophoresis". By these methods several antigenic components could be identified which migrate in standard osmophoresis toward the negative pole and thus avoid the meeting with the antibody [4, 6]. Supposedly the use of these methods can succeed in revealing antigenic components of adenoviruses not yet reported or determined. Publications on these works had aroused interest abroad, too. Among others the Center of WHO in Geneve offered its help for the continuation of this work, and from Ivanovsky Institute for Virus Research (Moscow) a collegue arrived for a longer stay in the Institute to perform studies on a common research subject [7].

Based on this technique a micromethod had been developed for detection of very small amounts of several different soluble antigens in very short time and the reaction had been found more sensitive than agar gel diffusion precipitation, thus it can be applied for rapid diagnostic purposes [1].

The purified hexons of different adenovirus types had shown only partial identity by the serological methods mentioned, which manifested in the phenomenon that immune sera could be completely absorbed only by homologous antigen. Absorption with heterologous antigen diminished the reactivity of sera only toward heterologous hexon types, but not to the homologous one, even in case of surplus heterologous antigen.

It was determined that the hexon, penton and fibre antigen of type 1 human adenovirus is characterized by diverse isoelectric points and amino acid composition [2]. Isoelectric points of hexon and penton are in the acidic domain, while that of fibre is slightly alcalic. The analysis of amino acid composition showed that in the hexon more asparagic acid can be found than in penton and fibre, while in the latters the concentration of lysine is higher. Hexons of types 1, 5, 7, 8, 9 and 19 were examined by chromatography and the elution properties of serotypes belonging to different subgenera were found to be different from each other.

Besides the physico-chemical differences it had been shown with appropriate serological methods that the hexon antigens of different serotypes, sooner regarded to be "common", exhibit only partial identity and contain type-specific components, too.

Experiments were made to compare the hexons of the supernate and of cells of virus cultures [8] and the polypeptides and immunoreactivity of empty viral capsids [9].

For the analysis of immunoreactivity of hexon antigens a passive haemagglutination technique had been developed by the use of tannic acid treated sheep erythrocytes coated with purified hexon, a method with sensitivity similar to that of ELISA [10], and proved to be useful for the examination both of monoclonal and of polyclonal antibodies, thus for diagnostic purposes [11]. A latex agglutination method had been also developed and used for investigation of the epitopes of hexons as well as the reactivity of polyclonal immune sera. With latex particles coated with purified hexon the antibody level of polyclonal sera can be determined, the sensitivity of the reaction corresponds to or exceeds in some extent that of complement fixation test, but it is much more simple and rapid (4–5 minutes), thus might be a useful tool in diagnostics [12]. Latex particles coated with a monoclonal antibody of appropriate reactivity proved to be a sensitive tool with high specificity for detection of small quantities of adenovirus antigens, thus they can be used in laboratory diagnostics, too [13].

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PATHOMECHANISM OF ADENOVIRUS INFECTIONS*

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The immunological studies on the pathogenetic role of adenoviruses following the multilateral investigations of humoral immunity were transferred later to those of cellular immunity, to studies on the pathomechanism of adenovirus caused diseases with uncleared etiology or probably polyetiological ones in which the classical virological criteria as virus isolation and significant increase of antibody level in the blood cannot be found in relationship with the etiological role of adenoviruses.

The role of lymphocytes

As it is well known certain serotypes, mostly types 1, 2, 5 and 6 of adenoviruses – similarly to herpesviruses – can be present latently in the organism, first of all in the lymphatic organs, from where they can be isolated only rarely, using special and long-lasting techniques. In the same time the specific viral protein-antigens can be detected in the cells of local lesions or in the circulating lymphocytes, or the latters can be sensitized toward the respective viral antigens. In diseases of supposed viral etiology the role of viruses in the accomplishment of pathological changes cannot be proved unambiguously by conventional virological methods, the approach to elucidate etiology is possible only with the introduction of novel analytical procedures as detection of virions or specific viral antigens in the cells by immunofluorescence assay or electron microscopy, as well as the demonstration of the patients' sensitized lymphocytes by transformation tests with the respective viral antigens inducing lymphoblastic transformation of the cells [1, 2].

The possible pathomechanism and immunobiological background as well as the correlation between the immunological functions and latent carriership of viruses in the

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supposed adenovirus infections were studied in several different diseases and clinical manifestations.

It was found that patients suffering in different recurrent diseases, like recurrent ulcers of oral mucosa, asthma bronchiale, uterocervical metaplasia, irregular gynecological haemorrhage, grippe abdominalis, colitis ulcerosa etc. can be considered as latent virus-carriers and adenoviruses, mainly the latent serotypes – and often also herpesviruses – are present in the cells of local lesions and in the patient's circulating lymphocytes. From these cells infective viruses had been isolated in low percentage [3, 4]. A much more characteristic phenomenon was, however, that in spite of failing to cultivate infective virus from the patients, specific viral antigens were present in high percentage in the cells of local lesions or in the circulating lymphocytes detected by immunofluorescent methods. The sensitization of lymphocytes toward the respective viral antigens was also characteristic [5–15].

These investigations were made with manifold methods on more then ten thousand different clinical specimens of about three thousand patients belonging to different disease-groups. The incidence of adenovirus antigens and sensitization of lymphocytes in patients of different diseases and in healthy control persons given for better understanding in percentage, was the following: in aphtha recidivans carriership of viral antigens was 63% in patients and 3% in controls, lymphoblastic transformation in patients 43%, in controls 5%. Infective virus could be also successfully cultivated and virions were detected by electron microscopy in cells of local lesions and circulating lymphocytes. In colitis ulcerosa patients viral antigen-carriership was found in 71% in contrast to the 4% of controls, while transformation of lymphocytes was in patients 80%, that in controls 6%. In grippe abdominalis viral antigen carriership occurred in 65% of patients and 13% of controls. Infective viruses were cultivated form the appendix, lymph nodes and pharyngeal lavage. In Down's syndrome antigen carriership was found in 45% of patients and in 13% of controls, while the rate of positive blastic transformation was 73 and 35%, respectively, and infective virus was also found in the patients saliva. In cases of irregular gynecological haemorrhage the antigen carriership was 72% in patients and 1% in controls and from abrasion material infective virus was isolated. In uterocervical disturbances viral antigens were found in 44% of patients and 16% of controls.

It is obvious that the occurrence of demonstrable viral antigens and lymphoblastic transformation was significantly higher in the patients than in the healthy controls. In patients suffering from recurrent oral ulcers in more than half of the cases viral particles were observed in local lesions by electron microscopy, but even in apparently healthy mucosa cells, too.

It is remarkable that antigen presence and lymphocyte sensitization of control groups was only in children (grippe abdominalis, Down's syndrome) over 10%. The only exception was the 16% occurrence of viral antigens in healthy persons' cervical cells. On the other hand there are observations that the sooner apparently healthy persons became sometimes the later patients.

From these data direct etiological conclusions can be drawn only for certain diseases, i.e. grippe abdominalis. In these patients seroconversion was found, too. In other diseases the antigen carriership and lymphoblastic transformation give a basis for the supposition that the increase in viral antigens might be the cause of pathogenic alterations,

but the possibility that the pathogenic condition had given ground to the increased virus production could not be excluded [1].

Considering that in the diseases investigated adenovirus and herpesvirus often occurred simultaneously human amniotic cell cultures were doubly infected with adeno- and herpesviruses to determine whether in a culture the two viruses are able to multiply in the presence of each other. The time shift of the two infections corresponded to the individual multiplication rates of the viruses. Cells analysed by immunofluorescence indicated that the two viruses are able to replicate in the same cell culture, even in the same cell. Viral antigens were traced by immune sera produced in rabbits and conjugated with fluorescent stains of different colours. Thus anti-adenovirus serum, conjugated with fluorescein-isothiocyanate gave yellow-green, while anti-herpesvirus serum conjugated with rhodamin gave orange-red fluorescence in the course of binding to the respective antigens in the cell. But the presence of both kinds of antigens could be proved by their localization, too: adenovirus antigens were found mostly in the nucleus, while herpesvirus antigens mostly in the neighbourhood of nuclear membrane in the cytoplasm of cells [16].

The phenomenon observed in patients concerning the high affinity of adeno- and herpesviruses to lymphatic elements and that a permanent virus-carriership can develop had been confirmed by infection of unsusceptible experimental animals, too. In intravenously inoculated rabbits the localization of viruses had been traced by cytological and immunofluorescence methods and by electron microscopy. It was found in case of both viruses that they appear first in the circulating lymphocytes, subsequently in the thymus and spleen and only very rarely in other organs. Adenoviruses were detectable in the lymphatic organs of animals even after 60 days [17–20].

Further data concerning the pathomechanism of adenovirus infections were obtained by incubation of mononuclear leukocytes of healthy persons *in vitro*. Latent adenoviruses could not replicate in infective form, but the antigens were detectable. However, if the cell was previously stimulated by phytohaemagglutinin infective adenoviruses could replicate in them. This mechanism may occur also in the human organism. In latent virus-carriers the disease or recurrence can manifest itself if some factors induce the stimulation of lymphocytes. This way virus-carrier lymphocytes may play an important role in the pathomechanism of diseases caused by adenovirus or other viruses. With respect on the oncogenic potential of adenoviruses the possible role in oncogenesis of viruses or their components with long lasting presence in the organism should be considered [5, 7].

Reactivation of latent adenoviruses and their possible role in oncogenesis

Experiments concerning the pathomechanism of adenovirus infections had drawn the attention repeatedly to the observation that lymphocytes of a significant part of healthy persons – about 15–20% – displayed the presence of latent adenoviruses. DNA of latent adenovirus types was integrated in the cellular nucleic acid or was found as free viral DNA. In several cases, only a fraction of the whole genom was integrated. It had

been also observed that in certain circumstances the latent viruses become reactivated and the apparently healthy virus-carriers became sick [1, 21, 22].

For further investigation of latency and the possibility and mechanism of reactivation arteficial latent virus-carriership was produced in HEp-2 cells with adenovirus type 5 and with two temperature-sensitive mutants ts18 and ts19 *in vitro*. Studying of several chemical and natural compounds the native and radiodetoxicated endotoxin of *E. coli* 089 was found to be able to reactivate all the three viruses on permissive 32 °C temperature, thus it induced virus replication from the latent state. According to the experimental results reactivation of the wild strain and one of the ts mutants could be induced with type F prostaglandine (PGF-2α), supposedly by promoting phosphorylation of viral proteins [23, 24].

The *in vitro* effect of adenovirus on healthy human lymphocytes was investigated, too. It was found that adenovirus type 5 induces a considerable decrease of E-rosette formation and reduces to about one tenth their stimulability with phytohaemagglutinin, thus their blastic transformation. The virus-infected lymphocytes produce lymphokines, which diminish the phagocyte function of polymorphonuclear granulocytes [12, 25, 26]. These findings indicated that adenovirus infection can influence many important properties of lymphocytes leading to different immune disturbances which may have a role even in tumourigenesis with special regard to the oncogenicity of the virus and to its latent presence in the organism. Namely all adenoviruses have an oncogenic DNA region on the left side of the genome and its organization is approximately similar in every adenovirus type. The oncogenic region consists of two parts, called E1A and E1B. The E1A region contains regulatory genes functioning in the immortalization of cells and taking part in the development of malignant transformation, while E1B region encodes the early proteins maintaining the completely transformed phenotype [27–30]. These early antigens are not parts of the virus, but are virus specific and there are specific antibodies to them in the blood of tumour-bearing organisms.

Incidence of antibodies to adenoviruses was examined in the sera of more than 500 patients, most of them with urogenital disorders. In 53% of patients suffering from malignant urogenital tumours antibodies to the early antigens of the highly oncogenic type 12 adenovirus have been found. Other urological patients displayed 18%, while patients from other disease groups 4% of the same antibodies [29, 31, 32].

In vitro complementation experiments were also performed in tissue culture with the temperature-sensitive mutants mentioned by addition of tumour extract simultaneously with the virus. Temperature-sensitive mutants can namely multiply on restrictive 39 °C temperature only when somehow they get the protein missing due to the mutation. Complementation was successful nearly in 60% of cases of histologically proved bladder and kidney carcinomas, in spite of the fact that the infective virus was never revealed in the tumour cells. Complementation was successful in 23% of non-malignant urological control cases. These data did not allow to draw etiological conclusions, only a continuous operation of a region of adenovirus genome could be assumed, resulting in the production of certain viral proteins in the respective tumour cells. This could explain the successful complementation, while the fact that infective virus particles are not produced in the tumour cells could be explained by the lack of the existence of the total viral genome in them [33–35].

Investigation of problems mentioned above called the attention to the possible role of viruses in abortion and male infertility, too [36, 37].

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STUDIES ON TUMOUROUS AND OTHER UROGENITAL PATIENTS WITH RESPECT TO ANTIGENS OF ONCOGENIC ADENOVIRUS*

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The possible connection of viruses with tumours was investigated by serologic examinations. Concerning the presence of antibodies against adenoviruses, especially those against the early non-virion antigens of oncogenic adenovirus type 12, approximately 4000 tests were made with sera of 446 urogenital patients with and without tumours and 70 ones with internal diseases. It was found by complement fixation tests that antibodies against non-virion antigens of adenoviruses were present in 53% of urogenital patients suffering from malignant tumours and prostatic hypertrophy, in 18% of non-tumourous urological patients and in 4% of patients with internal diseases, respectively. The results suggest that adenoviruses may play a role in tumourous diseases of the urogenital organs.

Since the beginning of this century over 60 viruses have been proved to have tumourigenic capacity. They can induce benign and malignant tumours in a wide variety of animal species [20–22]. One of the recent advances in tumour – virus research has been the finding that human adeno- and herpesviruses are also capable to induce malignant transformation of human and animal cells under experimental conditions [6, 14, 16, 17, 19, 23–25]. Though there are numerous observations and studies suggesting that the oncogenic feature of these viruses can also be effective in humans, no direct and unambiguous evidence has yet been found. The verification of virus etiology has only been successful so far in some human benign tumours, for example verruca vulgaris, condyloma acuminatum, molluscum contagiosum and certain papillomas. In case of

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malignant tumours one of the reasons for the lack of direct proof may be that obtaining infectious virus from human malignant tumours is usually without success, just as infectious virus particles cannot be found in cells that are made experimentally malignant by human adeno- and herpesviruses [22, 26]. In the latter cells, however, there are always substances of protein character present that evidence direct connection with the virus (T-antigens and early non-virion antigens that are meant to be virus "fingerprints"). This explains the effort to demonstrate in human tumours, too, the existence of such tumour-specific protein components or antibodies produced against them. Concerning herpesviruses such experiments have become almost conclusive in certain cases of tumours [7, 11, 12, 18, 27]. Similar data relating adenoviruses can hardly be found in the available literature.

For this reason studies have been performed as to whether antibodies against latent and oncogenic adenovirus types occur, and if so in what proportion, in sera of patients suffering from malignant or benignant urological tumours, prostatic hypertrophy and other urological diseases as well as in the relevant control sera. Furthermore, studies were carried out in search for antibodies against the early non-virion and tumour-specific antigens of viruses.

Results

The sera of over 500 patients and controls were tested; the majority with eight different antigens, thus about 4000 tests were performed. A total of 516 patients were grouped according to their diagnosis as follows: 253 cases of urogenital tumours, 193 cases of non-tumourous disease of the urogenital system, 70 cases with inflammatory internal diseases (control group).

The urogenital tumours consisted of 57 bladder, 32 prostate, 15 kidney and 23 other types of malignant tumours and regarding the benign tumours 22 urethra polyps and 89 prostatic hypertrophies completed the 253 cases.

Sera were examined with complement fixation (CF) test and the following viruses or early antigens were used: adenovirus type 1 and 5 (latent serotypes), 12 and 18 (oncogenic serotypes) as well as the early non-virion antigens of the same serotypes.

In the CF tests almost identical results were obtained with types 1 and 5 viruses of latent character; with the oncogenic types 12 and 18 we got very similar results.

In case of urogenital tumours the antibodies against the early non-virion antigens of the oncogenic adenovirus type 12 as well as adenovirus type 1 of latent character occurred in 53% and 56%, respectively. In case of the type 12 virus this frequency was 18% regarding the non-tumourous urogenital patients and 4% in case of the controls with internal diseases. In case of the type 1 adenovirus 23% of non-tumourous and 33% of the control patients had antibodies against the early antigens.

It deserves attention and supports the specificity of the studies that antibodies against the structural antigens of adenovirus type 12 were observable in only 13% of tumorous patients, in 6% of patients suffering from non-tumourous diseases of the urogenital system and in 2% of patients with internal diseases. Antibodies against the

structural antigens of adenovirus type 1 were found in 34% of the non-tumourous patients. This can be explained partly by the tendency for latency of this virus and partly by the fact that these patients were younger than those with tumours. The same may also relate to the occurrence of antibodies to early type 1 antigens in case of control patients suffering from inflammatory diseases.

Discussion

The possible connections of benign and/or malignant tumours with viruses have been approached by means of manifold investigations [13–15, 18, 21].

Viruses and their antigens, nucleic acid fragments or genes, enzymes related to the viruses in the tumours and antibodies against the virus or its components have been searched for. The early non-virion antigens produced in the infected cell but not identical with the structural elements of the virus, as well as the antibodies against the latter have also been studied. Connections between several kinds of human tumours and viruses could have been demonstrated by means of the above-mentioned methods. Thus, connections in tumourous cases of the urogenital tract with herpes simplex, cytomegalia, papova viruses and C-type virus particles have also been observed [9, 10]. Viral nucleic acid sequences have been consistently observed *in vitro* in prostate cells cultivated for longer periods [21]. Concerning the herpes simplex virus there are data available according to which antibodies against the non-virion viral antigens can be found almost in 100% of patients suffering from tumourous diseases of the urogenital tract and the oral region [1–3, 10, 14]. It has also been investigated to what organs or organic systems the oncogenic viruses have greater affinity. Accordingly, it has been found that herpesviruses occur frequently in the male urogenital organs [12]. Persistence of cytomegalovirus for quite a long period has been observed in the human semen [4, 5]. There are many observations indicating that adenoviruses have affinity to the urogenital organs, but no specific data are known as yet. Our former investigations have suggested a connection between the female genital organs and adenoviruses. While studies on the cervix epithelial cells by immunofluorescent technique indicated that in addition to herpesvirus antigens adenovirus antigens might be present in 7% of otherwise clinically healthy women, the occurrence of virus antigens in the malignant cervix epithelial cells could reach more than 20% [13–15]. Latent adenoviruses have, however, been discovered in 14% of patients with irregular haemorrhage, during the examination of epithelial cells of curettage. It has been observed in animal experiments that the virus in case of pregnant mice infected with human adenovirus can transplacentally permeate into the embryo and persist in the ovaries of the pregnant animals [8].

According to these results, antibody against the non-virion antigen of oncogenic adenovirus type 12 can be observed in more than 50% of the tumourous cases of the urogenital system, contrary to the 18% of non-tumourous urogenital patients and to the 4% of the internal disease cases.

The data suggest that the defective form of oncogenic adenovirus or its genes is present and functions in the organism of quite a high percentage of patients with tumours

in the urogenital system, since only non-virion antigens can be induced by them. Furthermore the presence of non-virion antigens is proved by the specific antibodies produced against them.

Based on the results obtained from over 4000 tests it seems that – besides other already tested viruses – adenoviruses may also play a role in tumourous urological diseases.

It may also be noteworthy that while during our recent investigations the antibodies against the non-virion antigens of oncogenic adenoviruses were primarily and with a high percentage found in patients suffering from tumourous diseases, the antibodies against the non-virion antigens of latent adenoviruses were observed mainly in the sera of patients with internal diseases, namely in 33% of the selected controls. The diseases of the controls were of inflammatory character and in such clinical cases antibodies may always occur or latent adenoviruses may be present in the organism [25].

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MOLECULAR BIOLOGICAL CHARACTERIZATION OF ADENOVIRUS DNA*

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The agarose gel electrophoresis of DNA, the ethidium bromide fluorescence detection of DNA fragments and restriction endonucleases were discovered at the end of the '60s. The methodological progress enabled institutions equipped with less sophisticated technology to achieve also unique experimental and scientific results in the field of viral DNA research. The team working on virus DNA within the adenovirus research group has constructed several new restriction endonuclease maps of the genomes of human and animal adenoviruses; contributed to the methodology of the determination of specific endonuclease sites, and genome polarity; discovered new restriction endonucleases, adenovirus subtypes, new empty capsid, and genome subpopulations; participated in cooperations leading to novel, although hypothetical approaches in AIDS therapy, taxonomic definition of viruses, and evolutionary origins of adenovirus replication and encapsidation strategy.

The direct examination of the viral DNA has been initiated at the beginning of the seventies in the Institute of Microbiology, Semmelweis Medical University. Andrea Bánrévi, Péter Geck, Jr., György Fejér, Csaba Jeney, Kang Wi Gyu (Korean Ph.D. student), Alexi Kiss, Péter Medveczky, Árpád Molnár, László Palkonyai, Zsolt Ruzsics, Éva Szomolányi, and Mária Takács participated in the work for longer or shorter periods in addition to the authors. Other members of the Institute of Microbiology, members of

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other Hungarian institutions and foreign cooperative partners contributed temporarily to the research activities. At present most of the participants entered other Hungarian and foreign institutions, and the majority of them has continued research in virology.

Restriction endonucleases and restriction endonuclease maps

Restriction endonucleases have been discovered in 1969. The genome of adenoviruses is a linear, double-stranded DNA of 30 to 50 kb length. The research work started with isolation of restriction endonucleases, and the first results were obtained on the physical localization of specific sites within the DNA of different serotypes, which had not been studied earlier [1–5]. New restriction endonuclease maps of the DNA of serotypes 1, 6 and 8 have been published [2–5, 22].

As the final stage of this work an intermediate adenovirus serotype was characterized [6]. The experiments revealed the molecular basis for the peculiar biological properties of the virus strain.

Numerous technical difficulties had to be overcome during the beginning years. Contaminating DNase enzymes were shown to be present in both restriction endonuclease preparations and purified adenoviruses. In order to detect such contaminations disturbing the experiments, a superhelical, covalently closed circular (ccc) bacteriophage DNA (PM2) had been introduced for the detection of aspecific endonucleases [7]. A considerable number of specific restriction enzymes were unable to digest the DNA preparations. The aspecific endonucleases, however, could be detected by these target molecules.

The restriction enzymes were purified to higher degree, and several new purification procedures had been introduced in order to improve the purity and quality of the preparations [8–11].

Even two new restriction endonucleases have been purified and recognized as the result of the lucky combination of molecular biological methodology and oral microbiology. Different serotypes of the *Streptococcus mutans* were shown to be resistant to DNA transformation. The phenomenon was thought to be the consequence of a restriction endonuclease.

From serotype C of *Streptococcus mutans SmuCI*, from serotype E the *SmuEI* were isolated. The enzymes were found to be isoschisomeres of *Ava*III and *Ava*II, possessing recognition sequences of 5'-GG/W/CC-3' and 5'-ATGCAT-3', respectively [12–14]. In order to determine the recognition sequences comparative enzyme digestion of DNA preparations of known nucleotide sequence (SV40), the computer analysis of theoretically possible specific hexanucleotides of SV40 DNA sequences were done [12, 13]. Optimal conditions of enzyme activity and the physical map of human adenovirus type 1 DNA were also determined with the new enzymes [13]. The sticky ends produced by the *Smu*EI (ATGCA'T) were also measured [13, 14].

The sequence analysis programme has been also developed at the Institute (M. Takács, unpublished) using a 'basic' programme package as summarized later [15].

The determination of new restriction endonuclease maps proved to be an important step in the adenovirus research, since that time these data were the only molecular markers in the identification of viruses on the basis of the biochemical properties of viral DNA. The number of the (seven) specific cuts was determined first, which resulted in the production of 8 restriction endonuclease fragments [12, 13]. The physical sequence of the fragments has been detected next, using different known endonucleases. In those days the findings resulted in an important step for both adenovirus and SV40 research, since two new physical maps and a new enzyme became available for the characterization of markers of these genomes [12–14].

It is an important problem in the restriction endonuclease mapping of the viral DNA molecules, which end of the linear DNA is coding for the transforming genes [1]. This has been also determined by a new experimental approach [2] using the relative TM determination of the fragments. The technique was later on successfully used for the physical mapping of the DNA of avian adenoviruses.

"Bent" DNA fragments were also identified within certain regulatory regions of the adenovirus 1 genome (A. Bánrévi, unpublished).

The most important result of the physical mapping project was that in contrast to the generally accepted opinion, according to which serotypes can be subdivided using restriction endonucleases, it has been concluded that the maps characterize adenovirus subgenera from the biological point of view. The variability detected is characterizing the taxonomic subgenera [15].

Molecular cloning of adenoviral restriction endonuclease fragments and biological application of physical mapping

The isolation and identification of restriction endonucleases made it possible to link them with the DNA of bacterial plasmids and bacteriophages. Thus the production of viral DNA became much cheaper and more efficient in *E. coli K12* than in virus-infected tissue culture cells.

DNA fragments of adenovirus serotypes 1, 8 and 35 were cloned into pBR322. The recombinants were used even for the construction of restriction endonuclease maps. By radioactive labelling of individual DNA fragments the DNA homology of different genomic regions became available using Southern blot hybridization [16–22]. Difference was found in the DNA homology of the E1B region of adenoviruses of different oncogenic potential. It could be shown that the fragments of lower and higher intertype DNA homology possess mosaic-like distribution in the genomes of different serotypes, indicating the mosaic-like recombination events during evolution [16, 17].

Considerable differences were found in the DNA homology of adenoviruses of different animal species. No detectable homology was found between the genomes of subgenus C human adenoviruses and those of serotype 2 of canine adenoviruses. The DNA of the bovine adenovirus type 2, however, possessed well detectable homology to both human subgenus C, and canine type 2 genomes [18]. These results provided the first

possibility to identify the location of the oncogenic region within the genome of canine adenovirus type 2.

In addition to the differences of the oncogenic regions, virus-strain-specific differences were also detected in the E3 region of bovine adenovirus (BAdV) type 2 isolates [19, 20]. These differences were associated with the host specificity of the viruses. Bovine and ovine subtypes of BAdV serotype 2 could be differentiated on the basis of molecular and biological properties (haemagglutination properties, antigenic changes, asymmetric recombination within the E3 region). It was found that the genomes of adenoviruses of different species are different in length [21, 22]. The linear DNA molecules of bovine and canine adenoviruses are by 3 to 6 kb shorter than those of human adenoviruses. In some cases the length difference was found to be virtual due to the different bending of the genomes (BAdV type 4), and in some cases "space filling" DNA repetitions can enlarge the DNA (e.g. 8–12 times repetition of a 135 bp fragment) of canine adenovirus type 2 (6, 21, 22). It has been suggested that an enhancer might be amplified by the repetition in this part of the genome. Nevertheless, the more efficient encapsidation of the longer genome can be also a selective advantage for these structures. The question, why members of the second sub-group of bovine adenoviruses (Bartha) possess different genome structures than any other adenoviruses, (except that of mice) had not been further tested until now [15, 21, 22]. The encapsidated genome populations, and the density of virus particle populations were shown to be different from that of other adenoviruses. The detected genome-heterogeneity was found to be different from the deletions appearing after high-multiplicity passages of human adenoviruses [15, 21].

The byproduct of these works was the discovery of two populations of adenovirus empty capsids, one of which still contained the 100 K protein as integral part of the capsid population 'A' [23].

Activities in the fields of theoretical virology, and biohazard associated with recombinant technology

In the middle of the '80s the era of genetic analysis using restriction endonuclease analysis and hybridization with restriction endonuclease fragments became anachronistic. The nucleotide sequences of the complete genomes of several adenoviruses and later on that of the herpes simplex virus became available. That time the nucleotide sequence analysis had not been possible at the Institute. The research work had to be concentrated to biological questions.

It was shown that different cloned adenovirus genes may influence the activities of certain genetic functions of the vector molecules [24–26]. Adenovirus promoters could reactivate adjacent, silent pBR322 promoters driving the expression of antibiotic resistance genes (ampicillin). That time two explanations could be put forward [15, 25]) in order to understand the mechanism of activation: 1.) the activity of viral promoter, and 2.) the structural modification of the plasmid promoter due to the inserted bent DNA fragment of the virus. Both explanations possess scientific basis even today.

MAGYAR
TUDOMÁNYOS AKADÉMIA

As early as the beginning of the seventies a modification of the taxonomic classification of the viruses, as autonomous, extrachromosomal genetic elements together with bacteriophages, plasmids, viroids and DNA elements of the cell organelles was proposed [26, 27]. The replication mechanism of adenoviral DNA has been the basis of the argumentation, since bacteriophages and plasmids also were shown to possess the same replication mechanism avoiding the formation of Okazaki fragments [27]. The asymmetric DNA replication had been discovered also in connection with the process of (DNA-) transformation of bacteria, and had been shown to be functioning during bacterial conjugation. All 3 mechanisms possess many similarities, even the appearance of a protein-primer, initiating replication. Therefore, it has been hypothesized that these prokaryotic mechanisms might serve as evolutionary origin of the adenoviral DNA replication machinery [27].

Biohazard, and dangers associated with recombinant technology had not been tested thoroughly in experimental systems. The available gnotobiotic mice provided opportunity to test directly the faith of *E. coli K12* bacteria carrying pBR322 in the gut of gnotobiotic mice. The mice were fed with the bacteria, but no antibiotics were used to select for carriers of the plasmid. In spite of this fact the bacteria were shed continuously during the six weeks of the experiment, and at least 10% of them was harbouring the plasmid, too [24].

Six weeks later, when the sterile conditions were suspended, the gut flora of the mice became normal within 7 days indicating that under everyday conditions the laboratory work (or potential contamination) with arteficial, non-natural recombinants may not compete with the natural flora of the gut [24].

The AIDS epidemic began in the '80s. Although the prevalence of the disease was very low in Hungary, and no technical facilities were available, a group of medical virologists had to be interested in the problem. A completely new approach of the therapy has been elaborated and published. The essential idea of the hypothesis was the superinfection of the virus-carrier patient with genetically modified defective viruses, which could replace 'wild' virus variants, and slow down or stop the progression of the disease [28].

New applications of recombinant DNA technology

Monoclonal antibodies could be used successfully for the analysis of complex antigenic structures. In order to replace the tedious technology of hybridoma induction and selection, cloning of cDNA of antibody mRNA was introduced. From producer mouse-hybridoma cells mRNA of hexon specific adenovirus type 1 IgG was isolated. The antibodies were specific to genus-specific epitope of the hexon capsomer [29].

The mRNA preparation was performed with oligo-dT-Sepharose chromatography and reverse transcribed. Variable regions of both H and L chains of IgG were amplified using PCR primers specific to conservative IgG sequences. Two amplimeres were obtained of 340 (H) and 325 (L) bp, respectively. Both amplimeres were ligated, and cloned into the pCANTAB5 phagemid vector, and expressed as fusion proteins inserted

into the g3p protein of phage M13. The variable regions specific to the adenovirus hexon epitopes were located on the outer surface of the phage particles produced by the *E. coli* host carrying the recombinant phagemid. More than 100 clones had to be examined in order to identify hexon-reactive clones. The screening for the antigen-reactive clones was done using ELISA. The paratopes of phage-antibodies were found to be identical to those of the original monoclonal antibodies [29].

In the second half of the '80s S.C.L. Woo has put at the disposal of the research group a recombinant carrying a region of human phenylalanine hydroxylase (PAH) gene. In cooperation with the 2nd Clinic of Pediatry of the Semmelweis Medical University and with 15 other European institutions patients suffering from the recessively inherited disease phenylketonuria (PKU) and their healthy family members were examined using restriction fragment length polymorphism (RFLP) [30–32]. The application of the PAH probe enabled the partners participating in the cooperation to identify more than 40 haplotypes of the PKU in the European population. It has been shown that the migration of the Europeans had been more complicated in ancient times as thought earlier on the basis of historical and anthropological data [32].

A truncated variant of the PAH gene has been also identified [31]. This haplotype (No. 4) occurred only in Hungary and Slovakia indicating that the nonsense mutation has occurred during recent centuries within the geographical region of the Karpathian Basin.

One of the group (Gy. Fejér) obtained a fellowship and has worked 3 years in the laboratory of M. S. Horwitz [33, 34]. As the main success of this fellowship the technology to create transgenic mice has been introduced in the research work of the team. The characterization of mice manipulated in the United States containing the early region 3 (E3) of adenovirus type 5 became possible, and continued at home. It has been shown that pancreatic islet allografts survive for longer periods in the E3 transgenes in comparison to control animals indicating the immunomodulatory effect of the constitutively expressed E3 genes [34].

Discussion

During more than the last two decades the members of the team have constructed several new restriction endonuclease maps [2–6, 15–19, 22] of the genomes of human and animal adenoviruses HAdV 1, 8, 35, and BAdV 2. The team also contributed to the methodology of the purification of endonucleases [7, 9–11, 14] determination of specific endonuclease sites [12, 13], and genome polarity [15, 18, 21]; discovered new restriction endonucleases [12, 13], adenovirus subtypes [6, 19, 20] new empty capsid [23], and genome subpopulations [18, 21]; participated in cooperations leading to novel, although hypothetical approaches in AIDS therapy [28], taxonomic definition of viruses [15, 27] and evolutionary origins of adenovirus replication and encapsidation strategy [27]. Cooperation of pediatricians, molecular biologists, and virologists led to new results in the field of non-infectious epidemiology i.e. phenylketonuria [30–32].

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MOLECULAR STRUCTURE OF THE TWO-DIMENSIONAL HEXON CRYSTALLINE ARRAY AND OF ADENOVIRUS CAPSID*

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Nearly 45 years passed since the discovery of adenoviruses and nearly 130 adenovirus types of human and animal origin were recognized. These naked viruses show regular icosahedral shape and the virus capsid is composed of 252 capsomers. The 240 hexons mean about 95% of the capsomers and they represent approximately 53% of the total bulk of the virion. In the adenovirus capsid hexons have six nearest neighbours, i.e. they form a regular hexagonal structure. There is such a strong though not covalent cohesive force among the three polypeptide subunits constituting the capsomer that the hexons may be recovered in intact form from the infected cells and each of their characteristics corresponds to the hexons building up the virion capsid.

Structure of the two-dimensional hexon crystals

Highly purified human adenovirus type 1 hexons preparations were crystallized using a dialyzation process against 0.5 M acetate buffer [1], and the crystallization process was followed by serial electron microscopic examination. A few hours after dialysis, small regular groups of hexons were found and with the progress of the process two-dimensional crystalline arrays consisting of several thousand of hexons were developed. In this network the hexons organized in regular rows form a single layer [1, 2]. Their

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relation was similar to the hexons situated on the triangular facets of the icosahedral virion. Consequently each hexon has six nearest neighbours according to the hexagonal structure. Every second row was shifted along the longitudinal axis, this distance being about half the diameter of one hexon particle [3, 4]. Analysis of the crystal structure revealed 15–20% local irregularity (short range disorder) and about 10% deviation in the values of the lattice constant determined in different directions of the hexon rows. Angles formed by non-parallel hexon rows deviated by a few degrees from the regular hexagonal order, i.e. the position of the hexons in dense two-dimensional crystals is slightly skew and irregular. In some cases extra hexon row (dislocation) developing between two hexon rows diverted them from their original direction [3]. Electron microscopic studies also showed the development of three-dimensional microcrystals of multilayer appearance showing a regular internal structure.

Shape and intrahexonal location of the hexon polypeptides in the crystalline array

The hexons consist of three polypeptide subunits and accordingly they have threefold symmetry axis. Analysing numerous two-dimensional crystalline arrays it was found that the bulk of the hexons display ringwise shapes with an approximately round hole in their centre [5]. Nevertheless, 2–4 electron dense spots could be observed in the ring-wall, especially on underfocussed high resolution pictures. On the other hand, in some cases, the number and shape of the polypeptides they consisted were clearly discernible. The three main polypeptides constituting the hexons of one type were either like round cornered parallelepipeds or slightly bent parallelepipeds. These oblong polypeptides were linked by their thinner sides and thus the threefold symmetry and the approximately triangular hole enclosed by the three polypeptides were clearly visible. The linked polypeptides form a triangle in this type of hexon. Since the junctures of the polypeptides are approximately as large as the longer sides of the polypeptides, these hexons are hexagonal. The other hexon type showed an even more expressed triangular shape and clearly consisted of three main polypeptides. The area enclosed by its six nearest neighbours, in which it is located, is hexagonal. This type was much less frequent than the former one. Its shape is approximately an equilateral triangle, while the slit in the centre is not round but narrow, Y shaped. The profile of polypeptides is rhomboidal [6]. These two types of hexon ends correspond to the hexon ends facing inside the virion, and virion surface, respectively.

Mutual orientation of hexons and polypeptide subunits within the crystalline array

By computer processing of the electron micrographs, it was shown that the polypeptides of two neighbouring hexons in the crystalline array are situated so that one polypeptide of a hexon is nearest to two polypeptides of the neighbouring hexon, i.e. a

polypeptide is always linked to two other ones [7]. Thus, the hexon polypeptides are linked according to "one-to-two" pattern similarly to the linkage within a complete hexon, with the difference that the polypeptides converge in the hexon. This "one-to-two" orientation means that the longer side of a hexon polypeptide subunit is connected to the end of two polypeptides of the neighbouring hexons. The hexons in this "one-to-two" arrangement orientated identically as regards the position of the polypeptides and have an equivalent environment corresponding to the hexagonal packing and threefold symmetry axis. Irregularities occur if some hexons rotate a few degrees from the position of the rest. The greatest deviation can be caused by a rotation of 60 when "one-to-one" or "two-to-two" linkages ensure between the neighbouring hexons. Further rotation means a gradual return to the original position, since a further rotation of 60° restitutes the original orientation, as follows from the threefold symmetry axis.

Inter- and intrahexonal connections between hexon polypeptides in the crystalline array

In a tight two-dimensional hexagonal crystalline array there are 1.5 to 3 nm spaces between hexons. The obtained values are slightly different in the three non-parallel hexon rows. High resolution electron micrographs proved the presence of fine connections bridging the interhexonal spaces between neighbouring hexons. The length of these connections corresponded to the interhexonal distances, their diameter ranged from 0.5 nm to 1.5 nm [8].

The interhexonal connections show various forms. In general, like straight bridges they link the neighbouring hexons but their middle or end often become thinner. Interhexonal connections deviating from the average size are not rare, these are probably double connections joined along their longitudinal axis.

The connections set out radially in several points of one complete hexon and reach the six neighbouring hexons, thus enclosing a certain angle with one another. In same cases, two connections seem to be parallel, and three hexons are linked by six elements. Those six connections divide the empty space into four parts enclosed by three hexons, the connections in a central position form a triangular space with an adjoining quadrange, square or parallelepiped, to each of its sides enclosed by parallel connections. The triangular space in the middle emerges due to the V shaped lines, joining at approximately 60°, described by the central connections from any hexons to its two neighbours. Y shaped connections, with 120°, linking the three next hexons are also frequent. Their thickness is generally above the average. They divide the space enclosed by the three next hexons into three, almost oval parts. In general, 6 to 10 connections are clearly seen between hexons and its six nearest neighbours.

The complete hexons are formed by three polypeptide subunits according to the threefold symmetry. In the negatively stained samples the three main units building up a hexon are seldom clearly discernible, due to a shift in the position or bend of one end of each polypeptide in relation to the other end and also to staining. In the preparation made from the crystallized hexon, fine connections were detected linking the main structural

polypeptide subunits. The intrahexonal connections are 0.75 nm thick and 1.05 nm long. By joining the three polypeptides they build the complete hexon with a hole or a channel in its centre. The polypeptides in a hexon seem to be linked by doubled intrahexonal bridges. Inter- and intrahexonal elements extend between the ends of polypeptides either of two neighbouring hexons or within one hexon. The main polypeptides of a hexon form a parallelepiped, sometimes slightly bent and their joinings indicate the almost triangular shape of the hexon. The corners of a triangle are probably situated where two polypeptides meet, its sides being the longer side of the polypeptides. In any two neighbouring hexons the polypeptides are orientated in such a way that in regular cases they meet corner to edge.

Molecular structure of virion
Arrangement of hexons and polypeptide subunits in the adenovirus capsid

Direct examination of electron micrographs of negatively stained preparations revealed several virions in the capsid of which the triangular profile of the hexons facing the virion surface as well as the three hexon-building polypeptide subunits and their orientation were well discernible [9]. With the help of Markham's rotational procedure the image of the profile of peripentonal hexons improved on both ends of the three edges bordering the given face. Two of the three polypeptide subunits forming the triangular profile are situated near to the penton and the third one on the edge of the capsid towards the penton situated farther. The localization of peripentonal hexons on the three bordering edges shows a threefold symmetry in respect of polypeptide subunits as well. One side of each peripentonal hexon i.e. two polypeptide subunits, are facing the penton and one polypeptide is located on the edge in radial direction from the nearer penton towards the farther lying one. The five peripentonal hexons surrounding one penton display a position corresponding to fivefold symmetry. The orientation of peripentonal hexons was identical both by rotation from the threefold symmetry axis and from the fivefold symmetry axis. The mutual orientation of every peripentonal hexon i.e. of their polypeptide subunits to their six neighbours is as follows. Two polypeptide subunits i.e. one side of the triangular profile are oriented towards the penton, and the same two polypeptide subunits are linked also with two neighbouring peripentonal hexons thus in the case of the neighbouring hexons the polypeptide orientation shows a "one-to-one" pattern. The third polypeptide subunit shows "one-to-two" orientation with a hexon similar orientation belonging the "group of nine" (GON) hexons which takes part in the constitution of the edge of the capsid. Its fifth neighbour is a hexon which belongs to the same GON but is situated on the triangular face nearest to the given peripentonal hexon being in " two-to-one" linkage to it. The sixth neighbour is a hexon belonging to the other GON and linked with the given peripentonal hexon displaying with a "two-to-two" orientation [10, 11].

The profile and orientation of each of the six hexons building the triangular face was also determined. The mutual orientation of six hexons belonging to the same GON and building one triangular face as well as that of their polypeptide subunits show always

the "one-to-two" type orientation within the GON. Every polypeptide subunit corresponding to one of the hexon vertices points in anti-clockwise direction.

Each GON is situated on the triangular face of the capsid in such a way that two hexons of each of the three vertices are linked with two peripentonal hexons. The mutual orientation of the GON is that one hexon of each neighbouring GON participates in the formation of the common edge. Three hexons of each of the two neighbouring GONs are in connection at five contact points. Two of them display "two-to-two" and three a "one-to-one" orientation. One of the latter is situated in the middle of the longitudinal axis of the common edge built by two GONs, i.e. exactly on the twofold symmetry axis. As each GON participates in the formation of three edges and has thus three neighbouring GONs, these observations are valid to all the three sides of the GON. Therefore the polypeptide subunits of the hexons reach an equivalent orientational position in the whole capsid, taking into consideration the spatial deviation between the planes of the triangular faces.

On the base of the electron microscopic studies of the virions it was shown that the distance between the edge and triangular faces are wider than those among the hexons on the faces and on the edges. The distance separating the pentons and peripentonal hexons appears similarly wider. This phenomenon may be explained by the deviating spatial orientation of the hexons constituting the faces and edges as well as of the pentons on the vertices, which follows from the icosahedral structure of the capsid [11]. The mutual spatial orientation was characterized by the angle enclosed by the longitudinal axis of the hexon studied and that of the neighbouring capsomer used for comparison, in open position towards the virion surface.

Electron microscopic analysis of the virion and model experiments lead to the conclusion that the mutual spatial orientation of GON hexons can be parallel (their longitudinal axes are parallel) or the direction of their longitudinal axes is different in one or two planes, thus they form different angles with the longitudinal axes of the neighbouring hexons [11].

Linking components between capsomers in the adenovirus capsid

Direct analysis of high-resolution electron micrographs of the virion revealed that the distances separating the hexons in the adenovirus capsid were bridged by fine linking elements. Owing to these connective structures forming bridges at some sites between capsomers the slit may appear as holes in the capsid [12, 13]. These connections between a given hexon and its six neighbours were clearly seen. The distance i.e. the length of the connective elements between the capsomers, is about one third of the diameter of the hexon. By the use of a 120 rotation (threefold symmetry axes) of a triangular face of the virion a regular network of connective elements were observed among the six hexons situated at the face of the virus capsid. By the rotation of a penton according to fivefold symmetry the connective elements were detectable among the penton and peripentonal hexons. Usually, hexons are linked to their neighbours by not one but two connective elements. It was shown that a central hexon is connected to its six nearest neighbouring hexons by six pairs of approximately parallel connections, i.e. by 12 elements.

By analysis the structure of two-dimensional hexon crystalline array it was shown that certain individual hexons within the crystal lattice were significantly displaced from the theoretically correct position. Nevertheless, each hexon stays within a certain definite distance as compared of its equilibrium position determined theoretically in the lattice. This proves that the interhexonal connecting elements must be flexible and elastic to a certain extent. This consequently refers to the interhexonal elements in the virus capsid, as well. It means that the bend and torsion arising from the icosahedral structure can be tolerated by the connective elements.

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INTERTYPE SPECIFIC EPITOPE STRUCTURE OF ADENOVIRUS HEXON*

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During the years passed since the adenoviruses have been discovered, a great number of information accumulated on the antigenic structure of the major virion component, known as hexon. The antigenic determinants had been found on the hexon molecules in a variety of which the determinant common to mastadenoviruses is called genus specific antigen. The presence of type specific determinants had been demonstrated as well. The probable existence of subgenus specific determinants were also described. These informations on the antigenic structure of the complex viral antigens originated from the studies carried out with multireacting polyclonal antisera, but the use of polyclonal antibodies limits the appropriate antigenic analysis to consider the total antigenicity of proteins.

In the middle of 1970s a new method i.e. the use of monoclonal antibodies was introduced to study the epitopes of the proteins of different origin. From that time, the widely used monoclonals gave a powerful tool for antigenic analysis of adenoviral antigens, too, and a map of all epitopes on adenovirus hexon might finally be achieved with monoclonal antibodies.

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Monoclonal antibodies to adenovirus hexons of different origin

Three panels of monoclonal antibodies (MAbs) were produced to adenovirus hexons of different origin. To develop the specific antibody producing hybridoma cell lines, mice were immunized with crystallized human adenovirus (HAdV) type 1 hexon of subgenus C [1], with purified hexon of HAdV-35 (subgenus B) [2], as well as purified virions of bovine adenovirus (BAdV) type 2 [3]. In this latter case, the hexon specificity of the MAbs was determined by immunoblotting method. With these three panels of MAbs the antigenic structure of human and animal adenovirus hexons were extensively investigated. For the studies of the epitope composition of different hexon types, for the investigation of the presence of identical or related epitopes according to the three polypeptide subunits of a complete hexon, as well as for the determination of antigenic sites on the hexon molecule, different enzyme linked immunosorbent assays (ELISAs), and passive haemagglutination method were used [4, 5]. The hexon specific MAbs were studied in the supernates of the hybridoma cells and in the ascitic fluids produced by mice. The number of investigated hexon types varied between 10 and 21. All the hexon types of human and animal origin used in these experiments were purified.

Number of the different epitopes on the hexons

For studying the possible number of the epitopes present on the surface of the hexon molecule, the cross-reactivity (CR) of the MAbs has been tested with purified hexon preparations of different hexon types with indirect ELISA and passive haemagglutination techniques [1]. There was some difference between the CR determined by the two methods, but mostly the type of CR pattern of a given hybridoma antibody was identical with the panel of heterologous hexons in the two assays [6]. The difference in the reactivity patterns (RPs) using the two assay systems could be due to the different adsorption behaviour of the hexons to the polystyrene plates and to red blood cells causing steric blocking of some epitopes, as well as to the difference in the amount of absorbed hexons in the two assay systems.

It was found that hexons carry numerous epitopes, which are more or less similar to each other, and they grouped the different AdV serotypes showing identical intertype specificities. The epitopes can be overlapping or separate and these intertype (IT) specific antigenic specificities (probably epitopes) are present on the hexons of different human and animal serotypes in various combinations [7]. Their presence is independent of the subgenera to which the investigated hexon types belong. In addition to these IT specific epitopes, MAbs recognized genus specific and type specific epitopes, too [2, 6, 8, 9–11].

The distinction and marking of the different epitopes recognized by the MAbs were carried out by the determination of the composite CR pattern, the titre and the correlation coefficient of all the MAbs with more than 20 different hexon types representing all the six human subgenera, as well as different bovine and simian adenoviruses [12]. The three panels of MAbs recognized 18 different bi- and multilateral IT specific epitopes. It was found that the total number of the different IT specific

epitopes on the hexons varied between 2 and 18, and the distribution of the distinct specific epitopes was different. With their IT specific spectrum the individual hexon types could be characterized. By pairwise analysis human hexon types formed three epitope clusters showing identical epitope spectra, but there were also human and animal hexon types which could not be clustered due to their epitope composition. However, they displayed a closer or looser antigenic relationship among each other and to the members of the epitope clusters [13].

The degree of antigenic relationship was expressed by the similarity/dissimilarity percentage calculated from the number of the identical and different epitopes present on any two given hexon types [13].

Differentiation of epitopes with agar gel diffusion

MAbs directed against HAdV-1 hexon were tested in agar gel diffusion experiments with the homologous and heterologous hexons [14]. A part of the MAbs investigated formed a single precipitine line with the homologous hexon and other MAbs were able to precipitate the antigen only in a biclonal combination with an appropriate other MAb. One group of MAbs formed a continuous line of complete identity when tested simultaneously against homologous and different heterologous hexons, while another group of MAbs formed a line of double partial identity (double spur). Based on this phenomenon, the first group of MAbs was characterized by recognition of identical epitopes, in these experiments such reaction appeared between MAbs and the genus specific epitope of hexon molecules. The second form of reaction (spur formation) appeared between epitopes and MAbs with partial identity. These latter ones are the probable IT specific epitopes. With a mixture of selected MAbs the sterically independent epitopes have been determined [14].

Antigenic relationship among adenovirus serotypes

Detailed analysis on the antigenic relationship of hexon epitopes was carried out using adenovirus serotypes of subgenus C [15, 16] and a part of serotypes of subgenus D [17]. It was of interest to determine with a relatively large number of MAbs having intertype specificities whether antigenic diversity or homogeneity exists among the investigated serotypes belonging to two different subgenera. In the case of hexon types of subgenus C a remarkable homogeneity was shown according to the number of reacting and non-reacting MAbs as well as on the basis of a similar level of titres and of identical CR pattern of the MAbs. The 14 different epitopes corresponding to these specificities are uniformly present on the surface of all subgenus C types, but they are present or absent in different combinations on the different hexon types belonging to other subgenera. It means a close antigenic homogeneity among hexon types of subgenus C, but also more or less antigenic relationship with hexons of other subgenera.

Epitope composition of subgenus D hexons was partly different from subgenus C hexons, as well as the epitopes in subgenus D hexons showed different variations. Based on the comparative results, the epitope structure of 5 from the 8 characterized subgenus D hexon types seemed to be much more similar to each other than the epitope composition of the 3 remaining ones [17].

Completing these experiments with the results of one hexon type of subgenus F, as well as with that of subgenus A, B and E hexons, it was concluded that a gradient of antigenic relationship exists among the human and some animal hexon types.

Number of the identical epitopes on the hexons

The complete hexon capsomer consists of three identical polypeptide subunits. This trimer structure suggests that the different epitopes on the surface of the hexon particle have to be present in more than one copy. For studying the presence of multiple copies of identical epitopes on the hexon molecule, a double MAb sandwich ELISA has been developed and used [18–20]. In these assays the MAb used for antigen capture was the unlabelled form of the same MAb, as the peroxidase labelled detector MAb. Consequently, the hexons are able to bind to the capture MAb only with the epitope specific for the given MAb, and it could react with the peroxidase labelled second MAb only in the case, if the given epitope is present in more than one copy on the hexon molecule [18]. Results indicated that presumably more copies exist of similar or closely related epitopes on the different hexon polypeptides. The differences found among the different hexon types could be explained with the lower frequency of a given epitope on the different hexon types.

Prediction of sequential epitope sites on hexon subunit

For determination of the probable number and the location of the potential sequential epitopes two types of prediction strategies were used, namely the identification of hydrophilic regions and the locations of β-turns on the known amino acid sequence of HAdV-2 and HAdV-41 hexons [21]. Based on the results obtained by these methods from 19 the predicted epitopes on type 2 hexon 16 could be considered to be intertype specific. The sequence numbers of predicted epitopes show their locations on the whole amino acid sequence and therefore it was possible to determine the approximate positions of epitopes on the subunit structure. The location of the predicted epitopes of HAdV-2 on the three dimensional model of hexon subunit was determined with the help of their sequence. Five of them could be found on the "lower" part of the pedestal domain P1 and P2 orientated toward the core of the virion. One of them could be the genus specific one and the rest might be perhaps IT specific epitopes with broad presence spectra. Predicted epitopes located on loop 1 and loop 2 corresponded to the two type specific epitopes. Data indicate that the IT specific epitopes are located between the type and genus specific epitopes in the three dimensional structure of hexon subunit.

Two peptides corresponding to potential IT specific epitopes of hexon protein were synthesized and then conjugated to branched polypeptide poly/Lys-(DL-$Ala_{3,4}$)/. For study of their immunogenicity, Balb/c mice were immunized with the conjugated polypeptides and the antigenicity of them was studied with MAbs in ELISA experiments. Epitope in peptide SP1 was predicted only on the HadV-2 sequence and no related sequence patterns were found in the corresponding regions of sixteen other adenovirus hexon types, while epitope in peptide SP2 was predicted in both HAdV-2 and -41 sequences and homologous or closely related sequences were found in fifteen other hexon types. In spite of this difference peptides SP1 and SP2 conjugates have shown similar antigenicity and immunogenicity. The explanation of the relatively weak immune reaction might be that for conformational reasons, peptides in their conjugated form are poorly accessible to MAbs raised against native hexons, and/or these predicted epitopes are not functioning as B cell epitopes in native hexons [21].

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OUTLINES OF THE IMMUNOLOGICAL RESEARCH IN THE INSTITUTE OF MICROBIOLOGY; SEMMELWEIS MEDICAL UNIVERSITY*

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The working group on immunology under the leadership of Pál Földes began its activity with poliovirus studies during the severe epidemics of 1957. It was he who first in Hungary isolated poliovirus strain from patients [1]. His colleague was Ilona Szeri who had gained her first experiences in virology at József Sinkovics's virus laboratory. Then Zsuzsanna Bános and Piroska Anderlik joined them and became permanent members of the working group on immunology. Since 1965 with the leadership of Ilona Szeri, they have been conducting basic researches into immunology for over three decades at the Institute, with a wide sphere of collaborators. Research has been intended to acquire more thorough and precise knowledge of the role in immunobiology of the thymus and lymphoid system and of pathogenesis of the wasting syndrome as well as of interactions of the microorganisms and the organism. The most significant results are going to be summed up in the following.

Investigations into poliomyelitis

Developments in methodology in the 1950s and the use of tissue cultures in research made it possible that investigations of poliomyelitis viruses could be increasingly started world-wide and thus also in Hungary to prevent the disease that caused still severe and feared epidemics. This is the field in which also the working group on immunology (later simply group) has started to work. To find out the conditions favourable for the epidemics and to introduce vaccination in the practice, they have dealt with actual problems of everyday life in the country.

*This paper is written to commemorate the fiftieth anniversary of foundation of the Institute of Microbiology, Semmelweis Medical University, Budapest.

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They have tested capability of the Hungarian made gamma globulin preparations that were available on the market for passive immunization. The results have been in good agreement with the effect of preparations from abroad [2].

It was after the poliomyelitis epidemic in the summer of 1957 that a mass intracutane inoculation with Salk vaccine was performed for the first time in Hungary. (According to their examinations, the paired sera out of a group of 15 children showed positive response such as 14 paired sera to type 1, the same number to type 2 and 13 paired sera to type 3 of poliomyelitis virus.) The outcome has raised trust in intracutane inoculation [3].

At the time of the poliomyelitis epidemic of 1957 in Hungary they made strain isolations and serologic examinations. They could isolate 21 cytopathogenic strains out of 56 faecal specimens from patients with paralytic poliomyelitis. Of the strains 13 were identified as type 1, one as type 2 of poliovirus, three strains proved to be type 11, one strain type 7 of ECHO virus and three were unidentifiable. All the 214 reconvalescent sera from patients with poliomyelitis diagnosis under orthopaedic follow-up treatment contained antibodies against poliomyelitis viruses [4].

Comparisons were made concerning antigenicity and storage at room temperature of Salk vaccine prepared in Hungary and abroad. Storage for a fortnight at room temperature did not cause a measurable decrease in the antigenic value of vaccine samples; antigenicity of both the Hungarian and foreign preparations met the minimum requirements for all three types of poliovirus [5].

The group has participated in nationwide studies concerning introduction into practice of Sabin type vaccine in Hungary. They have studied the possibilities for simultaneous inoculation with live attenuated poliovirus vaccine and BCG vaccine and interactions of the preparations [6]. Furthermore, they have tested for virus excretion and immune response of the newborns immunised with SABIN vaccines of type 1, 2 and 3 respectively [7]. Their findings and experiences have been used in the vaccination practice of Hungary.

In Hungary a vaccination programme with SABIN vaccine began nationwide in 1959 and as a result of it the poliomyelitis epidemics ceased following 1960. Interest of the group has turned then towards theoretical problems of immunology.

Investigations into the pathogenesis of wasting syndrome

Essence of the immune system and its function was not yet known in the late 1950s. There were two events in 1960 that opened a new era in immunology: one of them was the clone-selection theory of Burnet, and realisation of the role of thymus in immunobiology was the other. The latter is in relation to J. F. A. P. Miller's report according to which neonatally thymectomized mice are unable for specific immune response in the course of their latter life. This report meant also the realisation of the connection between the thymus as primary lymphatic organ and the wasting syndrome.

Miller has shown that whenever a mouse is thymectomized within 24 hours after birth then the animal develops normally, but at a later stager general atrophy takes place

(wasting syndrome); the animal is hindered in development then it takes a typical humped posture and its hair becomes unkempt as well as diarrhoea appears and finally perishes in a few weeks. General atrophy of the lymphoid system and reduced immune reaction is typical of the syndrome.

Thymectomy in newborn age. To know biological role of the thymus and the lymphatic system, the group succeeded to apply neonatal thymectomy to mice already in 1961, first in Hungary. Investigating consequences of several thousand neonatal thymectomies their first findings were the followings:

1. Interactions were observed between micro- and macroorganisms in mice that had been neonatally thymectomized and intracerebrally (i.cer.) infected with lymphocytic choriomeningitis (LCM) virus [8, 9, 10].

a. The otherwise fatal lymphocytic choriomeningitis that is based on the cellular immune reaction has not been developed in thymectomized mice and the animals survived the infection in virus carrier state.

b. In virus carrier mice the fatal wasting syndrome has been developed sooner and in greater proportion than in those that were thymectomized only. The reason for that has been explained by the LCM virus that adversely affects the lymphoid system and causes immunosuppression. They have proved and first reported that LCM virus through its lymphoid atrophy producing effect promotes development of the wasting stage.

2. They have identified a formerly unknown symptom of the wasting syndrome following neonatal thymectomy. It has been proved that response to stress of mice with wasting syndrome differs from that of normal mice; they do not exhibit lymphopenic reaction and their stress sensitivity increased [11].

The results mentioned above have shown that neonatal thymectomy affects not only the specific immunoreaction capability but also the general adaptation. They have also shown that persistence of a pathogen, the LCM virus, in a thymectomized organism while further damaging the lymphoid system promotes wasting and early appearance of the biological processes that are typical of it, and finally leads to greater mortality [12].

Immune depression states due to different reasons. Their further studies have revealed the same consequences in mice being in state of immunological areactivity because of other reasons than neonatal thymectomy.

Besides inadequacy of the adaptive immune response ability it has been established that general adaptation is also disturbed in mice

– treated with antilymphocyte serum [13],
– suffering from Graft-versus–Host reaction [14] and
– being in germfree condition [15].

Interactions between microorganisms and macroorganism were similar in neonatally thymectomized mice and in mice in state of immune depression due to different reasons namely

– in mice suffering from Graft-versus-Host reaction, treated with antilymphocyte serum or with lymphotropic cytostatics [16–18],

– in germfree mice with underdeveloped lymphoid system because of the poor antigenic stimuli of their environment [19, 20],

– in aged mice being in state of physiological involution of the thymus [21, 22].

The observed interactions have been the followings:

– after LCM virus infection, no lymphocytic choriomeningitis has developed and the animals survived the infection in chronic virus carrier state,

– persistence of LCM viruses has induced irreversible lymphoid atrophy and led to the development of fatal wasting similar to the wasting syndrome.

Based on their experiences in relation to the effect of LCM virus to promote development of wasting syndrome, they could clear up the pathogenesis of the wasting syndrome [23]. These results can be summed up as it follows. In mice characterised by inadequacy of thymus-dependent lymphoid system the additional effects that induce exhaustion of the T lymphocyte reserve, promote irreversible atrophy of the thymus-dependent lymphoid system. As a consequence a fatal wasting develops that can be characterised by symptoms that are typical of the wasting syndrome following neonatal thymectomy.

New findings deductible from the results.

1. In mice with inadequate T lymphocyte function, the persistent virus carrier state following the i.cer. LCM virus infection promotes development of fatal wasting. This effect of the LCM virus is independent from origin of inadequacy of the T lymphocyte function.

2. Lack or inadequacy of the T lymphocyte population is of decisive importance in the pathogenesis of wasting syndrome.

3. The lymphoid system's atrophy is primary in the process of development of wasting syndrome and it is followed by general wasting; therefore, the lymphoid system's atrophy is not a partial phenomenon or consequence of general wasting but precedes it.

4. From the point of view of pathogenesis wasting syndrome of different origin can be considered identical because in all cases the irreversible atrophy of the thymus-dependent lymphoid system is followed by wasting syndrome characterised by identical symptoms.

5. Such an irreversible damage of the thymus-dependent lymphoid system can be elicited also by a joint or consecutive effect of several factors, as the effects adversely affecting the lymphoid system are added up.

6. Through its immunosuppressive effect leading to the damage of the thymus-dependent lymphoid system, the LCM virus promotes development of wasting syndrome. Any kind of immunosuppressive virus or microbe species that similarly to the LCM virus induces inadequacy in the T dependent lymphoid system can promote development of wasting state.

Wasting syndrome analogue pathological states. Based on their observations, analogy has been supposed between wasting syndrome as a consequence of neonatal thymectomy and the premature neonates' and infants' atrophy due to infectious diseases. Behind the identical symptoms an identical background of pathology exists [24]. The idea has been extended over aged organisms as well. The immunosuppressive microorganisms' effects in an aged organism in state of thymus involution may promote

development of cachexia of old-age. Therefore, wasting syndrome can be regarded as premature ageing [10, 23].

Recent tendencies in research

Knowledge on the microorganisms' immunosuppressive attributes has been still insufficient in the 1970s. Studies on the lymphoid system of mice suffering from acute toxoplasmosis were made in the working group. Besides significant spleen hypertrophy in the period of deaths, severe thymus atrophy and a striking decrease of the lymphocyte count in peripheral blood has been found [25].

In the following years the group has extended researches on relations of the micro- and the macroorganisms in different fields, with the inclusion of a wide sphere of collaborators. Effects of the microorganisms and their substances on the macroorganism have been studied under Piroska Anderlik's direction. Under the guidance of Zsuzsanna Bános there have been studied various immunomodulants affecting the cellular immune response in mice of different age, on the basis of their effects on the course of LCM virus infection. It is László Berek who has studied the relation of the thymus function and skeletal growth.

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BIOLOGICAL EFFECTS OF MICROORGANISMS AND THEIR SUBSTANCES IN MICE*

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In our experiments we evidence from several aspects that the normal microbial flora has a permanent and life-long immune modulating role in conventional organisms and a stimulating effect both on specific and non-specific defence. However, in case of artificial interventions (stress, drugs) affecting the organism, existence of the normal flora may have an adverse effect on it (endotoxin effect, bacterial translocation).

The immunomodulants show a stimulating effect mainly in organisms with undeveloped immune system, and their effects are independent from the presence or absence of the microbial flora.

With ageing, effect of immunomodulants can change and become indifferent or even suppressive.

Dose-dependence of stimulating or suppressing effect of immunomodulants may be related to their non-immunological effects (endotoxin effect, bacterial translocation).

Finally, on the basis of our experiments, we consider the Gf mouse suitable for examining the effect of a given agent in the practice, on the one hand, and for observing the host organism's reactions, free from the influence of the normal microbial flora, on the other.

Along with the known physiological and pathological events, our results draw also attention to as distant fields as drug sensitivity, drug interactions influencing drug sensitivity. The authors put emphasis on importance of germfree environment during immunosuppressive treatments in humans and when making special examinations under experimental conditions.

Our knowledge of microorganisms and their substances has very much broadened in the last decades because of the new potentials provided by researches in molecular biology and *in vitro* investigations. To know, however, the effects of the microorganisms and their substances on the whole organism and the factors influencing them, *in vivo* experiments are necessary to observe the organism's reaction. This is why we performed *in vivo* experiments in research.

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We aimed at investigating the following mechanisms:

– role of the normal microbial flora in the non-specific defence mechanisms of the organism;

– manifestation of the effect of microbial and other immunomodulants on defence mechanisms of organisms whose immune function varies because of the presence or absence of normal flora and the physiological changes with age or because of artificial interventions, and

– whether presence or absence of the normal microbial flora and the immunomodulant effects influence the organism's other biological functions and reactions through modulation of immune reactions.

According to these aims and relying on our experimental results we intended to conclude on the wider biological role of the normal microbial flora; effects of immunomodulants influencing the non-specific defence reaction of mice; factors influencing the immunomodulant effects.

Materials and methods

Experimental animals. The animals used came from different inbred and outbred stocks of mice and were different from the point of view of their

a) microbiological state, namely they were

– germfree (Gf) (with undeveloped immune system because of the poor environment in antigen stimuli),

– specific pathogenic free (SPF) and conventional (Cv) (slightly developed or normal immune system);

b) age,

– suckling (physiologically undeveloped immune system),

– adult (developed immune system) and

– old (with physiologically regressed immune system) and

c) thymus function, such as

– euthymic (normal thymus function) or

– athymic (thymectomized in newborn age and athymic because of genetic reasons [nude]).

Immunomodulants. We performed comparative experiments to study how natural and artificial immunomodulant effects and substances such as

– presence or absence of normal microbial flora,

– microorganisms and their substances, namely bacterial preparations [*Bordetella pertussis* vaccine (B. pert), native endotoxin of *Escherichia coli* (LPS) and its detoxicated variant, the Tolerin (T), live attenuated Newcastle Disease virus (NDV) vaccine, zymosan (Mannozym, M), conventionalisation, monocontamination with *Peptostreptococcus* (PSTR)],

– hormone derivatives [Glutaurin – a bioactive material isolated from parathyreoid (Glut), TP-4 – a synthetic tetrapeptid, thymopoietin sequence analog],

– drugs [dianhydrodulcitol – a lymphotropic cytostatic in the alkylating group (DAD) as well as chlorpromazine (CPZ) – a calmodulin-antagonist],

– biological effects (stress, cold, shaking) affect the defence mechanisms and reactions of mice with different immune function.

In our experiments effects of the preparations widely used in the human or veterinary practice for therapeutic and preventive purposes in Hungary were studied.

Examination of defence mechanisms. Studying the non-specific defence mechanisms, changes of the inflammatory reaction and plasma fibronectin concentration (pFN cc) as well as lymphatic organs indices and additionally drug sensitivity and degree of bacterial translocation (BT) were registered.

Other methods. A number of conventional methods of biology, bacteriology, immunology, haematology, surgery, pharmacology, histology and statistics were used.

Results

Role of the normal microbial flora. Comparing the results obtained by the use of mice with and without normal microbial flora we could prove the followings:

– reduced acute phase protein content of Gf mice [1],

– lower pFN cc level in Gf mice [2],

– pFN cc being reduced in SPF mice, with a higher level, however, as compared to Gf mice [3],

– the pFN level of neonatally thymectomized or nude mice does not differ significantly from the control value [3],

– the increase of pFN cc with age both in Gf and Cv mice [2].

Effects of microbial and other immunomodulants on the non-specific defence reactions. Microbial immune stimuli (M, NDV and PSTR) increased the pFN cc in Gf mice and conventionalisation exerted similar influence on SPF mice [4]. Mannozym, a cell wall derivate of a fungus displayed a definite pFN cc increasing effect, independently from the presence or absence of thymus and the mice's microbiological state [5]. Additionally, it was demonstrable that substances isolated from normal microbial flora which stimulate a cellular immune response in the presence of thymus, could stimulate the pFN cc – playing a role in non-specific defence – independently from the thymus [4, 5].

We studied the relationships between the effects of immunomodulants leading to splenic hypertrophy as well as immune modulations and the animals' microbiological and immunological state. It was pointed out that effects of B. pert, T and M inducing spleen hypertrophy could be effective independently from the mice's microbiological state, and no direct relation with the immune stimulating effects was shown, and thus the B. pert induced splenic hypertrophy was associated with definite immunosuppression and Br, CPZ and TP-4 could stimulate the immune responsiveness, causing no spleen hypertrophy. Splenic hypertrophy can develop without thymus, but the cellular immune response can be stimulated only in the presence of thymus [6–11].

Effects of microbial and other immunomodulants on the organism's non-immunological reactions

Examination of drug sensitivity. It is known from literary data that Gf mice as compared to the Cv ones show an increased resistance to anti-tumour preparations such as cyclophosphamide and mustard-nitrogen as well as gamma irradiation. In our experiments a lymphotropic cytostatic, the dianhydrodulcitol (DAD), and its stereoisomer, the dianhydrogalactitol (DAG), belonging to the alkylating group and inducing immunosuppression and injury of the cellular wall, were used. Sensitivity to

chlorpromazine (CPZ) evoking long-lasting and deep dream and dose-dependently immunosuppression intestinal atony was also studied. Our experiments based on determining LD50 values showed that Gf mice had a lower sensibility to DAD or CPZ than the conventional ones [11, 12].

In contrast to these results, opposite changes of drug sensitivity were experienced in Cv conditions. Increased sensibility to DAD and DAG or CPZ was observed in mice

– exposed to stress [13],
– treated by B. pert [14],
– treated by DAG and CPZ in combination [15],
– in young animals that were T treated [16],
– in old mice in state of physiological thymus involution [17].

B. pert and T treatments, similarly to Cv mice, resulted in an increase of DAD sensibility of Gf animals as well, though to a less extent.

Investigating the possible reasons for differences in drug sensitivity, we found that drug sensitivity had no direct relation to the actual immune response capability, namely

– enhanced drug resistance accompanied the known and otherwise inadequate immune function in Gf mice,

– the inadequate immune function originated by spontaneous reason (ageing) or interventions (stress or B. pert treatment) was accompanied with increased drug sensitivity in Cv animals and

– the T in addition to its immunostimulating effect and B. pert along with its immunosuppressive effect increased drug sensitivity both in Cv and Gf mice.

Examination of the endotoxin effect. T treatment increased both Gf and Cv mice's sensitivity to DAD. In the intestinal walls of Gf mice treated with T alone alterations (oedema, inflammation, infiltration) indicating endotoxin effect, typical of DAD treated Cv animals, can be revealed with histological examinations. In Cv mice T slightly contributed to the DAD caused alterations in the intestinal walls; in Gf mice, however, definitely increased them [12, 16].

Examination of bacterial translocation (BT). It is known that in some pathological states members of the intestinal flora can permeate the intestinal wall and translocate into the otherwise sterile inner organs. Therefore, we investigated whether the BT phenomenon can play any role to develop the actual degree of drug sensitivity in Cv conditions. The following results were obtained:

– spontaneous BT was observed in 0–7% of young adult and healthy Cv mice [15, 18],

– spontaneous BT in 13% of old Cv mice with physiological thymus involution was registered [18],

– after stress, in comparison to the spontaneous BT, a significantly higher frequency was found both in young and old Cv mice that became even more explicit in 48 h after the stress effect [18],

– 90–100% positive organs (i.e. BT) were observed at the time of death after DAD LD50 treatment. BT could also be indicated even after chronic or subtoxic dose of DAD [19, 20],

– similarly, highly frequent BT or positive organs of 70–90% were found at the time of death, following the treatments with a dose of CPZ close to the LD50 value. This dosage caused intestinal atony as well as long and deep dream for 48 h in addition to its immunosuppressive effects [11, 15].

From the results described above in the chapter of *Effects of microbial and other immunomodulants on the organism's non-immunological reactions* we can draw conclusions. We can explain the different drug sensitivity of Gf and Cv mice as well as the Gf animals' enhanced drug resistance in the following manner.

In case of Gf mice when intestinal flora is absent, the BT phenomenon cannot aggravate the effect of DAD or CPZ on the wall of intestines, and the acute damaging effect of endotoxin otherwise present in the intestinal flora fails to come about.

In Cv conditions there can be found a minimum BT, however, the immune system is capable to eliminate or kill the bacteria being translocated.

In immunosuppressive state such as in old age as well as both in young and old age after stress, the BT increases. After DAD treatment, BT takes place on a higher level as the drug effects can be added to that after stress or the spontaneous BT of old age, and additionally the acute damaging effect of endotoxin from the normal intestinal flora can also be effective. All these effects together can lead to enhanced drug sensitivity in Cv mice, as compared to the Gf animals.

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MODULATION OF THE CELLULAR IMMUNE RESPONSE TO INTRACEREBRAL LYMPHOCYTIC CHORIOMENINGITIS VIRUS (LCMV) INFECTION IN MICE*

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Certain viruses do not kill the cells they infect. The immunological response of the host in such situations may be involved in the development of pathologic changes and clinical illness. Pioneering work by Rowe has shown that death associated with acute LCMV infection in the mouse is resulted from the immune response. Many investigators using a variety of techniques including neonatal thymectomy, irradiation, or treatment of adult mice with antilymphoid drugs or antithymocyte sera have confirmed and extended Rowe's observations. The study of LCMV and the disease it causes in its natural murine host has provided the initial findings that open new fields in viral immunobiology, viral immunopathology, and cell-cell recognition.

LCMV-induced acute immune response disease

The fatal lymphocytic choriomeningitis following intracerebral (i.cer.) lymphocytic choriomeningitis (LCM) virus infection is the consequence of the cytotoxic reaction of LCM virus antigen specific T lymphocytes to cells expressing viral antigens on the leptomeninx. The course of virus infection thus greatly depends on the cellular immune responsiveness of the animal. Acute lymphocytic choriomeningitis develops in adult mice with intact, developed immune system and the animals die on the 6-8th day following virus infection. The death rate in these mice is 100%. In mice with insufficient T lymphocyte function, as genetically athymic (nude) or newborn mice with undeveloped

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immune system, the lymphocytic choriomeningitis fails to develop and the mice surviving the infection become lifelong virus carriers.

In case of suckling mice, mortality rate and the time between inoculation and death depend on the host's age at infection. The rate of mice surviving the infection in a virus carrier state decreases with progressing age and maturation of the immune system, and old mice die sooner than young ones. In adult mice, the occurrence of fatal meningitis can be greatly reduced by thymectomy in newborn age or by other treatment resulting in lymphoid depletion and immunosuppression. The effect of substances causing lymphoid hypertrophy on the course of LCMV infection was not known before 1970. Therefore, in the early experiments we have investigated the influence of substances (phytohaemagglutinin or *Bordetella pertussis* vaccine) resulting in lymphocytosis and spleen hypertrophy on the outcome of LCMV infection in mice of different age with intact or impaired immune system. It has been found that manifestation of LCMV infection in the form of fatal meningitis is altered in mice having received treatment causing lymphoid hypertrophy. Furthermore, LCMV infection seems to be a suitable model to study the immunomodulatory effect of different substances.

Experiments and results

Influence of a great variety of substances was studied on the course of intracerebral LCMV infection in mice with normal or inadequate (undeveloped, involuted or damaged) immune system. The following substances were studied: phytohaemagglutinin (PHA), dianhydrodulcitol (DAD), a lymphotropic cytostatic agent, the calmodulin antagonist chlorpromazine (CPZ), TP-4 synthetic product (tetrapeptid analogue of the natural thymopoietin sequence), microorganisms, namely *Bordetella pertussis*, *Brucella abortus*, Newcastle Disease Virus (NDV) or microbial substances (*Escherichia coli* lipopolysaccharid (LPS) endotoxin, Mannozym, a zymosan containing cell wall derivative of *Saccharomyces cerevisiae*), etc. Alteration of the outcome of LCMV infection by different treatments as a modulatory influence on the cellular immune response has been considered. While immunostimulatory effects accelerate and increase, the immunosuppressive effects delay the time of appearance and decrease the rate of fatal lymphocytic choriomeningitis. Experiments were performed on suckling (1–4-week-old) mice with undeveloped, on young adult (6-week-old) mice with developed, and on ageing (12–24-month-old) mice with regressing immune system as well as on adult germfree gnotobiotic mice with undeveloped immune system due to antigen deficient environment, moreover on mice of different age having received DAD treatment causing lymphoid atrophy and immunosuppression.

The mice were i.cer. infected with the WE strain of LCMV being maintained in our laboratory by serial passages in mice.

During the experiments development of neurological symptoms (tremor, spasms) that are characteristic of LCM was observed and deaths were registered.

Histological examinations of the animals' brain sections from the infected groups were performed to demonstrate existence or absence of typical LCM and degree of

lymphocytic infiltration. Development of asymptomatic virus carrier state was shown by reisolating LCMV from the brain of mice that survived the observation period.

Stimulation of cellular immune response. In our experiments on suckling mice with physiologically immature immune system the insufficient cellular immune response was stimulated by microbial preparations such as inactivated *Bordetella pertussis* vaccine (B. pert.), both native and radiodetoxified LPS (rdLPS), live attenuated NDV vaccine, Mannozym (M), moreover by PHA and by CPZ [1–6]. In addition, B. pert. stimulated the cellular immune reaction in DAD treated mice with damaged lymphoid system [7].

In young adult mice with intact, mature immune system, stimulatory effect of inactivated *Brucella abortus*, rdLPS, PHA, CPZ and TP-4 was proved [6, 8–12]. It was also demonstrated that PHA stimulates the cellular immune response not only in intact, but also in DAD treated mice with impaired lymphoid system [13].

We found that rdLPS, live attenuated NDV vaccine and Mannozym stimulated the reduced cellular immune response of ageing mice having physiological thymus involution [14, 15].

In adult germfree mice with undeveloped immune system and reduced cellular immune response, stimulatory effect of inactivated *Brucella abortus*, rdLPS and CPZ was demonstrated [6, 8, 9].

Suppression of cellular immune response. Treatment with DAD [16] or with a large, sublethal dose of CPZ decreased the cellular immune response both in suckling and young adult mice [6].

However, B. pert. stimulated the cellular immune response in suckling mice, the same treatment inhibited it in young adult mice [17]. On applying DAD and B. pert. simultaneously in conventional young adult mice, the suppressive effect increased [18]. Both B. pert. and a large dose of CPZ inhibited the cellular immune response in adult germfree mice [6, 19].

Conclusions

In suckling mice both DAD and CPZ treatment has inhibited the development of meningitis, consequently, the cellular immune response to the virus. The course of LCMV infection in 2-week-old mice having received suppressive treatment has been found similar to that of younger, 1-week-old untreated mice. As a result, the immune suppressive treatment has delayed the development of cellular immune response in suckling mice. After immunostimulatory treatment the LCMV infection in suckling mice has run its course similarly to that of untreated young adult animals. This indicates that immunostimulatory treatments accelerate the development of the cellular immune capacity in suckling mice.

Our experiences have proved that direction and degree of the different immunomodulatory effects can be influenced – besides dosing the substances – by age of mice and presence or absence of normal microbial flora. Depending on CPZ doses the immunomodulatory effect was two-directional: large doses were suppressive and small doses were stimulatory, independently from the fact, whether the immune systems of

mice were developed or undeveloped due to their age or environment poor in antigen stimulus. The direction of immunomodulatory effect of substances examined by us has not differed in conventional and germfree mice, but the immunosuppressive effect of B. pert. has been different in degree. Therefore, the cellular immune response has delayed in the conventional- and inhibited in germfree mice.

Our results indicated that all the microbial preparations that were examined by us and being used also in practice stimulated the cellular immune response in suckling mice with physiologically immature immune system. Among them there were the *Bordetella pertussis* vaccine and the radiodetoxified endotoxin of bacterial origin, whereas NDV vaccine contains live attenuated virus and Mannozym is of fungal origin. In adult mice of mature immune system only rdLPS and *Brucella abortus* showed a stimulatory effect and B. pert. suppressed the cellular immune response, whereas NDV vaccine and M had no influence on it. In old mice with regressing immune system and insufficient immune function B. pert. had no influence, the NDV vaccine and M, however, similarly to rdLPS, stimulated the cellular immune response.

Several microbial preparations examined in our experiments are being used in Hungary such as the NDV vaccine for preventive immunization in the veterinary practice and the Mannozym that is applied in human therapy to stimulate the non-specific defensive capacity of the organism and to protect the organism against harmful side-effects of radiotherapy. Radiodetoxified endotoxin preparations (TOLERIN) may be a suitable product to increase non-specific resistance (including activation of bone marrow in immunosuppression, immunodeficiencies) and protection against various types of shocks (radiation injury, septic/endotoxin shock) as well as increase the immunogen effect of antigens as an immunoadjuvant. Clearly in the course of their administration their effect stimulating cellular immunity may also be active and may widen the practical value of preparations.

In addition to the actual state of the immune system, direction of the immunomodulatory effects can be related to the different mechanisms of the modulatory effects. The different immunomodulants can affect the immune system of similar state and function in a different way and degree, and can have the same effect on the immune system of definitely different state.

Our results draw attention to the fact that the organism's actual state can influence the direction and degree of the effect of immunomodulatory treatments in several ways, and this fact should be taken into account in the medical practice during immunomodulatory treatments.

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CORRELATION BETWEEN THYMIC FUNCTION AND SKELETAL GROWTH*

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Experimental and clinical data that deal with the physiological significance of the thymus come from the middle of the last century. In the beginning paediatricians approached its size by physical examinations and surgeons tried to produce experimental thymusless conditions but with poor and contradictory results. Some researchers observed retardation of the skeletal growth in thymectomized animals and others did not remark similar manifestations.

During the last decades, function of the thymus has become elucidated as one of the central lymphatic organs. Importance and significance of this gland in conjunction with wasting and immunological areactivity as sequelae of thymus extirpation right after delivery has been realized. The lethal wasting is characterized by a general retardation of the development and necropsy has revealed characteristic atrophic changes in the spleen and lymphoid system.

Investigations at the Microbiological Institute of Semmelweis Medical University have started to reveal the consequences of neonatal thymectomy and symptoms of wasting being unknown up to that time. In this article our experimental results referring to correlations between thymusless conditions and skeletal growth are summarized.

Experiments and results

Comparative experiments were done on inbred mice of different age (sucklings and young adults) whenever the function of the thymus dependent lymphoid system was

A. absent

1. following neonatal thymectomy
2. in congenitally thymusless (nude) mice

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B. underdeveloped

in germ-free mice having underdeveloped lymphoid-system because of the poor antigen stimuli of their environment

C. affected

1. following Graft-versus-Host (GVH) reaction
2. due to antithymocytic serum (ATS) treatment
3. by lymphotropic cytostatic agents:
   a) by dibromdulcitol (DBD) treatment
   b) due to dianhydrodulcitol (DAD) treatment.

Skeletal growth of mice was investigated by radiography and with bone histology. To study the lymphoid system and growth, lymphocyte counts in blood and body weights as well as relative weights of lymphoid organs were determined.

In the first experiments, we investigated the nature of osseal changes associated with wasting syndrome of neonatally thymectomized mice. This condition is characterized by a rapid weight loss, lethargy, ruffled hair, hunched posture, diarrhoea and finally death.

Radiographs of animals with wasting syndrome and sham-operated control littermates were taken during the 3rd and 6th–7th postoperative weeks. In the radiographs expressed differences were observed between the control and thymectomized mice. These appeared to be particularly significant in the long bones. Loss of bone minerals or bone atrophy was also remarkable in thymectomized mice. To measure bone atrophy radiography was used and the radiograms were evaluated by microscope with an ocular micrometer. The lengths of femora, the diameters and the sum of thicknesses of cortices were measured in microns. This method is named radiomicrometry. Comparing the data of thymectomized and control mice, longitudinal, diametric and cortical retardation in the thymectomized mice was determined. The femora of operated animals were shorter (retardation 24–22%), femoral diameters were smaller (retardation 16–16%) and considerable decrease of cortical thickness (retardation 37–45%) was seen in 3-week-old and 6–7 week-old animals.

The place of quickest growth, the distal ends of femora were chosen for histological study. In the histological sections from the control group the structure and thickness of the articular cartilage were normal. The epiphyseal plate showed continuous endochondral ossification. Similar histological sections from the bones of thymectomized mice displayed remarkable phenomena: in the distal epiphyses there were only 2–3 irregular, very thin fragments of osseal trabeculae, the cartilaginous surface was thin and fibrous and of irregular structure, and the epiphyseal plates showed a minimum or no sign of ossification. The sections revealed significant thinness and extreme sparseness of osseal trabeculae in the metaphysis as well. The hyaline pillars remained cartilaginous, with little or no sign of osteoblast activity. All diaphyseal cortices were strikingly thin.

In the matrix of epiphyseal plates of the wasted animals a marked PAS positivity could be observed. The diminished cellular activity and matrix production in the growth zone as well as preponderance of PAS positivity indicate the considerable alteration of oxygen processes of the epiphyseal plates.

The radiological measurements were in good correlation with the histological findings and the serious clinical condition of wasting syndrome. Combination of methods

of radiography and micrometry represented a useful way to estimate differences of the osseal changes on X-ray sheets more exactly than visual means allow [1–3].

Our previous studies showed that a single dose of dianhydrodulcitol (DAD) effected irreversible harm to the lymphoid system in newborn and suckling mice with underdeveloped lymphoid system. It produced lethal atrophy similar to the wasting syndrome caused by neonatal thymectomy and a decrease in the cellular immune responsiveness. These changes justify to consider the consequences of DAD treatment as "cytostatic thymectomy".

We studied the skeletal growth in mice with thymo-lymphocytic deficiency caused by DAD applied on the postnatal 3rd–4th day. The mice treated with DAD lagged behind the controls in body weight and growth. Fifteen percent of the mice treated with DAD died by atrophy during the experiment. The surviving mice were examined on the 28th day following DAD treatment. The mean body-weight, lymphocyte count in the peripheral blood and relative weight of spleen were smaller than those of the control littermates. The radiograms showed that DAD treated mice were smaller as compared to the control littermates. Radiomicrometric measurements revealed that DAD treated mice had shorter and diametrically thinner femurs than control littermates (retardation was 27% and 32%, respectively). The most expressed alteration of the femur was the decrease in the total cortical thickness of femoral midshaft – the cortical retardation amounted to 46%.

Histology showed marked differences in the growth plate from the distal femoral ends of control and treated animals. The distal femoral epiphysis and the growth plate were of normal histological structure in the control animals. The normal zonal arrangement of the growth plate in DAD treated mice got almost totally disintegrated. The germinal proliferation was sparse and the palisading was irregular. Large acellular fields could be seen in the cartilage transformation zone. The zone of hypertrophic, calcifying or degenerating cartilage cells and of the osteogenesis was very thin as well. Also, the development of the methaphyseal primary spongy bone was sparse. The spongy medullar space was poorly cellularized [4].

It is known that the thymus dependent lymphoid system is not developed in congenitally thymusless nude mice and its function is lacking. Comparative examinations were performed on 4- or 6-week-old homozygous or heterozygous specific pathogenic free (SPF) mice. The mean body weights and the lymphocyte counts in the peripheral blood were less in nude mice than in the control heterozygous ones.

Radiomicrometric measurements revealed an 11% longitudinal retardation on femurs in 4-week-old mice while that of 6-week-old mice was 15%. The femoral cortical retardation amounted 28 and 26%, respectively. There was no significant difference in femoral diameter (retardation was 4 and 7%, respectively).

Histological examination was performed on specimens from the distal end of femurs of the 4-week-old mice. The growth plates of the control mice were normal. The epiphyseal plates of nude mice were smaller with sparse trabeculae and poor cellularization. The growth disc was almost half or one-third of thickness as compared to the controls, showing few divisioning chondrocytes, large acellular areas in the germinal proliferation. Hardly detectable columnar arrangement in the zone of the cartilage transformation with irregular columns built of few cells was seen. There were only 2 or 3

cells in the zone of degeneration. The hyalin pillars were slim, short, stagnating in chondral phase with hardly any osteoblastic activity on their surface. Medullar cytopenia was present [5].

Function of the lymphoid system is known to be highly influenced by environmental effects. Underdevelopment of the lymphoid system including the thymus as well as the low circulating lymphocyte count in germfree animals is well known. We performed comparative studies on the skeletal systems of germfree and non-germfree 4-week-old and 6-week-old mice. Retardation of skeletal growth in germfree mice was demonstrated with radiometric measurements and with histology [6, 7].

Due to the lack of microbial antigenic stimuli in germfree bred mice the lymphoid system and its function is underdeveloped, though the mice have intact thymus. Since the skeletal changes were of the same rate and character in both the nude and the germfree mice, we can conclude that disorder of the skeletal growth in nude mice is due not to lacking endocrine function of the thymus but to the underdeveloped T lymphocyte system.

We have attempted to answer the essential question whether the formerly demonstrated osseal changes of the growing period could manifest when the lymphoid system (including the thymus dependent lymphoid system) is developed and affected in different ways. Among these possibilities there is producing GVH reaction, ATS and cytostatic treatments.

GVH reaction was produced in 2-week-old and 3-week-old (C57BlxA)F_1 hybrid mice by injection of spleen cells from adult C57Bl donors. Three weeks later following the injections, establishment of the GVH reaction was confirmed by significant decrease of circulating lymphocytes and decline of the body weight, and then bone changes were studied in 5–6 weeks of age.

Three to four week-old mice were subjected to ATS treatment and 4-week-old mice were injected with DBD. Skeletal growth was studied at 6 weeks of age.

Radiomicrometric examinations of mice suffering from GVH reaction and treated with ATS or DBD revealed 16, 8, 11% longitudinal, 11, 8, 8% diametric and 41, 28, 27% cortical retardation, respectively. In all mice with affected lymphoid system the histological picture revealed retarded enchondral ossification, and alcianophilia was found indicating an alteration of matrix formation. Reduction of body weight and lymphocyte count was slighter in ATS and DBD treated animals than in mice suffering from GVH reaction. Thus, severity of bone changes, especially cortical retardation was directly correlated with the degree of disturbance of the thymus dependent lymphoid system [8, 9].

Discussion and conclusions

Disturbed enchondral ossification and retardation of the skeletal growth were demonstrated under each experimental condition.

Bone alterations of similar characteristics have similar backgrounds in thymusless mice as well as in mice with underdeveloped and hypofunctioning lymphoid system.

Disturbance of the skeletal growth was associated with complete or partial absence of the thymus-dependent lymphocytes. Degree of the osseal changes was in linear correlation with the disturbed functions of thymus-dependent lymphoid system.

It is generally accepted that the microbial flora of the environment considerably influences development and functioning of the lymphoid system. Microbes and viruses play a decisive role in the development of the generally lethal wasting syndrome in neonatally thymectomized or congenitally thymusless mice while no atrophy or wasting occurs if they are bred in SPF environment.

In our experiments the skeletal changes were accompanied by lymphoid atrophy in mice neonatally thymectomized or treated with DAD and bred in conventional environment; no atrophy was found in SPF nude mice. Our results suggest that presence of eventual pathogens increases the rate of skeletal alterations in mice with lacking or impaired function of the lymphoid system.

In general, it can be stated that normal skeletal growth requires an intact functioning T lymphocyte system.

Concerning human medicine a hypothesis can be formulated on the basis of the results: whenever functioning the thymus-dependent lymphoid system is insufficient or affected skeletal changes may develop [5, 10–12]. To our present knowledge skeletal changes result from deficiency of the thymolymphocytic system in the following pathologic cases:

1. Marasmus infantilis (atrophia infantum) with thymolymphocytic deficiency, and with decrease of the cellulare immune responses and with retarded skeletal growth;
2. Ataxia teleangiectasia with thymolymphatic deficiency and with skeletal hypoplasy;
3. Hereditary lymphopenic agammaglobulinaemia with short limbed dwarfism;
4. Swiss type agammaglobulinaemia and achondroplasia;
5. Cartilage-hair hypoplasia;
6. Serious osteopathy due to steroid and immunosuppressive treatment, and chronic haemodialysis.

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POTENTIAL ROLE OF MICROBIOLOGICAL AGENTS IN SUDDEN INFANT DEATH SYNDROME*

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The potential role of microbiological agents was investigated in 10 cases of Sudden Infant Death Syndrome in Budapest between September 1996 and December 1997.

Autopsy, histological examination and microbiological tests were performed on samples of blood, cerebrospinal fluid, pharyngeal and bronchial samples from infants under six months died suddenly, without previous diseases.

The multifactorial pathomechanism of SIDS was suggested by detection of Parainfluenza Type virus antigen, isolation of toxin producing *Staphylococcus aureus*, *Enterobacteriaceae* and *Candida albicans* strains in large number of more samples of the same infant.

Sudden Infant Death Syndrome (SIDS) is the sudden death of any infant or young child, and a thorough postmortem examination fails to demonstrate any adequate cause for death [1].

A number of hypothesis have been put forward to explain SIDS, in which consistent findings are a peak incidence of these deaths in the two to six month age and male infants are involved in large number. SIDS occurs more frequently during winter

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months and night-time (12 pm to 6 am). The maternal cigarette smoking, absence of breast feeding, prone sleeping position and poor socio-economic situation of the family are great risk factors for SIDS.

Infectious agents and inflammatory mediator response of the infants might contribute to a great deal to SIDS. The "common bacterial toxin hypothesis" is the only hypothesis that explains the characteristic age distribution of SIDS [2, 3]. The hypothesis states that a viral infection of the upper respiratory tract can lead to a "supra-infection" by commensal bacteria, and if this occurs at the same time when infantile immunoglobulins are at their lowest, the baby is at risk of SIDS. Epidemiological data concerning SIDS have provided support for the argument that common bacterial toxins are important in the syndrome [4].

There has been a significant decrease in infant mortality rates in Hungary in the recent decades. In the past years incidence of SIDS was not decreasing, it was 0.19–0.25 per 1 000 live births in Hungary [4]. Therefore SIDS is an outstanding problem in our country, and we have examined pathological changes and microorganism flora of SIDS infants.

Materials and methods

Ten infants who died in SIDS between September of 1996 and December of 1997 were examined by detailed autopsy, histological and microbiological tests.

1. *Autopsy and histological studies.* Samples from various organs (heart-blood, lung, liver, spleen, kidney and segments of small and large intestine) were obtained during autopsies. The autopsy was done within two days in sudden death cases. The bodies were stored at temperature of 4 °C before autopsy. Mild infections of the upper respiratory tract or middle ear, or positive bacteriological or virological findings unaccompanied by morphological findings were not considered to have been the cause of death. All tissues were fixed in 10 per cent buffered formalin for 3–6 days. Five μm thick sections were cut from each block of paraffin-embedded tissues and were stained with routine hematoxylin and eosin (HE) method. Samples were also used for identifying any degenerative or inflammatory changes present.

2. *Microbiological studies.* Pharyngeal secretions were obtained by pharyngeal swabs and liquor cerebrospinal fluid by punction of the fourth ventricle. The swabs were taken and placed into transport culture medium at the scene within two hours after the sudden death was discovered. The parent/s signed the informed consent, and they got detailed information about the purpose of our investigation in sudden death cases. Heart-blood, lung tissues were obtained during the autopsy. Virus antigens were detected in paraffin-embedded lung tissue.

2.1. *Bacterial cultural methods.* The specimens were streaked on the surface of blood and chocolate agar, incubated at 37 °C aerobically, anaerobically and 5% CO_2 atmosphere. Plates were examined 24 h and 48 h after the incubation. The strains were identified by Cowan–Steel methods [5], Gram negative rods were tested by bio-Merieux ID32E system or ID 32 GN system.

2.2. *Yeast cultural methods.* Sabouraud agar containing 10 μg/ml gentamicin was used for the isolation of yeasts. The identification occurred in bio-Merieux ID32C system.

2.3. *Immunohistologic procedures for detection of viral antigenes.* Paraffin-embedded sections were dewaxed in xylene and immersed in 0.1% trypsine in PBS to the release of overfixed antigenic sites and treated with 1% H_2O_2 in methanol. They were incubated with appropriately diluted monoclonal antibodies against the following viruses: Respiratory Syncytial viruses (A, B), Influenza

virus A, Influenza virus B, Parainfluenza viruses (Types 1, 2, 3), adenoviruses (ARGENE, Biosoft, France). After incubation (2 h, 37 °C) bounded monoclonal antibodies were detected with anti-mouse IgG-HRPO complexes (Sigma). Peroxidase activity was visualized using AEC substrate and sections were slightly counterstrained with hemalum.

3. *Epidemiological information about SIDS infants* were collected through the collaboration of parents and paediatricians.

Results

Autopsy and histology. Intrathoracic petechiae were detected in 9 SIDS cases, subpleural petechiae were found in 8 cases, subepicardial petechiae were detected in 6 cases, subcapsular thymic petechiae were found in 6 cases. Subconjunctival petechiae were not detected in any SIDS case. Mild or severe pulmonary edema was found in 9 cases. Lymphoid cells or macrophages in the upper respiratory tract were detected in 5 cases. The histological data of these cases are presented in Table I.

Bacteriology. In 2 cases *Staphylococcus aureus* (*S. aureus*), in 4 cases *Enterobacteriaceae* species, in 4 cases *S. aureus* and *Enterobacteriaceae* strains were isolated. In the 1st case *E. coli* 0:8 H:999, a non-enteropathogen serotype was isolated from blood, cerebrospinal fluid and throat specimen. The results of bacteriological cultures are shown in Table II.

Yeast isolates were *Candida albicans* (*C. albicans*). High number of *C. albicans* was cultured from pharyngeal secretions in case 1st, from lung tissue in case 3rd, from pharyngeal secretions and lung tissue in case 5th (Table II).

Table I

Autopsy findings in SIDS cases

No. of cases	Autopsy findings
1	edema (brain, lung), petechiae (subpleural, subepicardial, subcapsular of thymus)
2	severe pulmonary edema, petechiae (subpleural, subepicardial, subcapsular of thymus), few macrophages and lymphoid cells in lungs
3	edema (brain, lung), petechiae (subepicardial, subcapsular of thymus)
4	severe pulmonary edema, petechiae (subpleural, subepicardial, subcapsular of thymus) few lymphoid cells in the upper airways
5	mild pulmonary edema, petechiae (subpleural, subepicardial, subcapsular of thymus)
6	severe pulmonary edema, petechiae (subpleural, subepicardial, subcapsular of thymus)
7	subpleural petechiae
8	severe pulmonary edema, petechiae (subpleural, subcapsular of thymus), few lymphoid cells in the upper airways
9	severe pulmonary edema, few macrophages and lymphoid cells in lungs
10	severe pulmonary edema subpleural petechiae, few lymphoid cells in the upper airways

Table II

Isolated microorganisms from SIDS cases

No.	Throat specimen	Pulmonary tissue	Blood culture	Cerebrospinal fluid
1	*Staphylococcus aureus* *Escherichia coli* *Candida albicans*	*Staphylococcus aureus*	*Escherichia coli*	*Escherichia coli*
2	*Staphylococcus aureus*	*Staphylococcus aureus*	*Staphylococcus aureus*	sterile
3	*Escherichia coli*	*Staphylococcus aureus* *Candida albicans*	sterile	sterile
4	*Staphylococcus aureus* *Serratia rubidea*	*Staphylococcus aureus* *Serratia rubidea*	sterile	sterile
5	*Staphylococcus aureus* *Klebsiella oxytoca* *Candida albicans*	*Staphylococcus aureus* *Klebsiella oxytoca* *Candida albicans*	sterile	sterile
6	*Enterobacter aerogenes*	*Enterobacter aerogenes* *Enterococcus faecalis*	*Enterococcus faecalis*	sterile
7	*Escherichia coli* *Enterobacter aerogenes*	*Escherichia coli* *Enterobacter aerogenes* Parainfluenza virus	sterile	sterile
8	*Escherichia coli*	*Escherichia coli*	sterile	sterile
9	*Staphylococcus aureus*	*Staphylococcus aureus*	sterile	sterile
10	*Escherichia coli*	*Pseudomonas aeruginosa* *Enterococcus faecalis*	*Enterococcus faecalis*	*Enterococcus faecalis*

Virus antigen detection. In the 7th case Parainfluenza Type 2 virus antigen was detected with negative previous clinical symptoms and very poor pathological changes. All other SIDS cases were negative (Table II).

Epidemiological data. In our cases 3 female and 7 male infants were under six months, and the majority of them was under the age of three months. They were found dead at their homes, and only 1 infant died in hospital. In most of the cases the time of death was the early morning hours. Four infants had showed symptoms of mild cold before death, and 3 infants had high fever. In 7 cases the infants had not reached the average Hungarian birth weight (3100 g). SIDS cases were found in spring, autumn and winter months. In one of the cases a twin with low birth weight was the victim of SIDS. Bottle feeding, mother's cigarette smoking and average social standards were the main features in our material (Table III).

Table III

Relevant clinical details of SIDS cases

No.	Sex	Age	Clinical history	Birth weight (g)	Time of death	Site of death
1	F	2 months	mild cold	2600	5 am	home
2	F	6 months	mild cold, cysta colli with *Staphylococcus aureus*	3200	4 pm	home
3	F	4 months	mild cold	2800	3 am	home
4	M	1 months	negative	2620	5.30 am	home
5	M	2 months	mild cold	3250	11.40 am	home
6	M	3 months	negative	2770	10 am	home
7	M	2 months	negative	3300	6 am	home
8	M	4 months	high fever without medical treatment	2800	8 am	home
9	M	6 months	high fever	2950	0.30 am	home
10	M	6 months	anus atresia at birth, cold, high fever for 3 weeks before death, resuscitation	2700	5.10 am	hospital

F = female
M = male

Discussion

The first six months for the newborns are highly important and sensitive periods in their adaptation life. The temperature changes from 36.8 °C to the adult 36.4 °C at the age of 8–16 weeks [6]. The change of temperature may last until the age of 22 weeks at infants born with low Apgar value or receiving artificial feeding [7]. Among various physiological changes, a significant decrease in heart rhythm was observed during sleep as an introductory factor of this period [7]. Significant decrease of cortisol level of urine was detected in the early morning hours [8]. The delay of all these changes could result in immature state of infants including the decrease of maternal antibody level and gradual operation of the infant's immune system only at the age of two-three months [7].

In this period normal microbial flora of epithelial and mucous surfaces are developed in the newborns and this is a highly important factor of natural resistance [9, 10].

In our work microbial flora of infants died in SIDS were studied, and the role of isolated strains were assessed in the cases, in which the same species were cultured from various organs or pathogenic bacteria and yeasts were isolated in pure culture. In two cases *S. aureus* was isolated from the pharynx, lungs and blood. *Enterobacteriaceae*

species were isolated in addition to *S. aureus* in four cases. In other four cases *Enterobacteriaceae* strains in pure culture and *C. albicans* were isolated from the pharynx and lungs.

Our results seem to be in accordance with literary data as the isolation of *Enterobacteriaceae* strains and *S. aureus* was reported by several publications [11–13]. These microorganisms are transient in low number in the upper respiratory tract, though the large number of these toxin producing strains could be a decisive factor in the development of SIDS [1].

S. aureus colonization are showed in 40–50% of infants in the first few months of life [9]. The ideal temperature for pyrogenic toxin production of *S. aureus* is 37–40 °C [15]. In normal respiration the temperature of mucous membrane of respiratory tract is under 37 °C, therefore increase in local temperature (including prone position, increase of respiratory discharge), provides a favorable microenvironment for the production of toxin [16]. The superantigen feature of pyrogenic toxin of *S. aureus* was confirmed, and this can enhance the effect of bacterial endotoxins [17]. The significance of pyrogenic and enterotoxin producing strains of *S. aureus* in SIDS pathomechanism has been confirmed by several researchers [18, 19].

The LPS antigens of *Enterobacteriaceae* species provide a physiologic effect that cannot be eliminated by the immature immune system of the infants. Therefore, the classical effects of endotoxin can be observed [20]. Endotoxemia has not been detected yet in the blood of infants died in SIDS [3]. However, decrease of IgG endotoxin antibodies and decrease IgM endotoxin levels have been observed [21]. The vital functions of infants can be disturbed by yeasts or their toxic metabolic products [22].

Toxins of *S. aureus* and LPS of Gram negative bacteria may be linked to CD 14 antigens of alveolar macrophages and monocytes. Through TNFα induction they can cause fever and release of various inflammatory mediators including IL 1 and thrombocyte activating factors [23, 24]. The pathomechanism of toxins of isolated microorganism is known, and this can explain the triggering of SIDS process. However, the direct cause of death is the systematic reaction started by the toxins of microbes [25].

Strains of *Haemophilus influenzae* and *Bordetella pertussis* were isolated in high number from the nasopharynx of infants died in SIDS [26]. The increase of toxin producing bacteria in gastrointestinal tract, *Clostridium botulinum*, *Clostridium perfringens* and *Escherichia coli* has also caused the development of SIDS [27]. The bacterial colonization of respiratory epithelium is contributed by virus infection and monocytes and macrophages are stimulated to produce INFγ production [28].

Different kinds of viruses have been identified among SIDS infants including Influenza virus, Respiratory Syncytial virus, Rhinovirus and Rotavirus [29]. Although the Parainfluenza Type 2 virus has been isolated from infants and children with severe diseases (croup syndrome, bronchitis, pneumonia) [30, 31]. Its role in SIDS has not been published yet. We detected Parainfluenza Type 2 virus antigen in lung tissue without any histological reaction as acidophil inclusion body or syntitial cells. Parainfluenza Type 2 virus could spread quickly in immunocompromised newborns, since the fusion glycoprotein component of the virus could be activated by the bacterial protease enzymes.

Results of autopsy and histological examinations showed no special characteristic features. Inflammatory mediators, pyrogen, necrotic and endotoxins directly, or the

circulatory failure could cause petechial haemorrhages in the region situated up to the diaphragma. We have not found petechial haemorrhages in the conjunctive and in the organs in the abdomen. Our autopsy results have not suggested any special finding, which could be suspicious for violent death. Howat's pulmonary immunopathological examinations of SIDS cases have published similar results to our findings [32]. New sensitive flow cytometry and ELISA methods improved by Blackwell can detect bacterial toxin presence in tissues of SIDS cases [33].

Our epidemiological data have confirmed the existence of risk factors in the development of SIDS, including age, time of death, nutrition and nursing habits [3].

The exotoxins of Gram-positive bacteria and endotoxins of Gram-negative bacteria are important factors of pathologic changes in SIDS process. *S. aureus Enterobacteriaceae* species or *C. albicans* strain, producing toxins alone or jointly, were isolated in the majority of our cases. It can be assumed according to the hypothesis of Blackwell [3] and Morris [2] that the toxins of these microorganisms through the induction of mediators may cause heat or vascular shocks, hypoglycaemia, deep sleep and apnoe leading to sudden death during the period of adaptation to life.

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THE ORIGIN AND EVOLUTION OF VIRUSES*

(A REVIEW)

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Summary

Viroids and prions might have existed early at the border of inanimate and living worlds. Most extant viruses can be characterized as derivatives of ancestors originating

*Dedicated with best wishes to octogenarian virologists Dr. Elek Farkas (Budapest, Hungary); Dr. Vincent Groupé (St. Petersburg, FL) and Dr. Joseph Melnick (Houston, TX).

*This paper is written to commemorate the fiftieth anniversary of foundation of the Institute of Microbiology, Semmelweis Medical University, Budapest.

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from episomal elements of prokaryotes (DNA phages) and later from eukaryotes. Retroviruses very likely originated from cellular retrotransposons.

Retrograde evolution of some large viruses from obligatory intracellular bacteria is possible but the ontogenesis of extant bacteria does not include a viral form of existence (the filterable L forms are not viruses) and well-defined viruses do not regenerate back into vegetative bacterial forms. Biologists experimenting with the evolution of prokaryotic and eukaryotic ancient cells cannot ignore the earliest appearance of viruses within or outside the living matter. Viruses participated in and gave direction to the evolution and natural selection by coexisting with uni- and multicellular organisms for billions of years. The coevolution of viruses and their host cells is characterized by incessant attacks and counterattacks through gene rearrangements and mutations (induced in the virus by an immunological counterattack of the host or by transgression of species barriers by the virus) and recombinations. Recombinations occurred between viral and viral or viral and host genes. Acts of "molecular piracy" as practiced by ancient viruses endowed the virus with the expression of several host genes for the advantage of the virus in its replicative cycle and host-to-host spread. Probably the first immortalized and malignantly transformed cells were induced by viruses as viruses evolved anti-apoptotic measures. While infected cells resort to apoptotic death before the assembly of a new viral progeny, prominent are the anti-apoptotic measures viruses evolved in order to assure the completion of their full replicative cycle. Further, viruses may escape neutralization by host antibodies and may survive a counterattack by the host's T cells directed at virally infected cells of its own. Viruses may induce a form of tolerance and coexist with their host without inducing disease. Persistent and apparently or deceivingly apathogenic or even attenuated viral "quasi-species" populations may contain individual particles that regain virulence due to recombinations and/or gene rearrangements, especially when transgressing species barriers. Xenotropic viruses of animals may replicate in human cells and vice versa confounding experiments with xenotransplants or with use of veterinary viral vaccines for the treatment of human diseases.

Introduction

This publication is a sketchy overview of a topic worthy of a monograph of over 1000 pages and references. The origin of viruses remains speculative but conjectures should be presented within reason. The co-evolution of viruses with their hosts is now undergoing a major change: mankind is intervening! Changes in the ecosystems, domestication of plants, insects and animals and the production and wide application of antiviral vaccines (killed or live attenuated) forcefully alter the natural course of events but always in favor of *Homo sapiens*. The rapid and extensive mobility (travel and transportation) around the globe allows for the transmission of latent and impending viral diseases (and their vectors) of plants, animals (whether pets or for food, husbandry or exhibit, or medical experimentation) and of human passengers. Add the use of viruses as agents of biological warfare against insects, rabbits (the calicivirus going astray in Australia) or human inhabitants of a large metropolis. Blood products administered in a

hospital, mishandled foodstuff sold in a supermarket or in a restaurant and most liberal sexual practices convert sporadic viral diseases into world-wide epidemics: is *Homo sapiens* still in control?

ORIGIN

Abiogenesis

The definition of viruses excludes their existence before living cells: no viral replication could have had occurred without the cell [1]. Prebiotic organic chemical reactions advancing on the surface of the anaerobic archaic Earth resulted in the formation of amino acids. Water vapor, methane (CH_4), ammonia (NH_3) and hydrogen (H_2) can be made to react (and reproducibly so) and form formaldehyde (CH_2O), aldehydes (CHO), hydrogen cyanide (HCN) and glycine or other amino acids (the Miller-Urey experiments in Chicago); and adenine and other bases of RNA or DNA (the Juan Oró experiments in Houston). Other nucleotides (especially uracil) with ribose and phosphates form the catalytic ribozymes that can arrange the joining together of short chain oligonucleotides. The source of energy since ancient times is triphosphates.

Ribozymes of Cech [2] also could catalyze the formation of short peptide chains as well as the cleavage of peptide bonds. For the formation of nucleotides, cyanogen (C_2N_2) and cyanoacetylene (HC_3N), both present in the ancient environment, were essential. Without enzymes, clay (montmorillonate) can catalyse the formation of RNA oligonucleotide chains: adenine pairing with uracil and guanine with cytosine (the Watson-Crick base pairs). The prebiotic replication of those short RNA chains was possible when a guanine-bearing ribonucleotide served as template for the formation of a cytosine-bearing strand, after which the two strands separated (the Orgel experiments in San Diego). DNA is formed as thymidine substitutes for uracil and the sugar becomes deoxyribose.

Theories abound concerning the formation of the genetic code: how the identity determinants of the first tRNA molecules interacted with amino acids, how archaeal and eukaryotic tRNA introns are related through a common ancestor, how the final intron of one gene would recombine with the first intron of another gene thus creating a new coding sequence for example for enzymes; the roles of histones, the first RNA-dependent RNA-polymerase (RdRp) and the first membranes that encircled the protocells (different in archaea due to phospholipids, in protobacteria due to hopanoids and in eukaryocytes due to cholesterol dominating). Complex molecules coexisting with the first protocells but without the stamina to evolve into cells themselves might have "survived" until "cells" (with nuclei?) became available for them to replicate within. Plant viroids (or virusoids) might attest to the existence of an ancient "RNA world". The viroid's ssRNA genome forms G=C and A=U pairs without encoding a polypeptide and it is not larger than 375 nucleotides in length. Prions also extend from fungi (*Podospora* sp.): Het-s and yeasts: Sup35 to the mammalian and human brains [3–5]. Their place in the evolution of proteins directing differentiation and development remains most obscure.

According to the progene hypothesis [6] the first protocells possessed a DNA genome and the first viruses originated from this genome. From an ancient DNA virus, retrotransposons, retroviruses and RNA viruses evolved. The protocell's genome consisted of a polynucleotide (mixed anhydrides and trinucleotides) and the gene product polypeptide-processing polymerase. The archaeviral virion supposedly possessed dsDNA, structural proteins and lipoprotein envelope; was able to leave the protocell by budding and enter another protocell on account of viral envelope and protocell membrane interactions. Plasmaviruses of *Mycoplasmataceae* [7] resemble most these archaeviruses. In present day viruses more recently evolved genes encode isometric or helical capsids. Viruses with ssDNA genome supposedly evolved form dsDNA viruses; RNA viruses evolved from the mRNA of DNA viruses after the development of RdRp.

In contrast, the "RNA world" [8] concept considers RdRp-encoding RNA viruses more ancient than DNA viruses. Only after RNA dependent DNA-polymerase (RdDp) appeared followed by the evolution of DNA-dependent RNA- and DNA- polymerases (DdRp or Dp) [9] could DNA viruses be formed in the primordial habitat of the protocellular living matter. The enzyme DdRp is essential for transcription in cell division of both prokaryotes and eukaryotes and in the replication of cytoplasmic DNA viruses. Amino acid sequence homology for the virally encoded enzyme spans insect iridescent virus, fish lymphocystis virus and human molluscum contagiosum virus with the closest relationship in vaccinia and variola viruses. Did ancient viruses evolve their own enzyme or acquired it from an ancient protocell? Interestingly in prokaryotes DNA viruses (10 DNA, 2 RNA/12) and in eukaryotes RNA viruses (47 RNA, 13 DNA/60) dominate inferring as if DNA viruses were more ancient. In higher plants retroviruses do not exist (but retroelements and dsDNA- and reverse transcriptase-positive pararetroviruses are active); these retroelements can be lead back to a common ancestor [10, 11]. Could it be then that ssRNA viruses evolved only after the eukaryotes separated from prokaryotes in the course of evolution? Whereas dsRNA viruses parasitize eukaryotes, algae, fungi, protozoa, plants and the entire animal world; therefore the ancestor of dsRNA viruses with fragmented genomes must have had arisen before the separation of prokaryotes and eukaryotes (vide infra).

Endogenous derivation

It appears that prokaryotes and archaea co-existed before the development of eukaryotes. The eukaryotic cell on a higher level of evolution possesses cell membrane, cytoskeleton, cytoplasmic organelles, endoplasmic reticulum and nucleus where its DNA strands reside separated from the cytoplasm by a nuclear membrane. Experts estimate the distance in time between prokaryotes and eukaryotes to span 1 or 2 billion years, while the age of our planet is estimated to be over 4.5 billion years.

If an acceptable definition of life is that of a self-maintaining system that replicates itself, undergoes mutations, development and natural selection, then life appeared on Earth very slowly and gradually between 3800 to 3500 million years ago. The chemoton as envisioned by Gánti and presented by Szathmáry and Maynard Smith [12] resembles

the first systems that existed at the border of inanimate and living worlds (Fig. 1). These systems consisted of an autocatalytic cycle, a polymer capable of template replication and a membrane allowing the entry of "nutrients" (fuel for the autocatalytic cycle) and exit of waste; the template-replicating unit generated the membrane. Most primitive prokaryotes and archaea supposedly derived from chemoton-like systems and coexisted for another billion years when an encounter occurred – one such event in a billion years! [13, 14]. It consisted of the chimeric fusion of a prokaryote and an archaea condensing (uniting) their protogenetic material in the first nucleus-like structure. For the first time in evolution some surplus of protogenetic material occurred resulting in the expulsion of excess naked protogenes; these then might have returned to prokaryotes and archaea and replicated in them outside, or integrated into, the genetic material of these protocellular organisms. The origin of ancient phages can be envisioned by this concept.

The first eukaryotes (the chimeric fusion product) proliferated in an environment also populated by prokaryotes and archaea evolving toward gram negative bacteria and archaebacteria. At some point in time second and third encounters had taken place: some prototype eukaryotides engulfed cyanobacteria (blue-green algae) producers of O_2 and thus acquisitioned chloroplasts [15]. These cells evolved into plants that changed the Earth's atmosphere to what it is today. Some other eukaryotes gained mitochondria from endosymbiotic purple bacteria and evolved into oxygen-consuming animal cells forming the entire animal world extinct and extant. Those who so persuasively lay down the sequence of events for cellular evolution, conspicuously omit the origin and evolution of viruses from their drafts. Yet viruses are part of the evolution of the living matter from its beginning on (Fig. 1) [16–18]. Viruses accompanied their hosts be these bacteria, algae, fungi, plants, protozoa, insects or animals throughout their evolution and without doubt influenced the direction of evolution and natural selection of their hosts as they developed antagonistic or mutually acceptable strategies for eradication and/or coexistence.

The phage-bacterium relationship is the most archaic. Prokaryotes and Archaea and related microorganisms (mycoplasma, Bartonella) are all carriers of phages (the existence of rickettsial and chlamydial phages is still in doubt). Of the 3000 different bacterial viruses 95% are tailed phages with dsDNA and icosahedral head. Phage prototypes are λ, and P2 and 4 and T4 for *Escherichia*; MΦ29 for *Bacillus* sp; and LS for mycobacteria. The T4 phage has a self-splicing RNA sequence in its genome. P4 existed in a bacterial episome and spread by conjugation only until it acquired "packaging nucleotide sequences" through recombination with phage P2; with coat proteins now newly synthesized, P4 had evolved from an episomal element into an infectious virus. Lambdoid phage dsDNA genomes consist of 50000 base-paired strands with genes encoding lytic enzymes, tail fibers and structural proteins of the head assembly. These phages readily exchange genetic material with one another and their hosts by at least four different mechanisms. For example phage genes for transglycosylase and some murein hydrolases (lysozyme, amidase) were obtained through "illegitimate" recombinations. Phages infecting *E. coli* or *Haemophilus influenzae* carry very similar integrase genes suggesting preservation from a common ancestor. These phages build their protein shell first and guide their dsDNA into it under the direction of packaging signals. Tail fiber genes of *E. coli* phages are related and derive from a common ancestor. Lysogenic phages exist integrated within the bacterial genome. Cryptic prophages are structures in the

bacterial genome with which lambdoid phages readily exchange genetic materials. Lytic phages destroy the host bacterium. The host prokaryote can protect itself against phage-induced lysis through the works of a 69-nucleotide RNA encoded by an operon; it terminates phage nucleic acid transcription [19, 20]. In *Lactobacillus* sp. the phage resistance factor gene contains 1056 nucleotides [21].

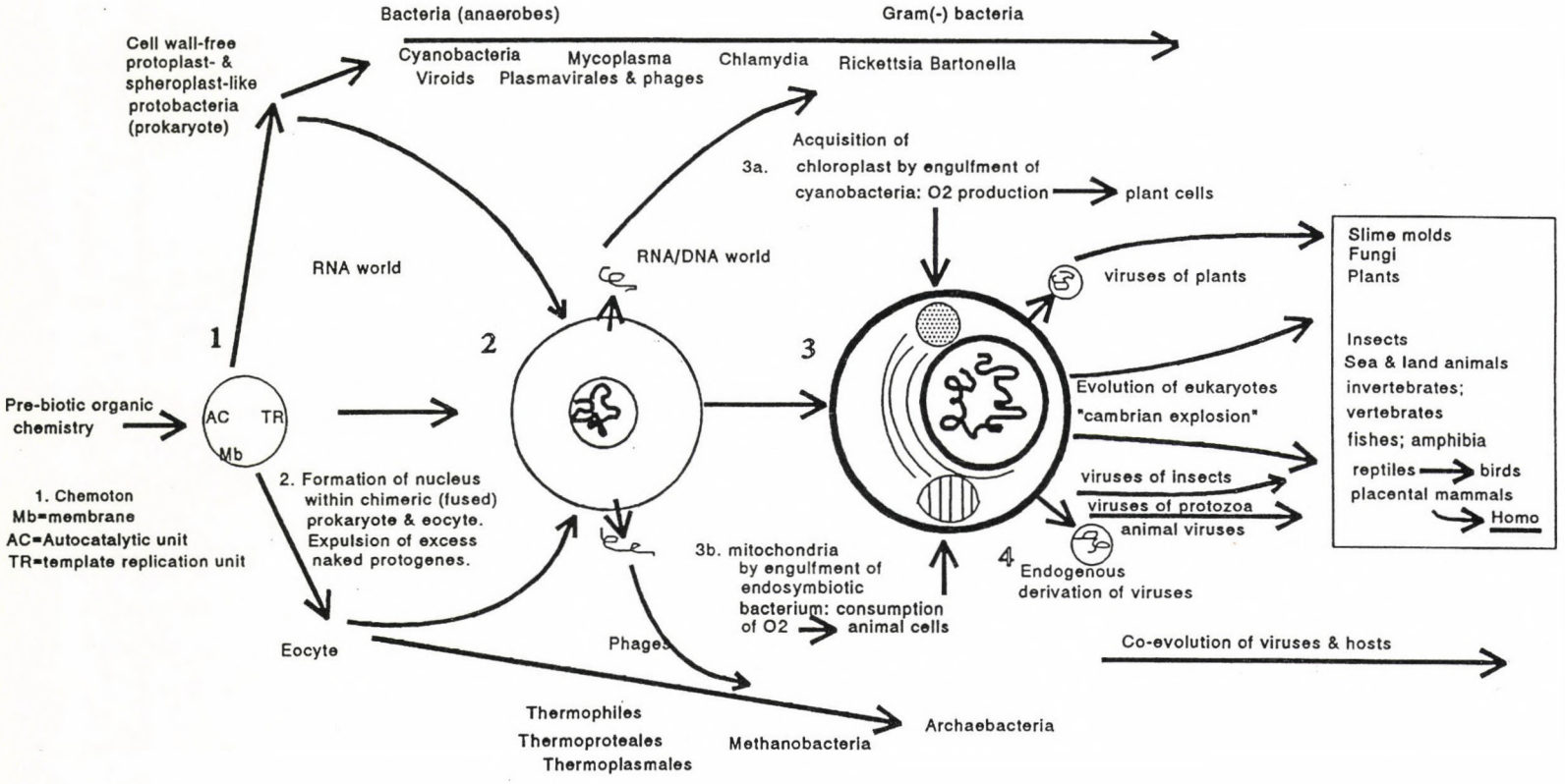
There is over 80% nucleotide sequence homology between phage and host cell amidase, DNA replication initiation protein, methyltransferase, transglycosylase, etc. genes [22, 23]. T4 phages display protein homology with both prokaryotic (methyltransferase) and eukaryotic (DNA polymerase, topoisomerase, ligase) cellular proteins due to "promiscuous" ancient recombinations. Icosahedral capsids built from coat proteins of almost identical eight-stranded "ß-barrel backbone folds" are shown in certain phages, plant viruses (carmo-, luteoviruses), picornaviruses (foot and mouth disease, poliomyelitis virus) and possibly in adeno- (the hexon capsid protein), flavi-, pesti- and hepatitis C viruses. Could it be that these divergent currently extant viruses possess the descendant of a common ancestral gene for these structures or that widely different ancient genes underwent "convergent evolution"? The lysozyme genes of T4 phages and eukaryotic avian species are especially similar [23].

Fig. 1. Origin and evolution of cells and viruses →

Pre-biotic organic chemistry: formation of amino acids and nucleotides.

1. *Chemoton of Gánti as presented by Szathmáry and Maynard Smith [12].*
2. *First eukaryote as chimeric fusion product of a prokaryote and ancient archaebacterium (eocyte) [13, 14]. Protogenes condensed into nucleus; excess protogenetic material expelled and might have returned as naked protogenes into prokaryotes (origin of phages? plasmavirales?). Encounter of ancient prokaryote and eocyte: once in a billion years! All life functions on Earth are monophyletic [14]: conservation of basic biochemistry from prokaryotes, archaebacteria and unicellular microorganisms (yeast) to most complex organized cell communities including animals and Homo sp. ("Saccharomyces sapiens": Yeast Genetics and Human Disease). (Conference, Baltimore MD Nov 14–17 1996); and the evolution of "bricolage" [16].*
3. *Acquisition of chloroplasts or mitochondria by ancient eukaryotes: development of O_2-producing plant cells and O_2-consuming animal cells.*
4. *Endogenous origin of viruses and their co-evolution with their uni- or multicellular hosts.*

Examples of ancient phage-host relationships: phage phi H in the archaebacterium Halobacterium salinarium; linear or circular dsDNA phages within lipid membranes in hyperthermophilic Archaea; mycoplasma virus L2 in Acholeplasma laidlawii; particle-associated protein-carrying phage in Bartonella henselae [17, 18].



Million years

3800	2000			570-425	400-290	250-100	65	3-2.5
Proterozoic	Precambrian		Cambrian	Devonian	Triassic	Tertiary	Quaternary
		Paleozoic	Ordovician	Carboniferous	Jurassic	Paleocene	
			Silurian	Permian	Cretaceous	Eocene	Pleistocene
					Mesozoic	Cenozoic	

In addition to diphtheria, cholera and streptococcal erythrogen toxins [24–28] well known to be encoded by phages, bacterial (Shigella, Salmonella, Haemophilus, etc.) virulence and morphogenic factors are also encoded by phages especially by the ssDNA filamentous phages [29]. Natural haploid gene transfers in bacteria occur by phages (transduction); conjugation; and uptake of naked DNA (transformation). Virulence and antibiotic resistance may thus be transferred among bacteria often in an interspecies setting.

A second ancient host-virus-relationship is that of protozoa and their viruses [30, 31]. Extant protozoa are widely infected with viruses. *Giardia lamblia* is one of the unusual eukaryotes that lack mitochondria; diplomonads, trichomonads and microsporidia emerged before the acquisition of mitochondria by the primitive eukaryotes [32, 33]. The presence of the mitochondrial chaperonin gene cpn60 in *G. lamblia* however suggests that unsuccessful abortive early attempts at acquiring mitochondria must have occurred in the evolution of eukaryotes. The dsRNA virus that readily infects *G. lamblia* may encounter resistance. Virus-resistant protozoa do not express the cell membrane receptor for virus attachment [34]. *Polysporoplasma* sp. of a marine fish (*Liza* sp.) is infected with a picornaviridae-related icosahedral virus [31]. *Leishmania braziliensis* (and other *L.* sp.) harbor single-segmented non-enveloped dsRNA viral pathogens [30]. *Babesia bovis* harbors a dsRNA virus [35]. It is not well studied if virally infected protozoa could produce ancestral interferons. It has been shown that extant protozoa resist infection with viruses of their host (example: failure to propagate herpes, HIV or poliomyelitis virus in *Entameba* sp.) [36].

It remains controversial if RNA viruses preceded DNA viruses. Negative-stranded RNA viruses with unsegmented or segmented genomes have their genomes transcribed before it is translated. The virally encoded RdRp is highly variable thus heterogenous viral progenies arise (quasi-species) amenable to natural selection under environmental pressures. Mononegavirales (rhabdo-, paramyxo- and filoviridae) are much more stable than negative strand RNA viruses with segmented genomes (bunya-, arena- and orthomyxoviruses). Here viral gene reassortments without recombinations are commonly practiced during the replication cycle. The emergence of recombinant, mutated and/or naturally selected (avian → porcine → human) orthomyxoviruses is commonplace [37–43]. Genetic exchange between a coronavirus and an orthomyxovirus has been recognized [44]. The ancestor of influenza D virus might have been propagated by an insect vector where it might have acquired an insert from a baculovirus recognizable in the influenza D viral genome today: the G64 protein [45, 46]. Positive strand RNA viruses (picorna-, calici-, flavi-, coronaviruses; hepatitis C and E viruses; plant viruses; bacteriophages, etc.) replicate without a DNA phase, in the cytoplasm of their host cells where they encode their own RdRp. These viruses receive and readily accept host cell genes encoding homologues of host cell molecules such as ubiquitin, helicase, proteases, capping enzymes and helical capsid proteins. These viruses might have had derived from an ancient mRNA. They are subject to point mutations and recombinations [40–43]. The most spectacular recombination is the one between Sindbis and eastern encephalitis viruses resulting in the virus first described as western encephalitis virus. The recombinant virus exhibits Sindbis viral envelope and EE viral capsid proteins [47, 48].

Retroviruses (onco-, lenti- and spumaviruses) are very different from pararetroviruses (hepadna-, caulimo- and badnaviruses) which possess dsDNA and reverse transcriptase [10, 11, 49]. Acutely transforming retroviruses operate through transduced cellular protooncogenes-oncogenes encoding most commonly growth factors and/or their receptors; they can immortalize and malignantly transform cells [50].

The *pol* genes of retroviruses encode the reverse transcriptase, a ribonuclease and an integrase thus accomplishing the integration of the viral genome in the host cell genome. Pararetroviral genomes are devoid of integrase-encoding sequences. Retroviruses differ from other retroelements of the normal cell in that the virus spreads horizontally and retrotransposons and retroposons are not infectious. If these structures acquire the genes *env* and *gag*, will they become infectious? Cellular retrons (Temin) or retrotransposons with *gag* and *pol* gene (Xiong and Eickbush) were the most likely ancestors of retroviruses [10, 11]. The human genome carries not translated endogenous retroviral sequences: our "retroviral heritage" [51]. Some incompletely transcribed endogenous retroviral sequences may contribute to autoimmune diseases including systemic lupus erythematosus (vide infra).

Of the large DNA viruses [52], poxviridae possess 150–380 kbp genomes, replicate in the cytoplasm and infect hosts from invertebrates to vertebrates including avian, simian and human pathogens. The completely sequenced genome of vaccinia virus includes genes encoding some 30 proteins with close sequence homology to host cell proteins, some with immunoregulatory properties. Large and medium-sized DNA viruses from poxviruses downwards through herpesviral (125–230 kbp), baculoviral (145–370 kbp) and adenoviral (32–48 kbp) genomes show viral and host cell DNA polymerase gene homologies (Table I). By thymidine kinase gene analyses alphaherpesviruses (HSV1–2, VZV) derive from a common ancestor. HSVs possess at least 12 genes that encode proteins with close homology to host cell proteins (uracil-DNA glycosylase, DNA-polymerase, DNA helicase, ribonucleotide reductase, thymidine kinase, and the RL1 neurovirulence gene). Human cytomegalovirus (hCM) represents the betaherpesviruses; it possesses some genes shared with alpha- and gammaherpesviruses in addition to more recently acquired genes without counterparts in other herpes viruses. These newer genes encode viral glycoproteins, G protein-coupled receptors and an HLA class I homologue; hCMV is a transactivator of many host cell genes [53].

Host range is readily transgressed by herpes viruses. Gamma herpesviruses are lymphotropic but the CD4 lymphotrope human herpes virus 6 (HHV-6) belongs to the group of betaherpesviruses; and the avian lymphotrope Marek's disease herpes virus belongs to the alphaherpesvirus group. HHV-6 can exist in a recombinant form incorporating adeno-associated type 2 viral *rep* gene [54]. AAV is a naturally defective parvovirus; its *rep* 78 gene acts as an antioncogene and is able to negate malignant transformation induced by oncogenic DNA viruses (adeno-, herpes-, papillomaviruses) [55].

Gammaherpesviruses [56] diverged distantly from alpha- and betaherpesviruses and are represented by Epstein–Barr virus (EBV) and by the Kaposi sarcoma-associated human herpes virus 8 (HHV-8) closely related to HV *saimiri* of New World (marmoset) monkeys. EBV can lyse host lymphocytes utilizing its own DNA polymerase for the

production of linear viral genome; or it may exist in latent state utilizing cellular enzymes to produce episomal circular viral DNA. The immunoevasive strategies of pox- and herpes viruses will be discussed elsewhere (vide infra).

Parvoviruses [57–60] contain linear ssDNA genomes and multiply in the nuclei of dividing cells of invertebrate and vertebrate hosts of a wide range. Viral transcription will not commence in resting cells. Early viral gene product proteins NS1–2 bring host cell DNA replication to a halt; mature virions can lyse host cells. Antioncogene p53 wild type when it suppresses the malignant phenotype, can confer resistance to parvoviruses. The gene product protein of p53 (or Rb) is often coupled by gene products of potentially oncogenic DNA viruses: large T antigens of SV40; E6 of HPV; EBNA-5 of EBV and E1B of adenovirus 5 [61, 62].

SV40 genomic sequences were found in Salk vaccine and in childhood brain tumors, some thyroid carcinomas and in mesothelioma (but without proof of relationship to the vaccine) [63–65]; HPV is implicated in the causation of uterine cervical squamous cell carcinoma and EBV is suspected to contribute to malignant transformation of lymphocytes in Burkitt's lymphoma; natural killer cell lymphoma; Reed-Sternberg cells of Hodgkin's disease and AIDS-associated lymphoma. In some of these and other (body cavity lymphomas) tumors EBV and HHV-8; and in Kaposi's sarcoma hCMV and HHV-8 co-exist (vide infra).

Transgression of species barriers is easier for the rapidly evolving RNA viruses (influenza A; HIV) than for the stable, species-specific small DNA viruses (polyoma-, papilloma- and parvoviruses). Benign persistent state of a virus in a cell community attests to genetic stability of the virus. Large quasi-species progenies are consequential to genetic instability. Tolerance (nonrecognition as non-self) to certain persistent viruses (hymenoptera to polydnavirus; mouse to lymphocytic choriomeningitis virus) exempts the partners from gene rearrangements. An immune reaction of the host (virus-specific T cells) would induce the development of "escaping viruses" due to rearrangements of the viral *env* gene (vide infra).

Table I

Examples of viral genes (other than protooncogenes) of host cell origin

Virus	Description
African swine fever	ORF P1192R encodes 46kbRNA → topoisomerase [106]
	Ancient gene formed before protozoa, yeasts and metazoa diverged
Adenovirus	Regulatory RNA [107]
Orf	Polypeptide homolog of IL-10 of ovine cell origin [97]
Vaccinia	Phospholipase D of host cell origin encoded by viral genes K4L and P1ßL: needed for spread. IFNαßR; IFNγR; soluble glycoproteins (see text)

Retrograde evolution

Exogenous pathogenic microbes can be arranged in a ladder descending from the top formed by large extracellular bacteria downward to obligate intracellular bacteria, rickettsia, bartonella-rochalimeae, mycoplasma, chlamydia and large viruses (pox- and herpesviridae). However there is a dividing line between chlamydia and poxviruses: the former microorganisms possess both RNA and DNA whereas viruses possess only either RNA or DNA but not both.

In the ontogenesis of bacteria pleuropneumonia-like developmental forms, protoplasts and spheroplasts and filterable elements appear [66]. "Pettenkoferien" of Preisz were not protozoa coexisting with bacteria; they were L forms observed first in the 1930s but unrecognized as such until Klieneberger-Nobel and Dienes clarified their origin and life cycle (cited in [66]). Some filterable elements regenerate into vegetative bacterial cells with cell wall, following fusion of the filterable elements [67–69]. This developmental cycle could possibly be interpreted on morphological (but not on molecular biological) bases to mean that modern or ancient viruses are filterable forms of bacteria. This concept envisions the gradual or sequential loss of excess genetic material leaving operational only those genes that are essential for self replication but only within the machinery of a cell. A large body of older literature contains a great deal of misinterpreted information when bacterial developmental forms of extant viruses were presumably identified.

There is no molecular biological proof to confirm or expand those morphological observations that led to the presumption that viral forms of bacteria existed and regenerated into vegetative bacterial cells (as envisioned by Hauduroy, Mellon, Nicolle and Soviet bacteriologists early in this century). The fascinating "ultravirus tuberculeux", Calmette; influenza virus → *B. pneumosintes*, Olitsky and Gates; poliomyelitis virus → globoid bodies, Flexner and Noguchi; poliomyelitis virus → streptococci, Rosenow; Vi-antigen carrier "typhus virus", Magrassi etc. appear now as ghost of a past era (cited in [66]). There is nothing in the phage-bacterium relationship that would indicate regeneration of a phage into a bacterium; endogenously derived phages had evolved to exogenous parasites of bacteria similarly to the origin and evolution of animal viruses. R Doerr who was born in Erdely (Transylvania), served in the Austria-Hungarian army and became professor of virology in Switzerland maintained in the 1930s that phages were endogenously originated viruses of bacteria [70].

EVOLUTION

Anti-apoptosis

The first genes lethal to the cell that harbors them could be traced back to bacterial plasmids. In the relationship of bacteria with their phages mechanisms had evolved that terminate phage nucleic acid transcription; or kill the prokaryote before the assembly of

the new phage progeny. This second mechanism is accomplished either by proteolytic enzymes activated within the infected cell or by proteins cleaving RNA or DNA such as an essential translational elongation factor. One example is the Rex protein encoded by a λ prophage that aborts infection by other unrelated phages while killing the cell [71]. Could it be that interferons evolved from the ancient mechanisms of viral transcription inhibition and programmed cell death (PCD) evolved from the genome of the λ prophage acting integrated in the prokaryote's genome and protecting the survival of the species by killing the first infected individuals through DNA fragmentation?

Descendants of unicellular organisms appearing 1–2 billion years ago clearly show the phenomenon of energy consuming PCD or apoptosis: it is operational in *Trypanosoma* sp., in *Tetrahymena thermophila* and in slime molds (*Dictyostelium* sp.) [72]. These very same species harbor their own viruses. Could it be that apoptosis established itself early in the course of evolution to protect the species from defective or infected individuals?

Insect cells infected with polyhedrosis viruses (baculoviruses) succumb to apoptosis except when the viral genes p35 or *iap* prevent it [73]. When arthropods act as vectors for arboviruses, these hosts apparently escape PCD while they allow full replication of the viruses in a persistent carrier type relationship for mutual survival. Sojourn in the insect vector does not diminish the pathogenicity of the virus for the central nervous system or elsewhere in its mammalian host. Do arbovirus-infected mammalian nerve cells die due to viral lysis or due to self inflicted PCD?

To the introduction of certain viral genes, host cells respond by activating the process of apoptosis [74, 75]. This suicidal act pre-empts the assembly of the new viral progeny. If completely successful, the emergence of a new viral pathogen in that host species is eliminated at the price of the death of the first infected individual cells. Adenovirus E1A gene is an inducer of host cell apoptosis [76, 77] mediated by the cell's proapoptotic p53 gene. The cell's *bcl*-2 gene counteracts E1A-induced apoptosis. Apoptotic death of infected cells or that in antiviral host T cells is induced by Sindbis, influenza, bovine herpes or chicken anemia parvovirus; LCM, mouse leukemia and HI viruses. In order to keep the host cell alive and functional until the maturation of a new viral progeny, viruses evolved genes that counteract apoptosis. The E1B adenoviral gene encoding a 19kDa and a 55kDa protein accomplishes inhibition of apoptosis by complexing with the proapoptotic cellular gene p53 [77]. Other viruses evolved gene homologs of the antiapoptotic cellular gene *bcl*-2: EBV's latent membrane antigen (LMA) or the early lytic cycle bcl-2 homolog bhrf 1 gene; or African swine fever virus gene emw5-he. Cowpox virus encodes a protein that inhibits the interleukin 1 converting enzyme (ICE) that is essential for DNA fragmentation in a cell undergoing apoptotic death (vide infra). The mammalian ICE gene has its homolog, the *ced*-3 gene, in the nematode *Caenorhabditis elegans* where the apoptotic process is an essential part of the worm's normal ontogenesis (as it is in all embryos including the human fetus). The homodimers bcl-2/bcl-2 protect against, while bax/bax execute apoptosis; bax/bcl-2 heterodimers are poised to act either way [78].

Sindbis virus induces apoptosis by activating caspases [79]. The IL-1ß-converting enzyme (ICE) is a cysteine protease (caspase) essential for the completion of apoptosis. Virally encoded serpins (serine protease inhibitors) inhibit ICE. Polyhedrosis (baculo-) virus genes p35 and *iap* inhibit apoptosis in HeLa cells overexpressing ICE- or Fas-

(CD95-) associated death domain (FADD) [73]. Serpins encoded by vaccinia (B13R; SPI-2) or cowpox viruses (CrmA=cytokine response modifier) inhibit apoptosis and protect Sindbis virus-infected cells from death converting lytic infection to viral persistence [80–83]. Serpins B13R (SPI-2) or CrmA protect cells from Fas- or TNFα- or TGFß-mediated apoptosis [84–87]. Apoptosis induced by granzyme B of cytotoxic lymphocytes (not readily antagonized by *bcl*-2) was inhibited by poxvirus serpins SPI-1,2,3 [88]. Myxoma-(leporipox-) virus infects also lymphocytes and encodes the T2 protein showing sequence similarity with TNFα receptor (R). Paradoxically this protein protected virally infected T lymphocytes from apoptotic death. Myxomavirus encodes immunosuppressive virokines and viroreceptors simulating the activities of host cytokines and their receptors due to shared homology. These factors include serpins, TNFαR and IFNγR [88–95]. The virally encoded IFNR inhibit host IFNγ activities. Vaccinia virus encodes receptors for IFNα, and a phospholipase homologous to the host cell enzyme [96]. Orf virus encodes a homolog of IL-10 [97] (Table II).

The avian Rev-T reticuloendotheliosis retrovirus causes malignant lymphomas of bursal lymphocytes; v-*rel* is the transforming oncogene. The cellular counterpart (on human chromosome 2p14–15) present in all eukaryotes is the gene product protein nuclear factor kappa B (NFκB). Upon activation it separates from its cytoplasmic bond IκB to enter the nucleus for DNA binding where it inhibits TNFα-induced apoptosis. Viruses known to activate NFκB to achieve antiapoptotic effects are HTLV-I; HIV-1; hepatitis B; adeno-, papova- and herpesviruses (HSV-1; hCMV; EBV) [98–100]. The cellular NFκB is overexpressed or amplified in human tumors (lymphomas-leukemias; Reed-Sternberg cells of Hodgkin's disease; breast, ovarian and colon carcinomas) [101–103].

Receptors, cytokines and growth factors

"We read in Stephen Hawking's book 'A Brief History of Time' (Bantam, 1988) that the glowing white heat emitted by the early and rapidly expanding universe is still detectable today in the form of microwaves..." [104]. If biologists look for the molecules that primordial life forms of the archaic Earth benefited from the most, it is among the protooncogenes-oncogenes and their gene product polypeptides of the complex cells living today where these ancient molecules conserved throughout evolution are to be found.

Protooncogenes-oncogenes had been conserved from the yeast (and beneath) up through insects to the mammalian and human cells and are presented by acutely transforming (oncogenic) retroviruses that recombined with them and incorporated them into their genomes. The very same protooncogenes expressed legitimately are switched on and off in an orderly fashion during metamorphosis of an insect (Drosophila) or in embryonic life (ontogenesis) of an avian or mammalian fetus; here protooncogenes direct cell communities to migrate, differentiate, form organs or become eliminated by PCD.

Table II

Viruses encoding homologs of cytokines or receptors

Product	Virus and gene	Description
	Vaccinia	
ICE inhibitor	B13R	B15R$^-$ virus: ↑ morbidity
IL-1ßR	B15R	
IFNγR	B8R	IFNγR$^-$ virus (Ankara):
TNFαßR	A53R; B28R; C22L	highly attenuated and immunogenic
	Cowpox	
ICE inhibitor	B28R → 48kD crmB	
TNFαßR	A53R → crmC (see text)	
	Variola	
TNFαßR	G2R; G4R (see text)	
	Myxoma	
IFNγR	M-T7	M-T7$^-$ virus: attenuated
TNFαßR	T2	
	Fibroma	T2$^-$ virus: attenuated
TNFαßR	T2 → 58kD	
	H Cytomegalo	Binds monocyte chemotactic proteins; MΦ inflammatory
GCR C-C	U 27,28,33,78	protein; RANTES; not IL-8
GCR C-X-C	Herpes simplex	Binds IL-8
GCR	Herpes saimiri	Binds IL-8. Cytotoxic T lymphocyte associated antigen
CTLA-8		
GCR	H Herpes 6	Chemokine receptor (Table VII)
	V12,51	
IL-10	Epstein–Barr	Pre-empts IL-10R by saturation
IL-6R	Hepatitis B envelope	Virus binds to hepatocytes expressing IL-6 [109]

See references in text [108–111]

Many of the c-protooncogenes as they appear in acutely transforming retroviruses are fused with viral (v-) genes; their gene product proteins differ from "self" (or undergo point mutations replacing just one amino acid with another) and elicit immune reactions thus raising the probability of active specific with vaccines, or passive with monoclonal antibodies, or adoptive with immune T cells, immunotherapy targeting fused or mutated oncoproteins of tumor cells. For example: in the treatment of Ph$^+$ chronic myelogenous leukemia immunization of the allogeneic bone marrow donor with the fusion oncoprotein

of the *abl-bcr* oncogene will result in delivery to the recipient not only of hematopoietic stem cells but also of anti-leukemia cell immune T cells [105]. Of numerous anti-oncogenes or tumor suppressor genes, the pro-apoptotic p53 and the cell cycle regulator Rb genes stand out. Oncogenic DNA viruses seek out these genes with their own gene product proteins made to fuse with that of the anti-oncogene [61, 62]. Immortalized and malignantly transformed cells result.

It is not only protooncogenes that ancient viruses transduced from their host cells [108–111]. Table II shows cellular cytokines and their receptors and chemokine G protein receptors (GCR) for which viruses acquired genes (transduced host cell genes) with >30% sequence homology to the cellular substance and preserved biological function. When encoded by viral genes the substances favor the virus against the host (block apoptosis by inhibiting ICE; bind IFNγ and TNFαß; preempt by saturation IL-10R).

During viral infections and vaccinations with attenuated viruses host cell- and viral genome-encoded cytokines, lymphokines and chemokines arise. For example rhinoviruses activate the cellular bradykinine gene; these viruses enter host cell through attachment to ICAM-1 serving as viral receptor. This cell adhesion molecule also works as ligand for lymphocyte function associated molecule-1 (LFA-1). Monoclonal antibodies binding to ICAM 1 prevent rhinovirus infection. Bradykinins mediate most of the symptomatology of the common cold. Table III shows some selected cases of double function cell receptors: a biological function and expropriation by a virus for attachment and entry [112–123].

The virally infected mammalian host first presents to T cells processed viral antigens in the grove of MHC complexes; then produces IFNαβγ, IL-2, -4 and 10. IFNαβ regulate NK cells; IL-2 CTL (CD8) cells. Both Th1 (IFNγ, IL-2) and Th2 (IL-4, -5, -10) type cytokines-lymphokines appear. Later Th2 responses dominate varying widely in different virus-host systems. Finally TGFß appears as a negative immunoregulator and inhibitor of NK cells and CD8 T cells expanding under the effect of IL-2. Early low levels of IL-12 benefit the virally infected host; high IL-12 levels late during viral infection can be detrimental to the host.

Table IV shows selected cytokines-lymphokines that promote or suppress growth of neoplastic cells. Notorious among them is IL-6 serving as growth factor for Kaposi's sarcoma (KS) and multiple myeloma cells; kidney carcinoma and melanoma cells. In the case of KS, HHV-8 encodes additional dosage of IL-6 since it possesses a homolog to cellular IL-6 gene (vide infra); in myeloma, if confirmed, the same virus residing in dendritic cells of the marrow contributes to elevated IL-6 levels. Virulent influenza viruses induce IL-8 in the bronchial tree; this lymphokine is known to be a major co-inducer of the potentially fatal adult respiratory distress syndrome. Attenuated influenza viruses recently recommended for intranasal active immunization apparently lost the faculty to induce this lymphokine [124]. However, attenuated live viral vaccines remain variable and subject to gene rearrangements and recombinations; they may induce lymphokines-cytokines in the vaccinated host favorable for antiviral immunity but unpredictable as to tumor enhancement or inhibition.

Negative cytokine regulation by viruses entails encoding by viral genes or inducing expression by host cell genes those cytokines-lymphokines that immunosuppress the host (Table IV) [150].

Table III

Selected examples of viruses interacting with cellular receptors to gain entry into cytosol

Virus	Receptor	Description
Rhino	ICAM-1	Natural ligand: LFA 1 [112]
Parvo 19	Erythrocyte P antigen	P blood group (Landsteiner) [113]
Parvo (aleutian mink)	FcγRII	Immunoglobulin binding [114]
HI	CD4	Helper cell differentiation complex (see text)
Measles	CD64	Complement regulatory protein (see text}
EB	CR2 (CD21)/HLAII	Complement binding (see text); co-factor
Friend spleen focus forming (truncated *env*)	Erythropoietin receptor	Erythroblast colonies in spleen → erythroleukemia (see text)
Foot and mouth disease	Integrinαvß3	Attachment → internalization [115]
Echo	Integrinα2ß1	Mediates interaction: cell ↔ collagen [116]
Herpes simplex	TNF superfamily 29 kDa type II transmembrane protein	Envelope gpD → cellular herpes virus entry mediator [117]
Herpes simplex 1,2	Heparan sulfate	Type-specific difference for specific sulfate groups [118]
Dengue	Heparan sulfate	Suramin inhibits virion attachment [119, 120]
Respiratory syncytial	TNF subdomain_55kDa	Viral protein G attaches to receptor [121]
Coxsackie (cardiovirulent)	Decay-accelerating factor (CD55)	Cardiovirulent strain CVB3(cs) enters myocardial cells [122]
Prion protein PrP^{c}	Laminin precursor 37-kDa	↑ Laminin precursor protein expression correlates with PrP^{SC} accumulation in disease [123]

For entry sites, textbooks of virology list acetylcholine receptor for rabies virus, ß-microglobulin for CMV, EGFR for vaccinia virus, laminin receptor for Sindbis virus and sialic acid residues for influenza virus: thus macromolecules essential for the functions of the cell are expropriated by viruses to gain entrance into the cytosol.

Table IV

Cytokines-lymphokines promoting or inhibiting growth of malignant cells in the human host

Cytokine-lymphokine	Growth promotion	Growth inhibition (→**A**)
IFNα		Hairy cell leukemia. KS. T cell cutaneous lymphoma. Melanoma. Kidney carcinoma [125]
IFNγ	Protects B-CLL cells against **A**. Autocrine GF [126]	
IL-2	HTLV-induced lymphoma-leukemia. ↑regulates IL-2R; shedding of sIL-2R [127]	Melanoma. Kidney carcinoma
IL-4	↑T cells in T cell rich B cell lymphoma. ↑T cell lymphoma [128] Synergizes c IFNγ for B-CLL	**A** induction in B-ALL [129]
IL-5	Hypereosinophilic syndrome in T cell lymphoma [130]	
IL-6	Diffuse large cell lymphoma; multiple myeloma; KS; kidney carcinoma; melanoma (cited in [131, 132])	
IL-7	Sezary cell leukemia (secretes it, expresses its R) T-CLL; RS of HD [133–136]	
IL-8	Chemokine: chemoattractant to T lymphoma cells (see text). Expressed by EBV-infected cells. Inhibits IFNα [137, 138]	
IL-10	B cell-derived T cell GF. ↑Th1 ↓Th2 (↓IFNy, ↓IL-2). Induced by EBV BCRF-1 gene. NFκB recognizes sequences on IL-10 gene (↓↑ regulation). Induces neutrophil density around RS cells. ↑Pyothorax-associated lymphoma [139–143]	**A**-induction in B-CLL cells by ↓*bcl*-2 [129]
IL-12	Produced by EBV-transformed B cells. NK cell stimulatory factor. Induces Th2 (↑IFNγ) [144]	
IL-14	B cell GF [145]	
IL-18	IFNγ-inducing factor; induces IL-8, IL-1ß [146]	
IP 10	IFNγ-inducible protein: C-X-C chemokine. Keratinocytes produce it: epidermotropism of T cell cutaneous lymphoma [147, 148]	
VEGF	Angioimmunoblastic lymphoma [149]	

Immunoevasive strategies

All lymphocytotropic viruses are immunosuppressive be of either RNA (bursal virus; retroviruses) or DNA (herpesviruses; myxoma virus) genomic class. The dsRNA bursal disease virus of Birnaviridae first isolated in the 1960s in Gumboro, Delaware USA and then world-wide has rapidly gained extraordinary virulence by genetic reassortments.

The virulent virus destroys B lineage lymphocytes developing in the bursa of Fabricius, cecal tonsils and spleen. There is antibody- and T cell-mediated immune response that often fails to achieve cure of the infected bird. Virally infected lymphocytes succumb to apoptotic death (resembling CD4 T cell death in human AIDS). A heptapeptide expressed in virulent virus strains is absent in attenuated virus strains (Univax BD; Bursa Vac 3). The highly variable VP2 gene of viral genomic segment A induces major antiviral immune reactions. Recently from Europe to Japan mutant highly virulent bursal virus strains emerged that infected preimmunized birds. The situation requires the development of newer vaccines. This highly variable virus apparently produces swarms of quasi-species and its host-virus relationship resembles that of human influenza A viruses or HIV [151–156].

Neonatal infection of mice with LCM virus results in the elimination CD8 T lymphocytes developing in the thymus to react with virally infected cells. The host-virus relationship is characterized by tolerance of the host to a persistent virus; there is no symptomatic disease. In the adult naive mouse, inoculation of massive doses of rapidly replicating LCM virus strains could induce a state of anergy due to exhaustion and depletion of T cell-mediated immunity or "high dose immune paralysis" [157, 158].

When LCM virus replicates in the CNS of naive mice, immune T cells kill virally infected cells and thus inflict lethal injury to the host. Recovery from the disease is mediated by virus neutralizing antibodies and immune T cells cooperating [159]. LCM virus may undergo mutations changing just one amino acid in its epitope; immune T cells thus blinded do not recognize host cells infected by the mutant virus. CTL escape variant noncytopathogenic viruses readily establish the state of viral persistence in their hosts [158].

Measles virus (MV) enters host cells through the complement regulatory protein CD64. Patients infected with MV develop anergy; even UV-inactivated MV can inhibit mitogen-induced proliferative lymphocyte responses. These lymphocytes gain resistance to Fas- (CD95-) mediated apoptosis. MV-carrier presenter lymphocytes elicit reduced IL-2 response of reactive peripheral blood lymphocytes. MV glycoproteins F and H induce the immunosuppression. MV infects dendritic cells and monocytes. When T cells react, instead of immune T cells, cell syncytia are formed; while IL-2 production is reduced, MV replication in the syncytia continues until these cells succumb to PCD. (HIV has a similar relationship with dendritic cells: vide infra). MV nucleoproteins bind to B cells and inhibit antibody production. MV-infected hosts do not express functional IL-2R α subunit (but ß subunits are formed) and IL-4 production is reduced; IL-6, IL-10 and IFNγ levels do not change; IL-12 levels were found elevated and reduced. Despite the availability of attenuated live measles vaccine this virus is far from being eradicated from the human community [160–168].

The AIDS virus was visualized in (but not cultured from) the tissues of patients in 1980-1 at Houston's VA Hospital; these transmission EM pictures were shown 1983 in Vienna, Austria (cited in [169]). HIV envelope glycoprotein gp 120 fuses with CD40 of T lymphocytes. Chemokine receptors CCRS and CXCR4 interact and viral RNA enters the cytosol. RT produces preintegration complex DNA copies of the viral RNA. Nondividing host cells do not allow productive infection with retroviruses. Retroviral preintegration complexes do not pass through the intact nuclear membrane but enter the nucleus during

mitosis. Lentiviruses (HIV) can infect resting cells especially macrophages and their derivatives, the microglial cells of the brain or dendritic cells of lymph nodes (or Langerhans cells of the subcutis). Infected macrophages harbor integrated HIV DNA. The *src* protooncogene $p56^{lck}$ exerts its tyrosine kinase activity within the cytoplasmic domain of gp120-bound CD40 [170, 171].

The role of chemokine receptors in the process of viral (HIV) entry is most significant because natural ligands of these receptors can inhibit infection of the cell. These ligands α (CXC) and ß (CC) chemokines signal through G protein-associated receptors. The physiological role of chemokines is activation and chemotaxis of NK, B and T lineage lymphocytes and macrophages (the MDC chemo-attractant which acts on dendritic cells as well). IL-8 serves as the chemokine of granulocytes and it inhibits antiviral effects of IFNα [172–174]. MIP-1αβ and RANTES attach to CCR5 and they or their analogs (aminooxypentane RANTES) inhibit HIV entry; stroma-derived factor 1 attaches to CXCR4. The chemokine receptor US28 is encoded by hCMV and serves as coreceptor for HIV entry. HIV strains may (using CXCR4 or "promiscuously" both CXCR4 and CCR5) or may not (using CCR5 only) induce syncytium formation of infected T cells (with or without dendritic cells within the syncytia) [175–177]. Macrophage- (M-) tropic HIV isolates show less variability of their V3 (third variable region of gp120) sequences than that of CD4- (T-) tropic HIV isolates; M-tropic isolates do not, T-tropic isolates do induce syncytia. Stromal cell derived factor (SDF-1) blocks cell-binding of T-tropic HIV isolates to CXCR4 (fusin). RANTES, MIPαβ (products of CD8 T cells) block M-tropic HIV isolates to CCR5. Early HIV isolates deriving from the asymptomatic stage of the disease preferentially use CCR5, whereas HIV isolates from advanced disease use CXCR4. It is an adverse prognostic sign if an early HIV isolate is already resistant to RANTES, MIPαβ and uses CXCR4. Through *in vivo* V3 mutations the virus escapes control by C-C chemokines [178–181].

Before the discovery of HIV and its replication in dendritic cells, a somewhat analogous theory was published on the origin of Reed-Sternberg cells of Hodgkin's disease [182]: the primary pathogen is a putative retro(lenti)virus infecting dendritic cells which fuse with naive or reactive T or B lineage lymphocytes at which point co-pathogen EBV enters; thus RS cell is a natural hybridoma.

The yield of viral progeny and T cell and macrophage tropisms vary with different HIV isolates. Viral polyproteins are encoded by *gag*, *pol* (RT), *env* and at least 6 more viral genes: *tat* product protein induces endothel cell proliferation; *vpr* is necessary for the infection of macrophages and it arrests cells in G2 phase thus preventing apoptosis before the assembly of the viral progeny. This gene is highly conserved in lentiviruses. The gene *vpx* is present only in HIV-2 and in SIV_{SM} and SIV_{MAC} (vide infra). A single ancestral gene evolved into *vpr* and *vpx* genes [183].

Further activities of HIV's *tat* gene product 86 amino acids protein are transcriptional activation of HIV's long terminal repeat; inhibition of the proliferation of antigen-specific T cells and potentiation of the antiapoptotic NFκB. This in turn allows TNFα overactivity without cell kill but with stimulatory effect on HIV replication [171]. HIV protease is encoded by the *pol* gene. The enzyme cleaves the large *gag-pol* polyprotein to liberate packaging proteins for the maturing virions. Protease inhibitors prevent cleavage essential for maturation and infectivity of HIV virions [184].

Attenuated SIV strains eliminated 4 amino acids from their *nef* gene product protein. The attenuated virus (pC8) protects monkeys against the virulent virus (pJ5) on account of virus-specific CTL induction; the virulent virus fails to induce this reaction while host lymphocytes undergo apoptotic deaths. Cells infected with the virulent pJ5 virus release excessively membrane-bound or soluble Fas ligands; this ligand is responsible for the apoptotic death of Fas receptor positive reactive CTL. Cells infected with the attenuated virus pC8 do not release Fas ligand [185, 186]. This phenomenon resembles one escape mechanism of tumor cells that kill $FasR^+$ defensive host lymphocytes (cited in [187]) and the immune privilege the $FasL^+$ trophoblast provides to the fetus by eliminating $FasR^+$ maternal lymphocytes that would attack fetal cells expressing paternal antigens (cited in [188]).

Other mechanisms that may be operational when HIV escapes CTL are rapid production of genetically different quasi-species viral progenies with diverse *env* genes; production of $>10^9$ virions per day leading to "immune exhaustion"; downregulation of MHC class I expression of processed viral peptides (blamed also on *tat* and *nef* gene product proteins). CTL-mediated selection pressure occurs during the long asymptomatic phase of AIDS during which time the virus gains CTL escape status. African HIV subtype O shows extraordinary heterogeneity of its progeny evolving more rapidly than older HIV isolates. HIV-infected CD4 lymphocytes induce first vigorous CTL response controlling infection and reducing virus titers for several months. During this time a new HIV variant species arises with amino acid sequence mutations exchanging the immunodominant epitope in gp160 to another residue unrecognizable to host CTL [189–192]. The escape of HIV and SIV from virus neutralization by envelope-specific antibodies is due to the N-linked glycosylation of the gp120 envelope epitopes; virus particles shielded by carbohydrates on their outer surface render virus neutralizing antibodies ineffective [193].

The murine spleen focus forming virus (SFFV) is replication defective due to its incompletely formed envelope glycoproteins (gp55). These envelope glycoproteins however bind to erythropoietin receptors (EPO-R), stimulate them and thus induce erythroleukemias [194, 195]. SFFV were first recognized within the populations of Friend and Rauscher mouse leukemia viruses (MuLV). MuLV recombine with endogenous *env* retroviral sequences harbored in the murine genome. The truncated envelope of SFFV is the result of such recombination. In a study, murine SFFV might have accepted envelope proteins encoded by a putative human retrovirus presumably present in Hodgkin's disease [182]. If such recombinations occur in nature outside the confinement of the laboratory, virulent leukemogenic recombinant retroviruses may arise.

When cytoplasmic tubuloreticular structures (TRS) were first observed in kidney glomerular cells of patients with systemic lupus erythematosus (SLE), they were considered to be incomplete viruses (unenveloped viral nucleoprotein strands) inducing autoimmunity (cited in [169]. Up to this date this notion remains unproven; instead endogenous retroviral genomic sequences were found in the human genome showing propensity toward defective maturation into incomplete viral structures and toward recombination between themselves or with exogenous retroviruses. Some of the gene product proteins (HERV-K10; HRES-1 etc.) of these sequences are closely related to antigens expressed by well characterized retrolentiviruses (HTLV-I; HIV). Patients do not show tolerance toward some of these proteins: antibody (and cell-mediated immune)

reactions are readily formed. Such reactions occur in patients with SLE, multiple sclerosis and other autoimmune diseases [196–200]. An extensive review concludes: hybrid retroviruses result from recombinations of defective endogenous retroviral sequences with each other or with exogenous retroviruses [200]. These sequences remain tolerated throughout evolution because they may protect cells by viral interference from infection with virulent viruses or because the cells may take use of some of the polypeptides encoded by the endogenous sequences. However, the potential for the development of new retroviral species exists, especially when transspecies recombinations should occur. Genomic sequences of endogenous retroviruses (ERV) are vertically transmitted and their expression is prominent in the primate and human haemochorial placenta where the *env* gene and viral long terminal repeat (LTR) appear to be conserved for at least 30 (if not 100) million years. Expression of the *env* gene product glycoprotein induces trophoblast fusion to form the syncytiotrophoblast. Placental ERV may mature into virions. ERV possess LTR which can activate host genes next to the insertion site of the ERV sequence (a protooncogene?) (cited in [51]). Host cells evolved mechanisms to restrict the maturation and horizontal spread of their ERV (loci Fv1,2; Rmcf in the mouse). The paradoxical situation may arise that the replication of a murine ERV is inhibited in mouse cells; however another distant species may be unprepared to restrict this retroviral element [202]. When in the early 1970s one of the authors (JGS) and Gyorkey xenografted nonvirus-producer human tumor cell lines established in tissue culture into nude mice, they observed the sudden appearance of type C virus particles in some of these tumors (but could not readily explain the phenomenon) [201). We know now that xenotropic murine retroviruses, and some cat, pig and baboon retroviruses can replicate in human cells. This phenomenon confounds experimentation with human xenografts: these transplanted cells may become infected with endogenous retroviruses of their new hosts. The proposed use of baboon or pig xenografts to treat AIDS entails the reverse risk: human recipients of these xenografts may become infected with endogenous retroviruses of the donor animal. An alarming possibility is a new disease spreading horizontally from the xenotransplant recipient in the human community (cited in [51]).

Adenovirus evolved the protein E3/19k that binds to precursor molecules of MHC in the endoplasmic reticulum; as MHC never ascends to the cell surface, viral peptides in the grove are not presented to T cell receptors [203]. RID, the receptor internalization and degradation protein of this virus forces the internalization of the Fas receptor; cells devoid of this receptor escape apoptotic death induced by the Fas ligand or by TNFα [204]. E1B$^-$ (defective) adenoviruses replicate in cells with deficient or nonfunctional p53 gene [205]; these are most cancer cells. Adenovirus variants genetically engineered to be E1B$^-$ may be used for the virus therapy of cancer [206].

Epstein–Barr virus (EBV) exerts extraordinary genetic activity. One of the most remarkable genetic expressions of this virus is that of the latent membrane protein (LMP-1). LMP-1 imitates the TNF receptor. In this case however signal transduction activates the anti-apoptotic NFκB and the epidermal growth factor receptor [207, 208]. As EBV envelope gp 350/220 attaches to complement receptor (CD21=CR2), signal transduction toward NFκB activation commences. Thus virally infected cells convert a physiologically cell death-oriented pathway to survival and replication. Viral genes encode nuclear antigens EBNA 1–5 that confer viral latency, cell immortality and anti-apoptotic effects.

The viral gene BARF-1 encodes a colony stimulating growth factor [209, 210]. The gene product protein of viral gene BHRF-1 is a homolog of the cellular antiapoptotic *bcl*-2 gene. Viral gene BCRF-1 encodes a homolog of IL-10 which serves as a growth factor for transformed lymphoid cells [211–213]. As EBV-infected B cells in infectious mononucleosis are readily cleared by immune CTL; EBV-reactive immune CTL are now used for the adoptive immunotherapy of EBV-associated malignancies (Burkitt's, NK cell, posttransplant, body cavity, Ki-1^+ anaplastic lymphomas, Hodgkin's disease, lympho-epithelioma-nasopharyngeal carcinoma, etc.) [214–217]. It is to be seen what escape mechanisms EBV may evolve in malignantly transformed cells in order to fend off CTL.

Growth factors (GF) of KS cells are numerous; prominent among them is the gene product protein of HIV *tat* [218]. Autocrine growth factors (with their receptors expressed) are IL-6, VEGF, bFGF and oncostatin M (OSM). Platelet-derived GF, IL-1ß, IFNγ and TNFα are contributory to growth of KS cells. Inhibitory is TGFß as it antagonizes CTL expanding under the effect of IL-2 or as it contributes to apoptotic death of KS cells (cited in [169, 219]). Glucocorticoids induce growth of KS cells by downregulating TGFß expression [220, 221].

KS is a "breeding ground" of herpesviruses: hCMV, EBV, HHV-6 and HHV-8 may coexist in these lesions. Occasionally budding virions of an as yet unidentified (activated endogenous?) retrolentiviruses are visible (cited in [169, 219]). HHV-8, the causative γ2 rhadinovirus of KS and contributor to the etiology of certain malignant lymphomas [222–225] pirated a large number of host cell genes unique for this virus [226–234]. Table V lists some important preliminary unfolding genetic activities of HHV-8. The genome of this virus consists of some 270 kb and contains at least 80 genes. In addition to inducing the customary herpesviral enzymes (thymidine kinase, dihydrofolate reductase, thymidine synthase etc.), it exerts enormous biological activity in KS lesions. Its place in the evolution of Herpesviridae is uncertain. Its relative rhadinovirus EBV very likely coevolved from the Australopithecines up through *Homo erectus* and *Homo habilis* to *Homo sapiens* in Africa following mankind's exodus from Africa. Its other closest relative *HV saimiri* however evolved in the New World where it causes polyclonal T cell lymphomas in marmoset monkeys. The African and S. American continents were one and united in the Pangaea 300 million years ago and separated from one another 200 million years ago. Ancestral viruses of EBV, SIV and HIV-1, -2 and HTLV-I and II remained in the African continent. Could it be that the ancestral virus of HHV-8 evolved in South-America and entered the New World and Africa after the 1500s? KS appeared as a new virulent disease and in Kaposi's time it was still frequently fatal as it was in the pre-AIDS era in Africa (and still is). The native population of South America (separately from the immigrated Portuguese, Spaniards and Italians) should be studied by seroepidemiology for their relationship to HHV-8.

Table V

Major genetic activities of HHV-8

Sequence	Viral genomic gene product	Description
K2	vIL-6	Autocrine growth factor for KS cells [see text; Table IV]
ORF4	VCBP	Complement receptor; C inactivator
K4-6	MIP 1αβ	Monocyte inflammatory proteins activators of chemokines, cytokines [226]
K9	VIFR	Viral IFN regulator; inhibitory protein of IFNß [239–241]
ORF16	v*bcl*-2	Homolog of c-*bcl*-2; inhibits BAX-mediated apoptosis without complexing [229]
ORF65	Small viral capsid	Target for seroepidemiology [237, 238]
ORF 71 K13 74 K1	vFLIP	Viral FLICE-inhibitory protein antagonist of Fas- (CD95-) induced apoptosis [239–241]
ORF 73	LANA	Latent nuclear antigen: target for seroepidemiology [238]
ORF 72, 74	vcyc	30kDa cyclin D for cell cycle control; indirect anti-Rb gene effect: phosphorylation and inactivation: cell cycle → S [235]
	IL-8R	G protein chemokine receptor [230, 234]
	GPCR	G protein coupled receptor; angiogenesis inducer: autocrine loop with VEGF. Acts as transforming oncogene; signals without ligand [233, 236]
T0.7 mRNA	kaposin	Latency associated nuclear antigen [241]
	sIL-6/IL-6R complex	With OSM: growth factor
	M associated mannose R	Cell surface lectin [242]

FI.ICE=FADD-(Fas-associated death domain-) like interleukin-1 converting enzyme

Transgression of species barriers

In 1986 the Literaturnaya Gazeta (May 7) and Sovietskaya Rossiya (Apr 27 and June 8) published articles accusing the Central Intelligence Agency USA with the laboratory manufacturing of the AIDS virus (HTLV and visna-viral recombination) for biological warfare. US ambassador Hartman protested to no avail. Soon thereafter the Wistar Institute's live attenuated poliovirus vaccine of the 1960s was blamed for the AIDS epidemic erupting in Central Africa where the vaccine was first used, since the poliovirus was gown in African green monkey kidney cell cultures which contained a

then unidentified simian retrovirus. These implications were extended to AIDS cases in Israel and in the USA where live attenuated poliovirus vaccine was used by private practicioners to treat genital herpes (in homosexual men) presumably to induce viral interference [243]. Re-examination of the original Lederle poliovaccine failed to detect HIV or SIV_{AGM} or SIV_{MAC} in it [244]. Wistar director Koprowski succeeded in proving the falsehood of these contentions [245, 246]. One of the authors (JGS) went to Belem, Brazil in November 1986 as invited speaker of the Amazonas Medical Society. At the questions and answers session the only questions he was requested to answer were if the AIDS virus was artificially constructed in Fort Detrick, USA as a genetic recombinant of HTLV-I and visna virus. It took an accurate description of the AIDS viral genome as it was known then to contradict the audience and explain that the AIDS virus evolved naturally, caused sporadic diseases first (probably in the 1950s) by transgression of species barriers from simian to human hosts and exploded into a pandemic disease on the wings of promiscuous hetero- (in Africa) and homosexual (in the Western world) practices. A Brazilian newspaper wrote: "the professor angrily refuted the allegations" concerning the origin of the AIDS virus as it was presented to him.

Archaic retrolentiviruses originated from cellular retrotransposons that acquired cellular membrane glycoprotein genes and operate now through the activities of error-prone polymerases; present themselves with extreme sequence heterogeneity and continue to evolve (faster than their host species!) through mutations and recombinations involving interspecies transfers [247–249]. Macaque SIV increase their pathogenicity through passages due to several point mutations [250]. In captivity SIV_{SM}- and SIV_{MAC}-associated lymphomas occurred [251]; SIV_{AGM} harbored latently in its natural host, when transferred to captive Asiatic macaques caused fatal simian AIDS [252]. Baboon endogenous retrolentiviruses are expressed and pass species barriers [253]. SIV_{AGM} strains are chimeric recombinants of the 4 subspecies of African green monkey SIVs. HIV-2 and SIV_{MAC} can be traced back to sooty mangabeys (SIV_{SM}). All extant HIV subtypes A-G originate from one simian → human encounter that probably took place in the 1950s. HIV-1 subtype O originated in Central Africa; it shows extraordinary heterogeneity of its *env* sequences; it is thought to be a recent simian to human transfer. HIV-2 derives from several recent human encounters with SIV_{SM} in West Africa [254–256]. HIV-1 and HIV-2 stand closer to SIV species than to one another. HIV-1 is the closest relative of SIV_{CPZ} (chimpanzee). HIV-2 is closest to the sooty mangabey SIV_{SM}. SIV of green monkeys is quite separate from HIV-1 or 2 [257]. Table VI shows the fatal consequences of interspecies transfer of SIV.

HTLV-I also emerged from primate reservoirs producing 3 separate human clades. Cosmopolitan clade 1 HTLV-I can be traced back to South Africa from where it entered the Americas including the Caribbean basin through slave trades. Clade 2 exists now in Central Africa (Zaire) and just recently moved to Italy. Clade 3 HTLV-I viruses spread in Asia, Australia and New Guinea. Current HTLV-I isolates can be traced back to STLV-I strains of African and Asian origin. Transspecies move of STLV from chimpanzee to man occurred recently in Zaire. Different interspecies transfers took place in Asia between monkeys and early humans. $STLV_{PAN-P}$ (virus of Central African pigmy chimpanzees) is the closest relative of HTLV-II [258, 259].

Table VI

Emerging new viral diseases through transgression of species barriers

Virus	Reservoir	Recipient species	Description
Irido	Exotic fish	Frogs	Virus-carrier exotic fish released in the wild in Australia: 14 species of frogs wiped out [260]
Corona	Mouse (hepatitis)	Hamster	Recombinant virus replicates in hamster cells [261]
Morbilli	Canine	Baikal seal (Phoca)	Fatal illness [262]
	Canine	Human	Paget's osteitis deformans [263]
	Rinderpest	Porpoise (Phocoena)	Fatal illness [262]
	Rinderpest	Dolphin (Stenella)	Fatal illness [262]
	Equine	Human	Fatal encephalitis (Australia) [264]
Filo (Ebola)	Simian	Human	Fatal illness [265, 266]. Rapid transfer from index case to contacts
Hanta (sin nombre)	Rodent	Human	Fatal to near fatal illness SW USA [267]
Arbo (Japanese encephalitis)	Feral pig ↓ Mosquito →	Human	Near fatal to fatal encephalitis (New Guinea) [268]
Influenza A	Birds and pigs →	Human	H1N1 1918–1957; H2N2 1957–1968; H1N1 and H3N2 co-circulate 1990s; H5N1 Hong Kong 1997 [269, 270]
$STLV_{PAN-P}$	Pigmy chimpanzee	Human	HTLV-II → Africa; Portugal
$STLV_{PAN}$	Chimpanzee (Pan troglodytes)	Human	HTLV-I clade 1 (cosmopolitan) → world wide slave trade $HTLV_{ZAIRE}$ clade 2 → Central Africa $HTLV_{MEL}$ clade 3 → Melanesia (Papua New Guinea) (see text)
SIV_{SM}	Sooty mangabey (Cercocebus)	Macaque (SIV_{MAC})	Fatal illness → HIV-2
SIV_{AGM}	African green monkey (Cercopithecus)	Pigtailed macaque (SIV_{MAC})	Fatal illness (see text)

(Continued on p. 374.)

Table VI (continued)

SIV_{CZP}	Chimpanzee (Pan troglodytes)	Human	HIV-1 → AIDS M (western world) (evolving world-wide; recombinant isolates!) O (Africa: evolving in Africa) (1950–1980) (see text)
Bovine spongiform encephalopathy	Cattle; sheep	Human	nv Creutzfeld-Jakob disease (England) [5] (no transfer from index case to contacts)

Table VI lists a few examples of virus diseases emerging newly and sporadically within isolated human populations (Ebola; hanta; Chinese avian influenza, etc.) with great potential to epidemics; all result from transspecies transfer. Some new paramyxoviruses and related pathogens are emerging (phocine distemper; equine paramyxovirus, etc.) through interspecies routes of transfer; during interspecies transfer of murine coronaviruses gene rearrangements and recombinations occur [260–269] (Table VI).

Some incompletely inactivated viruses used combined in vaccines may undergo "multiplicity reactivation" (recombination) and transgress species barriers with devastating consequences. Best example is the Russian flu vaccine used in Mongolia. The vaccine consisted of PR34 and Khab77 influenza viruses. Soon after the human administration of the vaccine fatal illness erupted among camels. The influenza virus isolated from camels consisted of the recombined genomes of the 2 flu viruses that were put into the vaccine [270–272].

Table VII shows examples of interspecies viral interactions: insertion of retroviral genetic sequences into hCMV or Marek's herpesvirus; transactivation of viral or host genes (LTR of HIV) or encoding a viral co-receptor as these features especially entail to herpesviral-retrolentiviral relationship in human pathology. It shows that highly pathogenic viruses can coexist and cooperate without viral interference or host defenses being able to eliminate them [270, 273–285].

DISCUSSION

In the last century of some 3 billion years long co-existence of living creatures on Earth, mankind now is forcefully intervening with the processes of natural selection and co-evolution of hosts and their viruses. Plants, insects and animals had been domesticated long ago thus altering their natural distribution and habitat; and we invent to apply killed and live attenuated antiviral vaccines. These interventions succeeded in eradicating or controlling viral diseases of plants and animals or the insect vectors of these pathogens, especially those (smallpox; poliomyelitis) that had decimated mankind in the past. As the *Homo* sp. has taken the Earth and its nonhuman inhabitants into its possession, efforts that eradicate diseases continue.

Table VII

Cooperative interactions of herpes- and retrolentiviruses

Coexistence in pathological entities	HTLV-II and EBV in leukemic reticuloendotheliosis (cited in [273]) HIV-1 and EBV, HHV-6, HHV-8 in AIDS [274] Unidentified retro(lenti)virus and hCMV, HHV-8, EBV in KS (cited in [169]) Unidentified retro(lenti)virus and EBV, HHV-6 in RS of HD (cited in [182]) Avian retroviruses and Marek's disease virus Feline IV and feline herpesviruses type I [275] Bovine retrovirus and bovine herpesvirus type I (antagonism: both CD4-tropic; retrovirus proliferative, herpesvirus cytopathic) [276] Human B lymphoblastoid cell line coinfected with EBV and JHK-3 retrovirus [277] Human haemophagocytic syndrome by EBV or HHV-6 [278]
Retroviral genomic insertions	Marek's virus ← avian reticuloendotheliosis virus [279, 280] hCMV1 ← avian retroviral oncogene (v-*myc*) [281]
Transactivations and encoding	hCMV → GCR (chemokine R) for HIV-1 (see text) EBV (primarily B-tropic) → LTR of HIV-1 → encodes CD4 for HIV-1 → infects NK cells [282] (see text) HHV-6 (primarily T-tropic) → infects NK cells and CD8 cells: cytopathic → suppresses IL-2 → induces TNFα and IL-1 → induces CD4 for HIV-1 [274, 283, 284] Cercopithecine herpesvirus 1 (herpesvirus B Sabin): fatal infection in cynomolgus monkeys carriers of type D simian retrovirus [285]; fatal human infections

While live attenuated viral vaccines imitate the natural exposure to the pathogen the most and thus induce the best immunity against virulent strains of that pathogen, the worldwide administration of these vaccines is not without dangers. As Sabin's live attenuated poliomyelitis vaccines just about eradicated the disease, gene rearrangements in the attenuated virus and/or its recombination with a few particles of a latent virulent strain of this virus can cause paralytic poliomyelitis in rare recipients of the vaccine [286–290]. (Would pre-immunization with the killed Salk vaccine prevent this?)

This reactivation of an attenuated virus into virulence is reminiscent of an early observation concerning mouse leukemia viruses. A weakly leukemogenic and almost completely attenuated but immunogenic mouse leukemia virus strain when co-inoculated in large dose with limit dilutions (very low dose) of the virulent mouse leukemia virus, caused rapidly high incidence of leukemias. We postulated that the "leukemogenic gene" (not identified in 1962 if it were an "oncogene") lost from the attenuated virus was produced in excess by the virulent virus and the re-acquisition of this sequence by the attenuated virus during co-infection with minute amounts of the virulent virus restored leukemogenic potency of the attenuated virus [291].

Thus whenever live attenuated viral vaccines are in use in large scale, unusual phenomena due to reactivation may take place. The benefit/risk ratio so overwhelmingly favors the former that the development and use of intraspecies attenuated live virus vaccines must continue. The use of live veterinary viruses for the prevention or treatment of a human disease started with Jenner. It was in Pittsburgh in 1962 [292] when veterinary viruses were adapted to human cancer cell lines in vitro and then released to the Roswell Park Memorial Hospital (Buffalo NY) for the treatment of human cancers. Early moderate success (partial remission inductions) was followed by relapses [292–294]. The inherent risks of the procedure outside the aegis of a medical research institution were recognized and the investigators refrained from recommending world-wide use of live veterinary virus vaccines for the treatment of human cancers. When Asada or Shimizu et al. [295, 296] in Japan succeeded in inducing partial and temporary remissions of human cancers (including glioblastoma multiforme and ovarian cancer) with live attenuated human mumps virus vaccine, they considered the procedure for wider use but health care agencies of that country disallowed the use of this vaccine for the treatment of human cancers (Asada, personal communication by correspondence to JGS).

The authors of this communication strongly recommend utmost caution before the release or sale of live attenuated viral vaccines for interspecies administration in large scale (for example, veterinary viral vaccines not previously investigated in the human host for the treatment of human cancers or viral hepatitides etc. as advertised in the Internet). Do we know if these viruses could assume "xenotropic" behavior and start replicating in human cells (normal cells, tumor cells, hepatocytes, macrophages, lymphocytes) and if so do they undergo some gene rearrangements? If these viruses leave their unnatural human host, are they infectious latently or overtly to other human beings? Why do these viruses fail to induce first mucosal then systemic immunity, since their repeated inocula are claimed to work as well as the first inoculum? Was antibody or immune T cell induction against these viruses ever tested for in the new human host? Do they truly induce the proper cascade of cytokines and lymphokines necessary, not to stimulate but to subdue the target: a cancer cell or a hepatitis B or C virus-infected hepatocyte? In a superb article this is shown experimentally to be the case in the mouse [297], but was it tested for in the inoculated patients? Many cytokines and lymphokines with tumor-enhancing properties are known that may be induced when viral vaccines are blindly administered. Truly, how effective are these viral inoculations in the treatment of hepatitis or cancer: were the good results so claimed confirmed in bias-free prospectively randomized clinical trials independently from the sponsors of the advertised procedures? In world-wide applications when consumed by thousands (if not by millions) of people, will these attenuated live veterinary viruses that are given repeatedly in large doses in order to forcefully transgress natural species barriers always interfere and never recombine? Should recombination just once with a latent human virus occur, an entirely new viral pathogen may emerge. Insertion of human genomic sequences into attenuated or "non-pathogenic" viruses could on occasions restore viral virulence (the "neurovirulence" gene of herpesviruses); or gene rearrangements within viral quasi-species transgressing species barriers (as it occurs in the avian → porcine → human transfer of influenza A viruses; or simian → human transfer of first human AIDS virus) can render a "nonpathogenic" virus pathogenic in a new species.

For these remote but serious considerations proper restraint and caution should be exercised when interventions in the co-evolution of hosts and their viruses are planned, especially when this entails transgression of natural species barriers. The standard therapy for hepatitis C is ribavirin and Hu-r-IFNα2b with 45% success rate: a pre-investigated, safe and approved treatment modality. Attempts at the investigational virus therapy of human cancer certainly should continue especially with genetically engineered viruses (subject matter of another review article) or with viral oncolysates (see a future issue of *Acta* by Horvath et al. [298]). The phenomena of viral interference may as well be explored for the treatment of ongoing viral infections but with the stipulation that at this time these interventions cannot be considered proven effective and safe; therefore they are not ready for large-scale applications in human communities. These investigational projects should remain within the confinement of medical research institutions operated by trained manpower of expertise, and equipped with laboratory facilities in order to discover immediately and manage properly and promptly events that these procedures inherently entail.

As to genetically engineered viruses with defined genomes: canarypox-, retrolenti- and adeno- or adenoassociated viruses in this order of usefulness, as reflected by articles in the recent issues of *Gene Therapy* (Liebelt Publisher) with removed virulence and inserted new genes (that encode a tumor antigen or a lymphokine-cytokine, etc.), the pre-required laboratory tests are most rigorous. Their clinical trials also include those essential tests by which the fate of the inoculated genetically engineered virus particles can be traced in their new host (subject of a separate review; see for adenoviral vectors: Nász and Ádám) [299]. This approach is the acceptable one: a properly pre-investigated and controlled intervention fitting into the first decade of the next century; whereas the blind inoculations of large viral populations across species barriers and without measures applied to confine them should they undergo gene rearrangements or recombinations, appear to be outmoded and not without considerable risks.

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NEW ASSAYS FOR THE QUALITY CONTROL OF LIVE ORAL POLIOVIRUS VACCINE*

(A REVIEW)

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The strains of all three types of poliovirus used in the production of live oral poliomyelitis vaccine have been shown to yield vaccines that are both immunogenic and highly attenuated when administered orally to susceptible children and adults. Experience obtained for close to four decades with the vaccines prepared from these strains indicates that laboratory and animal tests described in the World Health Organization's (WHO) Requirements for Poliomyelitis Vaccines (Oral) do ensure the consistency of virus characteristics during vaccine production [1].

Major advances in our understanding of the molecular basis of attenuation and reversion of polioviruses resulted in the development of a new generation of tests. These include an alternative *in vivo* neurovirulence test in transgenic mice that express the human poliovirus receptor and a new *in vitro* assay (mutant analysis by polymerase chain reaction and restriction enzyme cleavage, shortly MAPREC) that assess the consistency of vaccine production at a molecular level. A WHO collaborative study was initiated in 1993 with the objective of assessing transgenic mice as potential models for evaluation of the neurovirulence of type 3 vaccines. The results of the study showed that there is a very good correlation between the TgPVR21 mouse assay and the monkey neurovirulence test. Further studies are in progress to develop a statistical model for making regulatory decisions on accepting or rejecting batches of type 3 vaccine. A WHO collaborative study was initiated in 1991 to evaluate the MAPREC assay for type 3 vaccines. The results of this study showed that the MAPREC test was a sensitive, robust and standardized molecular assay suitable

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for process development for new manufacturers and for monitoring the consistency of existing vaccine production. Screening single virus harvests with MAPREC before pooling them into monovalent bulk vaccine will also improve the quality of the final product.

World-wide experience has shown that the live attenuated strains of the three types of poliovirus developed by Dr. Albert Sabin have yielded vaccines that are immunogenic, safe and efficacious when orally administered to susceptible children and adults. The safety of oral poliovirus vaccine (OPV) depends on several factors, of which the most important are strict adherence to the seed virus system and the period and temperature of incubation of production cultures. Cultures must be incubated at a constant temperature in the range of 33–35 °C and virus must be harvested no later than 96 hours after infection. Tests on the working seed viruses and vaccine lots derived from them for consistency of virus characteristics, including tests in monkeys for neurovirulence and other tests *in vitro,* are of great importance in the production of a safe vaccine. If the quality of consecutive lots of OPV consistently meets these requirements, there is a high level of assurance that the vaccines will be both safe and efficacious.

Twelve years of experience in various laboratories of OPV manufacturers and national control authorities has shown that the monkey neurovirulence test (MNVT) adopted by WHO in 1982 [1] is a reproducible and sensitive assay for monitoring the consistency of vaccine production that contributes to the safety of OPV in humans [2]. Nevertheless because primates are used, efforts to complement and eventually to replace the MNVT by newly developed tests is of considerable importance. Major advances occurred in the past years in our understanding of the molecular basis of attenuation and reversion of polioviruses that resulted in the development of a new generation of tests [3]. The aim of this review is to compare the results of the MNVT with those of the new neurovirulence test in transgenic mice [4] and a molecular assay (MAPREC) for quantitation of revertants in OPV [5].

Monkey neurovirulence test

Neurovirulence is a complex phenotypic virus marker and can be experimentally increased or decreased. Tests of neurovirulence by direct inoculation of poliovirus into the central nervous system of susceptible primates were crucial in development of vaccine strains since they enabled Sabin to compare accurately the neurovirulence of candidate vaccine preparations. Results provided by these tests were critical in the selection of the strains that are now used for vaccine production [6]. Historically a variety of MNVTs has been used in different laboratories but none of these tests was well standardized and gave reproducible results [7]. The need to standardize the MNVT internationally was recognized by WHO as early as in 1976 [7] and as a result of a 3-year WHO Collaborative Study a new standardized MNVT has been developed and adopted by WHO in 1982 [1].

In the MNVT a monovalent bulk poliovirus vaccine is tested concurrently with a homotypic reference vaccine. The reference preparations are available from the WHO

Biological Unit. For types 1 and 2 vaccines 12 monkeys, for type 3 vaccines 20 monkeys are required. Thus for the testing of a trivalent vaccine (including the monotypic reference vaccines) a total of 88 monkeys are used. For each vaccine preparation a single dose is inoculated intraspinally into monkeys. Monkeys are observed for paralysis and sacrificed after 17–21 days. Although the vaccine virus strains are well distinguishable from the wild strains, they cause histologically well defined lesions in the target cells of the central nervous system. Lesions are scored by hemisection of the cervical and lumbar spinal cord and brain using a standard grading scale. Mean lesion scores are calculated for each monkey and further for each vaccine preparation. A monovalent bulk vaccine passes the test if the test results demonstrate that the mean lesion scores calculated for the bulk vaccine do not exceed those of the reference vaccine. Criteria for acceptance or rejection of each vaccine lot are calculated according to WHO Requirements for OPV [1].

Although the MNVT is a complex assay, experience shows that good within-laboratory reproducibility can be achieved as there is little change in acceptance-rejection criteria with time based on a pooled within-test variance [7]. Probability curves show that there is a 99% chance of rejecting a monovalent bulk with just a twofold increase in neurovirulence compared to the reference [7], whereas there is a 1% chance of rejecting a "good" batch that is not significantly different from the reference [1]. Results from three national control laboratories demonstrated that certain manufacturers can consistently prepare OPV lots that pass the WHO MNVT [7]. Over a 12-year period a total of 204 vaccine lots were tested in 8000 monkeys. Of 81 type 1 and 34 type 2 lots none failed the test. Of the 89 type 3 lots 4 lots were rejected, therefore manufacturers switched to a new type 3 virus seed (RSO) that was found less neurovirulent in monkeys.

The WHO MNVT served as a useful tool to monitor the consistency of vaccine production and prevented the release of an unsatisfactory lot. What is the correlation between monkey neurovirulence and vaccine performance in humans? This is difficult to answer because no one would willingly use a "failed" vaccine lot in humans. However, there seems to be some indication that earlier OPV lots produced in the 1960s and early 1970s were more neurovirulent in monkeys than those prepared in recent years [2]. Consequently, the number of OPV-associated paralytic poliomyelitis cases has decreased in the U.S. and Canada. It is noteworthy that since the introduction of the WHO MNVT in Canada and the U.K., the number of vaccine-associated paralytic poliomyelitis cases continued to remain at a low level [2].

Development of a neurovirulence test for OPV in transgenic mice

It is highly desirable for ethical, health risk to animal keepers and economical reasons that monkeys should be replaced in the OPV neurovirulence test as soon as possible with another animal species. Only primates are susceptible to all three types of poliovirus. The host restriction is due to the absence of a cellular receptor found on human and primate cells. Since attempts to identify and isolate the genes for both human and primate poliovirus receptors (PVR) were successful [8–10], the establishment of transgenic mouse lines that express the human receptor (TgPVR mice) became a reality

[11, 12]. When infected with poliovirus, TgPVR mice developed flaccid paralysis, followed by the death of some mice, and histological lesions were found in the central nervous system, similar to those observed in monkeys. The first transgenic mouse line (TgPVR1), when mice were inoculated intracerebrally, was found to differentiate between OPV type 3 and wild Leon/37 strains but was not sensitive enough to distinguish between OPV type 3 lots that passed and those that failed the MNVT [13]. By contrast, results of tests performed in another mouse line (TgPVR21), when mice were inoculated intraspinally, were encouraging to initiate a WHO collaborative study in 1993 on various type 3 lots of OPV [14]. In order to ensure the continuous supply of these transgenic mice for the collaborative study, investigators at the Central Institute for Experimental Animals in Japan succeeded in developing TgPVR21 mice from a limited research tool into a large scale production source of high quality animals [15]. Recommendations for the maintenance, containment and transportation of TgPVR mice were given in a WHO memorandum on transgenic mice susceptible to human viruses [16].

Studies on type 3 OPV in TgPVR21 mice. The initial WHO studies performed by six laboratories were restricted to type 3 vaccines which historically are the most problematic type to control in the laboratory [14]. The intraspinal route of inoculation was found optimal by Japanese investigators [17]. The technique was further refined and a standardized assay was developed in the Food and Drug Administration (FDA) laboratory [18] that was successfully transferred to several laboratories. In this test the majority of mice, unlike monkeys, develop flaccid paralysis and a dose-response can be established [14]. Statistical analysis of the data of the collaborative study indicated that discrimination between the reference vaccine and the vaccine that previously failed the MNVT, was statistically highly reliable in mice when two clinical indicators (severity of paralysis and the day of onset of paralysis) were used in the assessment. Gender was found to be a significant source of variability for clinical criteria with males being more sensitive than females. Gender balance must be taken into account in future experimental design [14]. Analysis of the data also demonstrated that the most discriminatory doses for types 3 vaccines were 3.5 and 4.5 log10 TCID50 per 5 µl of inoculum. A WHO meeting held in September 1995 in Geneva that discussed the laboratory data of the collaborative study [14], recommended that the study be continued and extended to additional laboratories including manufacturers and national control authorities.

The WHO collaborative study continued in 1996 and 1997. Preliminary analysis of data obtained on a type 3 vaccine lot that failed the MNVT and had an elevated revertant virus content (3% 472-C measured with MAPREC test) showed that this lot was successfully discriminated from the reference vaccine in numerous mouse tests performed by participating laboratories.

Another vaccine lot that failed the MNVT but showed a lower revertant virus content (1.7% 472-C) designated as a 'marginal' vaccine, was also discriminated by the same criteria in several mouse tests conducted by various laboratories. Four additional 'marginal' type 3 lots were tested by the FDA laboratory and all lots failed the mouse test [4].

Results on a 'marginal' vaccine from four laboratories tested in approximately 500 mice showed that the use of histological examination of the central nervous system similar to that used in the MNVT, did not improve the discriminatory power of the mouse

test assessed on clinical data. These histological data should be further analysed to establish the nature of the dose response curve whether it is identical to that of the clinical response.

The role of 2493-U revertants in type 3 vaccine batches was much debated in past years [4]. Some manufacturers produced vaccines that contained low level (0.3%) 472-C mutants with 100% 2493-U revertants. These vaccine lots were not neurovirulent in monkeys but possessed discernible neurovirulence for TgPVR21 mice. However, these lots passed the mouse test when appropriate reference vaccines were employed [4].

The results of the WHO collaborative study on type 3 vaccines clearly demonstrated that there is a good correlation between the TgPVR21 mouse assay described above and the MNVT. The main goal of the project is to develop a statistical model for making *regulatory* decisions on accepting or rejecting lots of OPV in the mouse assay as an alternative to the MNVT. For this purpose various parameters such as vaccine dose size, number of doses and validity criteria should be further investigated. It is obvious that once the statistical model was developed, it has to be validated by further laboratory tests. Vaccines prepared in various cell substrates are to be tested along with the WHO reference vaccine and a 'marginal' vaccine (failed in MNVT with marginally increased 472-C revertant content) by various manufacturers and national control laboratories.

Studies on type 2 and type 1 OPV in TgPVR21 mice. Only limited experimental data are available in transgenic mice on both types of vaccine.

Three type 2 vaccine lots that passed and two lots that failed the MNVT were tested with the WHO reference vaccine by FDA investigators. In addition three experimental vaccine lots that derived from a 'passed' lot were tested [4]. A good correlation between the monkey and mouse tests was obtained with all but one sample. The latter passed the MNVT three times but failed the mouse test twice. These findings suggested that TgPVR21 mice are at least as sensitive to type 2 OPV as monkeys. Further collaborative studies – similar to those conducted on type 3 OPV – are needed to develop the standardized mouse assay and the appropriate statistical model for regulatory decisions to accept or reject the lot in question.

Preliminary results from two laboratories showed that the correlation between the MNVT and transgenic mouse test is difficult to demonstrate with type 1 OPV [4]. The main reason for this is that the Sabin type 1 vaccine is the most stable of the three serotypes and individual vaccine lots virtually never fail the MNVT. Consequently, there are no 'failed' type 1 vaccine lots available for the mouse test and the experimentally produced (vaccine virus grown at high temperatures) samples were found too virulent in both monkey and mouse tests [4]. Therefore, these experimental vaccines are not suitable for further studies.

Development of the MAPREC assay for the quality control of OPV

Genetic studies on attenuation and reversion of poliovirus vaccine strains. The genetic basis of the attenuated phenotype of each vaccine strain has been investigated extensively over the last 20 years. The nucleotide sequences of all three poliovirus serotypes were determined in 1984 [19]. Genetic differences between vaccine and virulent strains were described: the type 3 vaccine strain has 11 mutations compared to the parent virulent P3/Leon strain [20]; the type 2 vaccine strain differs from a virulent revertant strain (P2/117) at 23 positions [21] and the type 1 vaccine strain differs from its neurovirulent parent P1/Mahoney by 55 point mutations [22]. The identification of the principal genetic determinants of attenuation of the Sabin strains has involved new molecular approaches. Hybrid genomes containing parts from attenuated and virulent strains were constructed using recombinant DNA techniques. These recombinant viruses are assayed for neurovirulence in animals and compared with their parent strains. Genetic determinants can be narrowed down further by construction of site-directed mutants [14].

Development of the MAPREC assay and its use for type 3 OPV. Using the molecular approach described above significant determinants of attenuation were found in the 5' non-coding and capsid protein coding regions of the type 3 vaccine strain. When recombinants between the Sabin type 3 vaccine, P3/Leon parent and P3/119 revertant strains were used, it was concluded that the attenuated phenotype of Sabin 3 was almost entirely due to a U at base 472 in the 5' non-coding region, a U at nucleotide 2034 and a C at nucleotide 2493 [14]. The question arose how constantly can these determinants be detected in various batches of type 3 vaccine and/or in what percentage are the revertant sequences present? Investigators in the FDA laboratory developed a new molecular technique called Mutant Analysis by PCR and Restriction Enzyme Cleavage (MAPREC) for direct quantitation of neurovirulent mutations in batches of type 3 OPV [5]. This assay involves extraction of virus RNA, reverse transcription, PCR amplification of a region of the 5' non-coding region using primers that introduce a Mbol restriction enzyme site for genomes with a 472-C base, Mbol digestion, resolution of digests on a polyacrylamide gel, and quantitation of the proportion of digested (472-C) to undigested (472-U) fragments [14]. Using the new method it was shown that all batches of type 3 OPV contained measurable amounts of revertants with C instead of U at nucleotide 472. Batches that failed the MNVT contained significantly higher quantities of 472-C mutants than batches that passed the test. The MAPREC assay with coded samples identified 100% of lots that failed the MNVT [14].

Based on these promising results, the WHO initiated a collaborative study in 1991. The aims of this study were to determine whether the MAPREC assay could be established in a large group of laboratories by estimating the percentage of 472-C in the viral genome of coded commercially prepared samples. The study also aimed to determine whether samples that passed or failed the MNVT could be discriminated by MAPREC in a variety of laboratories and finally the suitability of candidate reference materials for use in standardizing the assay [14]. At a WHO meeting held in September 1995 in Geneva results of this study obtained by 14 laboratories in 9 countries, including OPV manufacturers and national control laboratories, were presented [14]. The results of

the MAPREC analysis of the study panel of samples were highly consistent within and between laboratories. Four of the study samples had passed the MNVT and all consistently passed the MAPREC assay whereas the two MNVT-failed samples consistently failed the MAPREC assay. A DNA reference sample was found suitable for use in standardizing MAPREC assays and this preparation was accepted by the WHO Expert Committee on Biological Standardization in 1996 as an International Standard (IS) for calibration of MAPREC assays of type 3 poliovirus. Attempts are made to complement this IS preparation with two viral references that contain slightly higher or slightly lower 472-C revertants than the IS. In further studies the MAPREC assay was successfully used by a number of OPV manufacturers and national control laboratories and was found suitable for monitoring consistency of vaccine production. It was also found to be indispensable for establishment of new vaccine production or monitoring changing conditions of existing vaccine production, including switching to a new seed virus. Screening single virus harvests with MAPREC before pooling them into monovalent bulk vaccine should also improve the quality of the final product.

Application of MAPREC to type 1 and type 2 OPV. The molecular basis of attenuation and reversion of type 1 vaccine is less well defined than that of type 3 vaccine. Nevertheless, MAPREC assays are available to quantitate variation at nucleotides 480 and 525 that form a base pair in the 5' non-coding region and were found not stable at higher temperatures of incubation [3]. Sequence heterogeneity for the combined positions was found to be in a range of 1 to 3% for 50 commercially produced monovalent lots, fairly constant for a particular manufacturer but did not correlate with monkey neurovirulence. In addition, the use of MAPREC assay was hampered – like the transgenic mouse assay for type 1 vaccine – by the lack of availability of commercially produced type 1 vaccine batches that consistently failed the MNVT.

A MAPREC assay had also been developed for base 481 in the 5' non-coding region of Sabin type 2 vaccine [3]. Commercially produced vaccine batches were found to contain 481-G mutations in the range of 0.4 to 1.1% with no clear correlation between 481-G content and monkey neurovirulence. The pattern of accumulation of mutations with cell culture passage differed in primary African green monkey kidney cells and Vero cells. This emphasizes that application of the methodology to monitor production consistency will depend on comparison with a historic database for each manufacturer [3].

Conclusions

1. Many years of experience in various laboratories of OPV manufacturers and national control authorities has shown that the WHO MNVT is a reproducible and sensitive assay for monitoring the consistency of vaccine production that contributes to the safety of OPV in humans. Nevertheless because primates are used in the test, efforts to complement and eventually to replace the MNVT by newly developed assays is of considerable importance.

2. Major advances occurred in the past years in the field of genetics and our better understanding of the molecular basis of attenuation and reversion of polioviruses resulted in the development of a new generation of tests.

3. Results of a collaborative study initiated by WHO in 1993 on type 3 OPV clearly demonstrated that there is an excellent correlation between the new TgPVR21 mouse assay and the WHO MNVT. The main goal of this study is to develop a statistical model for making regulatory decisions on accepting or rejecting lots of OPV to replace the MNVT. Once the statistical model was developed, it has to be validated by further tests in many laboratories.

4. The TgPVR21 mouse assay for type 2 vaccines was found by one laboratory suitable to discriminate between batches that passed or failed the MNVT. Further collaborative studies – similar to those conducted on type 3 vaccines – are needed to develop a standardized assay and the appropriate statistical model for regulatory decisions. Experimental data to establish a transgenic mouse assay for the neurovirulence testing of type 1 vaccines are limited due to the high stability of the type 1 Sabin strain.

5. Results of the MAPREC collaborative study on type 3 vaccines initiated by WHO in 1991 showed that the MAPREC test was a sensitive, robust and standardized molecular biological assay suitable for use by manufacturers and national control laboratories for quality control of vaccine production. This includes the establishment of a new vaccine production (by characterizing the seed virus and batches derived from it) and monitoring changing conditions of existing vaccine production. Therefore, the MAPREC assay could be used as a prescreen before the neurovirulence test.

6. The MAPREC assay was also shown to be suitable to monitor molecular consistency of production of types 1 and 2 vaccines. The nucleotides that should be monitored include 480-A and 525-C for type 1 and 481-G for type 2 vaccines. These nucleotides are associated with attenuation and may revert during manufacture, therefore the MAPREC assay would be a useful screen test before the animal assay. However, it is recognized that other mutations may also contribute to attenuation and reversion to virulence for both serotypes, thus regulatory implications are not applicable until further data become available.

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ADVANCE IN BACTERIAL TYPING METHODS*

(A REVIEW)

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We give a short review about the two main groups of bacterial typing methods for epidemiological purposes: the phenotypic and genotypic methods. The advantage of the phenotypic methods is their feasibility for the less equipped laboratories, their disadvantages are the variability of the phenotypic properties, and their high labor requirement. The advantages of the genotypic typing methods are the higher discriminatory power and applicability of the same method for different species of bacteria. The disadvantage of these methods is that they are expensive and labour consuming. Beside the short description, we show the results of different authors obtained by the use of the molecular methods in the epidemiological practice and we call the attention to the insufficiency concerning the stability.

Various methods based on different principles are used in bacterial epidemiological studies. Methods can be divided into two main groups: based on pheno- and genotypic properties (Table I).

Phenotypic methods are currently used, their epidemiological usefulness is proved by countless examples. However, the phenotypic markers are changeable and the methods based on them are labour-consuming: e.g. the propagation and preservation of the type phages. It is also a considerable disadvantage that each bacterial species needs its own typing method. All these facts induced the scientists to make efforts for elaborating a stable typing method applicable for possibly all species of bacteria. The new relative simple and quick molecular methods for the isolation and characterization of DNA, RNA and proteins and the reproducibility of these methods made possible the development of typing on the basis of the molecular properties of bacteria; this trend was named as *molecular epidemiology*.

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Table I

Typing methods

Phenotypic typing methods:
Serotyping
Biotyping
Phage typing
Bacteriocin typing
Antibiotic resistance pattern
Genotypic typing methods:
Plasmid profile analysis (PP)
Restriction fragment-length polymorphisms, RFLP
Pulsed-field gel-electrophoresis (PFGE)-pattern analysis
Ribotyping: ribosome RNA type determination
Amplification of genomic DNA: multiple arbitrary amplicon profiling (MAAP):
– Random amplified polymorphic DNA (RAPD)
– Arbitrarily primed PCR (AP-PCR)
– DNA amplification fingerprinting (DAF)
Multilocus enzyme electrophoresis (MLEE)

Typing methods

Plasmid profile (PP) analysis means identification of the number and molecular weight of the bacterial plasmids. The method is shortly as follows: molecular weights of the isolated plasmids are determined by electrophoresis in horizontal or vertical agarose gels (AGE). After appropriate visualization the molecular sizes of plasmids are estimated from the mobility of standard plasmids of known molecular size [1–2].

The advantage of the plasmid profile analysis.

– the same method is applicable for typing different bacterial species, – the method is quick, it is feasible in one day,
– it is possible to carry out a lot of strains at once,
– in case of lack of gene expression and of "rough" isolates, non typable by serological methods the PP analysis can be successful,
– the small quantity of bacterial cells needed is advantageous too, especially in the case of harmful species or strains.

The difficulties of PP analysis:

– when the supposed epidemic strain does not contain plasmid,
– when the test bacterium contains only species – specific plasmid,
– when evidently non-epidemic strains carry the same PP.

Restriction analysis of plasmid and chromosomal DNA (RFLP)

The digestion by restriction endonucleases [3] results in a controlled fragmentation of DNA molecules (plasmids or chromosomes), and the resulted fragments could be separated and analysed by their length. The chromosomal analysis is applied mostly in case of bacterial-species indivisible by phenotypic typing methods.

λ- RFLP profile determination. The λ-RFLP determination is based on the RFLP analysis performed by a λ-gene probe [4]. The purified λ-DNA is labelled e.g. by digoxigenin. The bacterial genomic DNA is prepared and digested e.g. by EcoRI. The restriction fragments are separated by agarose gel electrophoresis (AGE) and transferred to a nylon membrane, where hybridization is done with λ-DNA and the fragment is detected by immunologic method.

Pulsed field gel electrophoresis (PFGE). [5] All linear double-stranded DNA molecules larger than a certain size migrate through agarose gels at the same rate. The greater the pore size of the gel, the larger DNA can be sieved. The linear DNA molecules larger than 750 kbp cannot enter the gel. In PFGE method pulsed, alternating, orthogonal electric fields are applied to a gel so that large DNA molecules in the agarose gel are moved from their molecular-traps every time the direction of the electric field is altered. The larger the DNA molecule, the longer the time required for the realignment. The advantage of this method is that by its use the bacteria are typable by 100% and the results are stable. The disadvantage is that the apparatus needed is very expensive and the working process lasts 20–28 hours.

Ribotyping rRNA probe [6, 7]

Ribosomal RNAs are conservative molecules present in every cell. Their sequence is determinable and this is a usable tool for identification and/or typing of bacteria. The principle of the method is: after isolation, purification and digestion of the whole genomic DNA, DNA fragments are electrophoretically separated, and transferred from the agarose gel to nitrocellulose or nylon membrane, and hybridized with labelled rRNA probes (Southern blot-analysis). Hybridization signals are detected by autoradiography. The advantage of the method is that many of so far examined species of bacteria are typable in 100 %, but the method is expensive and labour consuming.

IS200 typing [8]. IS200 is a mobile DNA element of 700 bp found in Salmonella and Shigella but not in *Escherichia coli.* The distribution of IS200 has been used for identification and typing. The method is shortly as follows: The IS200 is inserted in a plasmid, which is isolated, purified, digested with restriction enzyme and electrophoresed in agarose gel. The appropriate fragment is isolated and labelled e.g. with ^{35}S-dATP or biotin for use as an IS200 probe. Genomic DNA is extracted from the examined Salmonella strain and the DNA is digested with enzymes, which lack restriction sites within the IS200 sequence. The genomic restriction digest is electrophoresed and blotted on to nylon membrane. Hybridization is carried out with the IS200 probe and the result of the hybridization is visualized colorimetrically or by autoradiography.

Random amplified polymorphic DNA (RAPD) [9–10]

Random Amplified Polymorphic DNA (RAPD) analysis, or AP-PCR and DNA amplification fingerprinting (DAF) [11] are novel DNA fingerprint techniques, using single short primers of an arbitrary sequence to amplify genomic DNA in a low stringency PCR. In general they are named multiple arbitrary amplicon profiling (MAAP). The method is shortly as follows: the DNA of the test-strain is isolated, purified. Generally a primer of 10 nucleotides and a temperature which ensures a low specificity of annealing (30–45 °C) is used.

For the AP-PCR technique longer primers are used, with a longer heating period. Amplification products are separated by AGE or PAGE. The above methods are used to epidemiological examinations, microbial genetics and eukaryotic genome examinations.

Multilocus enzyme electrophoresis typing [12]

The method is based on the electrophoretic mobility difference of the strains. The aminoacid differences of the corresponding proteins are reflected in the mobility differences. The aminoacid sequences are based on the sequences of the coding genes, therefore, this method is ranged among the genotypic methods.

Comparison of the results obtained by the different typing methods

Salmonella typing. Thirty-nine *S. typhi* strains were examined by phage typing (PT), RFLP and PFGE methods. Phage typing differentiated the 39 isolates into 9 distinct phage types, whereas PFGE analysis of XbaI- and SpeI-generated genomic restriction fragments established 22 PFGE types. Each of the five isolates with PT E1 originated from India were grouped as PFGE profile type 12. Of the five PT M1 isolates examined three had identical PFGE profiles. Out of three isolates of D1 PT originated from patient returning from India and Indonesia, two PFGE profiles were identical, the third strain was a clonal variant [13].

S. typhi strains isolated from 1977 to 1986 from typhoid fever epidemic in Chile were examined by PT, RFLP, ribotyping, IS200-typing and PCR fli-C gene amplification. The results suggested that the Chilean epidemic was probably produced by multiple sources of infection [14].

Among 68 *S. enteritidis* isolates from 16 Health Centres in Japan different typing methods were performed by comparing PT, plasmid profile types, antimicrobial resistance, RFLP and PFGE of *S. enteritidis* from outbreaks and sporadic cases. Among the isolates studied, 10 distinctive RFLP types were found with XbaI and four with NotI, while 12 combination types were identified among the 68 isolates. The RFLP type was subdivided by means of the plasmid profile and phage type. Conversely, plasmid profile and phage type was separated by means of RFLP [15].

From four Italian hospitals, 218 strains of Salmonella were analyzed by PT and PCR ribotyping. Among 57 *S. enteritidis* 46 PT A isolates were divided in 16 ribotypes. Ribotype was homogeneous by 7 serotypes: *S. goldcoast, S. agona, S. heidelberg, S. panama, S. anatum, S. london, S. enteritidis.* PCR ribotyping proved to be a good technique for subtyping strains of *S. derby, S. infantis* and *S. typhi-murium* [16].

Using PT, PFGE, ribotyping, plasmid virulence gene homology and LPS chain examinations of *S. enteritidis* strains originated from a single patient over a 6-week period strains with different phage types (PT4 PT 9a and PT7) were isolated. All these strains showed the same ribotype (by use of Pvu II) and the genotypes of the patient-derived strains differed from those of the reference strains [17].

S. typhi-murium isolates obtained from vaccinated and non-vaccinated poultry flocks as well as the commercially available vaccine strain Zoosaloral H and a *S. typhi-murium* reference strain were characterized by ribotyping, IS200-typing and PP. Five different ribotypes, 7 different IS200 hybridization patterns could be observed. Ribotyping, IS200 typing and plasmid analysis (Hind III fragment) allowed the differentiation between the Thyphimurium vaccine Zoosaloral H and the wild type of the same serotype [18].

S. heidelberg strains could be divided by IS200 typing. The IS200 sequence provided a highly discriminatory molecular marker of the *S. heidelberg* chromosome [19].

Escherichia coli typing. E. coli strains belonging to the same serotype, and isolated from extraintestinal infections were examined by ribotyping, fimbria and haemolysin type. *E. coli* 06:K5:H1 strains analysed with HindIII enzyme could be divided in ribotype pattern F, with SaII enzyme in ribotype "a". Strains with different serotypes can belong to the same ribotype pattern. This indicates that ribotyping cannot replace serotyping, but it is a useful tool complementing serotyping [20]. *E. coli* O157:H7 strains were characterized by PT, PFGE and PCR of verotoxin genes. PFGE proved to be a useful subtyping method [21]. Twenty-two isolates of *E. coli* 0157:H7 from bovine reservoir and 50 isolates from sporadic human infections were compared for plasmid profiles, DNA RFLP was identified with a bacteriophage λ-probe and Shiga-like toxin genotypes. Twenty-three λ-RFLP profiles, 4 Shiga-like toxin genotypes, and 8 plasmid profiles were identified. By these methods it was possible to demonstrate that isolates of *E. coli* O157 from bovine reservoir and from cases of human disease are similar [22].

Typing of Staphylococcus aureus. AP-PCR and PFGE typing of *S. aureus* strains was carried out in 7 laboratories. The examined 60 strains could be divided into 16–30 types. The reproducibility in the different laboratories depended on the technique (on the PCR apparatus). The PFGE type proved to be stable but the AP-PCR method gave variable results [23]. PFGE typing of 12 MRSA strains examined in different laboratories with different PCR apparatus resulted in only in four instances the same number of fragments [24]. It was found that it is necessary to standardise methodology using a standard set of strains.

Pseudomonas aeruginosa typing. P. aeruginosa strains were typed by RAPD and PFGE methods [25]. The isolates originated from chronically colonized patients with cystic fibrosis (CF).

Table II

Discriminatory powers of different typing systems to differentiate Chilean S. typhi *isolates [14]*

S. typhi strains	Typing methods	D*
138	Phage typing	4.77
149	Biotyping	0.50
47	PstI ribotyping	0.93
16	PstI ribotyping with *S. typhi* strains, 1977	0.96
13	PstI ribotyping with *S. typhi* strains, 1981	0.91
18	PstI ribotyping with *S. typhi* strains, 1990	0.92
16	ClaI ribotyping	0.93
31	IS200 typing	0.67

D* = discriminatory capacity

The 385 strains isolated from 20 CF patients could be divided in 35 RAPD types. Only two patients had *P. aeruginosa* strains of the same RAPD fingerprint. PFGE and RAPD typing gave corresponding results. In spite of the change of the phenotype the results obtained by RAPD method were stable in strains originated from the same patient.

Comparison of MLEE with RAPD and PFGE methods [26]. In case of *S. aureus, P. aeruginosa, E. coli* O157:H7 first the RAPD typing method is recommended, for further discrimination the PFGE method. RAPD method became remarkably useful for studies of epidemiology and microbial molecular genetics and in studies of higher organisms' genome organization.

Discriminatory ability of typing systems. For the calculation of species diversity within an ecological habitat Simpson developed an "index of diversity" [27]. This has been applied as discrimination index (D) for the evaluation of the different typing methods [28]. The discriminatory capacity of the different typing methods is demonstrated in Table II on the basis of typing of Chilean epidemic *S. typhi* strains [14].

Taking into account the data from the literature and our own experience about the different typing methods, we can conclude that there is no unique "most suitable" method. It is advisable to apply first the phenotypic methods (phage typing, bacteriocin typing) when these methods are available. Thereafter one should use a molecular genetic method which were proved to be the most suitable to discriminate within the given bacterium species.

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THE ACUTE PHASE REACTION SYNDROME: THE ACUTE PHASE REACTANTS*

(A REVIEW)

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The acute phase reaction of the organism, which determines the response that follows the injury of tissues and organs and helps the survival of the individual is reviewed. The main participants, the induction of the reaction and the regulatory mechanisms are shortly discussed, with special respect to the prominent role of interleukin-6. The relationship between the production and synthesis of the acute phase reactants under physiological and pathophysiological conditions is analyzed. The particular functions of orosomucoid and α2HS-glycoprotein are summarized. Finally, the common biological actions of IL-6 and the acute phase reactants are mentioned.

Processes causing tissue lesion such as trauma, burns, surgery, infection, hypersensitive immune reaction, autoimmune disease, toxic or septic agent, ischemic event, malignant tumor are accompanied by a response reaction involving the whole organism in order to eliminate the precipitating cause, to minimize the tissue damage and restore its integrity. The nervous system, endocrine, metabolic and cardiovascular systems, the connective and muscular tissues are also involved in this mechanism of host defense. The liver and the immune system have particularly important role in this process. Inflammatory signs can be observed locally at the site of primary tissue injury. The acute phase reaction, which is an exceptionally complex response of the organism is initiated by the Mo/Mϕ cells and controlled by the cytokine-neuroendocrine system [1–7].

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The development of the acute phase reaction

The pathogenic agents getting into the tissues contact the Mϕ and the Mo in the tissues and blood, respectively. Following phagocytosis, these cells start to synthesize TNFα, IL-1 and later IL-6 and other biologically active molecules are also secreted. This is the initiation of the APRSy. As a response to TNFα, IL-1, IL-6, a large number of very active agents are produced locally and distant from the lesion. These cytokines act like hormones in the circulation thus capable to activate different cell types. The activation of the central nervous system and the hepatocytes seem to be the most important effect of these cytokines [1, 2, 8–11]. Temperature regulation is altered in the HT and fever is generated. At the same time, the TNFα, IL-1, IL-6 influencing each other's effect, mobilize CRH, the HT-HP-GS axis is activated. The released CS-s, however, affect the production of the cytokines stimulating their synthesis. The metabolism of the liver cells and the regulation of the APR genes are simultaneously altered. As a result of these changes the liver serves as one of the main defense organ [8, 12–15]. At the same time, due to the effect of these cytokines on hemopoesis, the endothelial cells and among them especially the post-capillary venules become activated. After stimulation with LPS and cytokines the migration of Gra-s, Ly-s and Mo-s from the intravascular space into the tissues occurs as a result of the linkage of the adhesion molecules expressed on the membranes of the endothelial cells and those of the leukocytes. In addition to the chemoattractants, selectins, integrins and other adhesion molecules (ICAM-s, CD31, CD44) take part in this process. The local tissue events are controlled by these molecules [16–25].

The main effects of cytokines on the nervous system

TNFα, IL-1, IL-6 are the most important cytokines that generate fever. They also have a basic role in the stimulation of the HT-HP-GS axis. TNFα and IL-1 enhance their own synthesis and the production of IL-6, whereas IL-6 inhibits the synthesis of the former ones. The production of all these cytokines is suppressed by CS-s. The release of catecholamines during APRSy and stress situations is augmented, which results in enhanced CRH secretion and eventually in enhanced IL-6 effects (Figure 1). While the secretion of GH and vasopressin is increased, the production of TSH is decreased. This relationship between cytokines and the central nervous system supports the notion that these interactions determine the development, regulation, and termination of APRSy [12–15, 26].

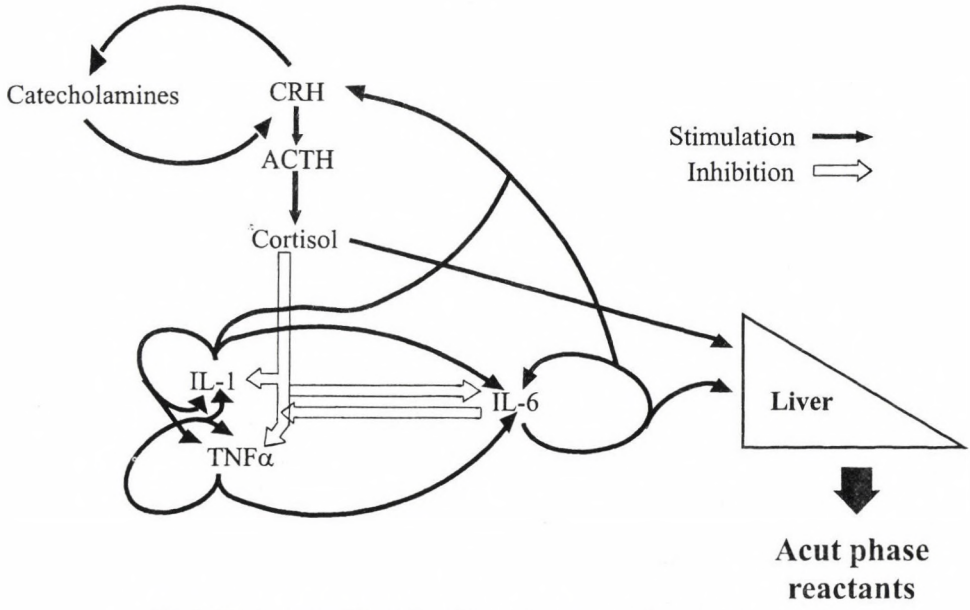

Fig. 1. Simultaneous effects of cytokines, hormones and the acute phase proteins during the acute phase reaction

The physiological and pathophysiological role of IL-6

IL-6 possesses an especially important role in APRSy. Until now this cytokine has been classified as a member of the proinflammatory cytokine group along with TNFα and IL-1. In the light of recent observations, however, this view has to be at least reconsidered [2–5, 9].

IL-6 is synthesized by most different cell types. In addition to cells participating in the immune response (Mo, Mϕ, mastocyte, endothelial cell, fibroblast, astrocyte) IL-6 is produced by the stromal cells in the bone marrow, synoviocytes, chondrocytes, keratinocytes, intestinal epithelial, vascular smooth muscle, HP folliculo-stellata, endometrial stromal cells, trophoblasts, and testicular Leydig's cells. Characteristics of IL-6 are explained by its unusual wide-ranged production pattern and the similar distribution and individual functional nature of IL-6R [2].

IL-6R is a transmembrane structure, which by itself, has no role in the signal transduction. The attachment of IL-6 to this receptor chain is followed by homodimerization of gp130, an other transmembrane structure, which initiates the transducing cascade. IL-6R has a soluble form, which is identical with the extracellular segment of the transmembrane receptor. After attaching to its receptor, IL-6 is able to link

to the membrane gp130, and to elicit transduction signals. IL-11, oncostatin M, leukemia inhibitory factor, ciliary neutrophic factor, cardiotrophin 1, and leptin share with IL-6 on gp130 as integral component of their receptors. Due to this structural relationship IL-6 is able to attach to the soluble IL-6R which is followed by linkage to gp130 on the myocyte, which can consequently lead to hypertrophy of the myocardium. IL-6R can virtually be expressed on all cell types [2, 5].

The most important physiological and pathophysiological effects of IL-6 can be summarized as follows. It stimulates the HT-HP-GS axis, enhances secretion of GH and vasopressin and reduces the release of TSH. It can induce the generation of fever, anorexia, fatigue, elevation of the basal metabolic rate, and decrease of serum lipid concentration. Under pathological conditions, it may have a role in the development of the CS withdrawal syndrome and also in the inappropriate vasopressin secretion. IL-6 also has a significant action in the regulation of the immune response by promoting the development and differentiation of Ly-s (especially B Ly-s) and the evolution of plasmocytosis, by enhancing the production of IgG-s (mainly IgG1), and by causing leukocytosis and thrombocytosis. IL-6 has significant effects on bone metabolism in both physiological and pathological circumstances. The development and function of osteoclasts is enhanced definitely, and so is, to a lesser extent, the development of osteoblasts. As a consequence, both in post-menopausal states and during the inflammatory processes osteoporosis may develop. The synthesis of IL-6, which is in close functional relationship with the neuroendocrine system, is inhibited by CS-s, estrogen and androgen hormones, while parathormone has a stimulating effect [2, 3, 9, 10, 27].

The acute phase reactants

The liver is one of the most important target organ of cytokines regulating the events of APRSy. Due to the effect of cytokines, the entire metabolic activity of the hepatocytes is altered thus providing the organism with indispensable quantity and quality of metabolites. According to this, the synthesis of a number of proteins and glycoproteins is increased while the production of others is decreased. The former ones are designated as positive, the latter ones as negative acute phase reactants, respectively. Their number is fairly large, over 40. They act together in the recognition and elimination of pathogenic organisms, in minimalization of tissue lesion, in the reparation of physiological structure and function of damaged tissues, mainly by inhibiting proteolytic enzymes and oxygen metabolites.

Although each of these reactants differ from each other in respect to their individual properties their overall action results in the processes described above [2, 3, 6, 28, 29].

The molecular mass, structure and serum concentration of the reactants are very different. It is noteworthy that with the exception of a few proteins (CRP, serum amyloid A), all of these molecules have N-linked (rarely O-linked) OS groups on the polypeptide chains. In case of some reactants, the OS chains comprise a few percent of the total

molecular mass, in others the ratio is around 20%, whereas 40% of orosomucoid consists of carbohydrates. Usually there is a sialic acid at the terminus of the OS chains, which have a role in modulation of the molecular conformation, in protection against proteolysis, and participation in processes of recognition and attachment. They might influence basic physiologic and pathologic processes. Orosomucoid, for example, can bind to selectins, which results in the modulation of leukocyte migration [6, 30].

The increase and decrease in serum concentrations of the reactants provided the base of their classification. The positive reactants have been classified according to the degree of elevation in concentration. The rise in serum levels of some proteins is about only 50% (ceruloplasmin, C3, C4), whereas the concentration of others can increase by 2–4-fold (orosomucoid, α2-haptoglobin, α1-antitrypsin, fibrinogen), while there can be a 100–1000-fold elevation in some reactants (CRP, serum amyloid A). Efforts have been recently made to define groups according to the cytokine that regulates the gene of the particular APR-s. The type I group contains proteins, which have TNFα and IL-1 as main regulators. CRP, serum amyloid A, and orosomucoid belong to this group. The type II group has IL-6 as the chief regulator. Fibrinogen, α1-antitrypsin, α1-antichymotrypsin, α2-macroglobulin (in the rat) are representatives of this latter group. It has been recognized, however, that glycocorticoids promote the synthesis of all the reactants, and that IL-6 can strongly synergize the synthesis of the type I proteins. Glycocorticoids have an outstanding role by enhancing the expression of IL-6R and the synthesis of the gp130 chain in hepatocytes. The true difference between the two groups may be that IL-1 acts as an inductor in group I, and more likely as an inhibitor in group II. The group III contains the negative reactants such as albumin, transferrin, AHSG, retinol-binding protein, which are regulated by IL-6 [1, 3–5, 9–11, 31].

The complement components can be classified in two groups. INFγ seems to be the main inductor of C3, factor B, C4bp, mannan-binding protein, C1 esterase inhibitor, and of factor H, nevertheless IL-6 and glucocorticoids are equally important in the induction of mannan-binding protein synthesis [8]. Data are available partly from in vivo animal, in part from in vitro experiments (physiological or tumor cell lines), and only partly from observations in humans, thus causing uncertainty. The synthesis of APR-s differs between hepatoma and healthy cell lines [32]. The pattern of change in concentrations of APR-s may significantly be influenced by the evoking mechanism. Reactions induced by surgery or LPS are strikingly different. The synthesis of the APR-s can be modified remarkably by physiological mediators such as insulin, hepatocyte growth factor, fibroblast growth factor, TGFβ, as well as by the dominance of Th1 or Th2 type immune response. As it has been mentioned above gp130 is a common transducing signal component of some mediators. The overlapping effects of these mediators, especially their collaboration with IL-6 in the induction of the APR can be explained on this basis.

The intracellular events leading to the initiation of the synthesis of APR-s induced by cytokines – such as IL-6 – cannot be discussed in detail but it is important to mention that on the surface of the hepatocyte the attachment of IL-6 and IL-6R is followed by signal transduction (through gp130), by activation of transcription factors and by alteration in transcription activity of the affected gene [35, 36].

The special functions of the individual APR-s cannot be discussed in detail now. Some of them can attach to the Mo/Mϕ-s through specific receptors and can modify cell

functions. Besides opsonizing and activating the complement system, CPR is able to induce the synthesis of TNFα, IL-1 and IL-6. This has both pro- and antiinflammatory consequences. Importantly, PBMC-s produce 5–10 times more IL-1Ra than IL-1β in the presence of CRP. This way of action resembles to those of immune complexes and aggregated IgG and contrasts with what is seen during the reaction elicited by LPS, in which the amount of IL-1Ra and IL-1β is much the same. CRP recognizes OS structures [3]. Only the chief functions of orosomucoid and AHSG are summarized below.

Besides being capable to bind a lot of drugs orosomucoid can modify immunological and inflammatory processes. It can influence the functions of Gra-s, Ly-s and Thrc-s. Its serum concentration multiplies during APRSy and the quantity and composition of the OS chains is characteristically altered. Molecules possessing one or two biantennary OS chains are particularly multiplied and have more focus residues. These alterations result in the significantly growing number of sLeX groups compared to physiological values. Structures having sLeX groups serve as ligands for selectins. E-selectin can be found on endothelial cells, P-selectin in the endothelium and platelets, and L-selectin in the leukocytes, respectively. Selectins make the migration of leukocytes possible from the blood to the damaged tissues through tethering and rolling these cells along the endothelial surface [16–19, 25]. Orosomucoid is able to influence these processes by the aid of its sLeX groups. It can inhibit the attachment of leukocytes by binding to E-selectin on the endothelial cells. After attachment of orosomucoid to soluble E-selectin or to the sLeX on the surface of the leukocytes, the binding of these two is blocked. The net result is rather antiinflammatory. Orosomucoid can enhance the production of IL1-Ra in PBMC-s although not as much as CRP or α1-antitrypsin. Remarkably, the productions of IL-1Ra and IL-1β are in the same range in the presence of LPS, whereas without LPS the secretion of IL-1Ra is dominant, suggesting that there is no more IL-1β needed. We have observed that orosomucoid inhibits the Con A-induced blastic transformation of peripheral blood lymphocytes at physiological concentration in healthy persons and in patients with LED. The secretion of IL-2 was moderately inhibited and the expression of IL2-R (CD25) was increased (data not published) [3, 30, 37, 38].

AHSG is a negative acute phase reactant. It has opsonizing properties, influences the lymphoblastic transformation, and can bind to Ly-s transformed by EBV. AHSG accumulates in the mineralized tissues such as bone matrix and dentin, particularly in fetal life. We observed that AHSG inhibits the mitogenic response of lymphocytes induced by PHA and PWM at physiological concentrations, and moderately inhibits the IL-2 production. These observations were made on peripheral blood Ly-s from healthy persons, from those taking oral contraceptives, and from patients with LED. These effects were markedly reduced at lower concentrations of AHSG. We have shown that AHSG inhibits the tyrosine kinase activity of the insulin receptor in a dose-dependent manner well below the physiologic concentration. The target points of the APR-s have not been identified yet and further investigations are needed. The OS components of the molecules merit special interest since mitogens recognize carbohydrate structures [39–42].

On the basis of the critical review of the available observations one can say that due to the effect of the APR-s the control and regulation of the inflammatory and repairing processes following tissue damage are promoted. IL-6 is the chief regulator of the APR-s. Besides its known proinflammatory effects IL-6 has an overall

antiinflammatory activity. IL-6 affects the acute and chronic inflammatory processes directly by inhibiting the production of TNFα and IL-1, by the intensive stimulation of the HT-HP-GS axis, thereby stimulating the secretion of CS-s, and by enhancing the release of IL-1Ra and soluble TNFαR. The induction of the synthesis of APR-s in the hepatocytes is an indirect but perhaps very important effect of IL-6 (Figure 2).

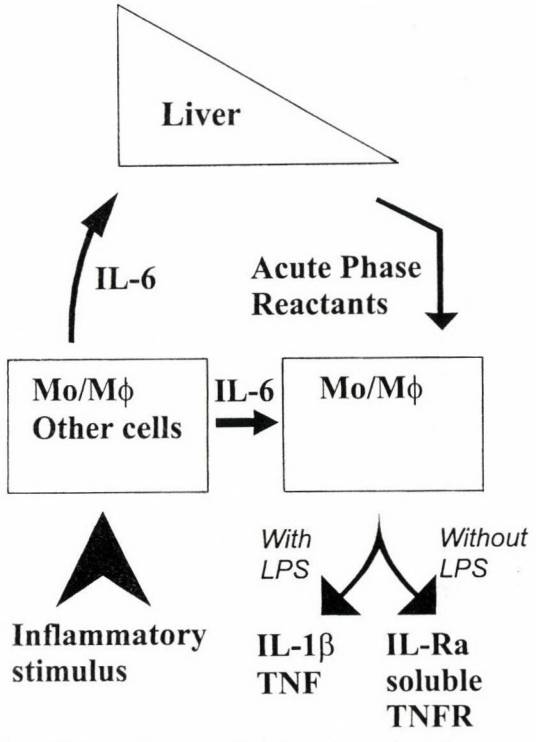

Fig. 2. The effect of IL-6 and the acute phase reactants on the inflammatory process (based on Tilg, H. et al., IL-6 and APP-s, Immunology Today, 18, 428–432, 1997)

Finally, human HSV8 contains the IL-6 gene in its genome. The virus can be found in Kaposi's sarcoma, Castleman's disease, body cavity lymphoma cells and in stromal cells of the bone marrow in multiple myeloma. IL-6, which is encoded by the virus but synthesized by the infected cells plays as a partly autocrine, partly paracrine growth factor in these pathological conditions. The investigation of these processes provides possibilities to establish a correlation between IL-6 and the growth of tumor cells and the regulation of the APRSy [43].

Data providing the base of the present view on APRSy are heterogeneous both quantitatively and qualitatively. The investigative work at present and in the future may

essentially modify or complete our knowledge on the pathological issues of APRSy. The application of the knowledge on APRSy in the clinical practice may particularly be fruitful.

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Abbreviations: AHSG, α2HS-glycoprotein; APR, acute phase reactant; APRSy, acute phase reaction syndrome; Con A, concanavalin A; CRH, corticotrophin releasing hormone; CRP, C-reactive protein; CS, corticosteroid; C3, C4, complement components 3 and 4, respectively; EBV, Epstein–Barr virus; GH, growth hormone; GS, adrenals; HP, hypophysis; HSV8, herpes virus type 8; HT, hypothalamus; ICAM, intercellular adhesion molecule; INFγ, interferon γ; IL, interleukin; IL-R, interleukin receptor; IL-1Ra, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; Ly, lymphocyte; Mo, monocyte; Mϕ, macrophage; OS, oligosaccharid; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; LED, systemic lupus erythematosus; sLeX, sialyl-LewisX; TGFβ, transforming growth factor β, Thrc, thrombocyte; TNFα, tumor necrosis factor α; TNFαR, tumor necrosis alpha receptor; TSH, thyroid stimulating hormone.

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ISOLATION OF HUMAN α2HS-GLYCOPROTEIN SYNTHESIZED BY SF9 CELLS*

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The human α2HS-glycoprotein is a negative acute phase protein synthesized by hepatocytes. Because of its fragility and the difficulty of its purification, we used recombinant DNA techniques to produce the protein in order to investigate its biological effects. The cDNA coding for the whole α2HS-glycoprotein from human liver library had been cloned into the baculovirus expression vector system using the pVL 1392 transfer vector and Sf9 cells. The recombinant protein was synthesized in the Sf9 cells and was isolated on a hydroxiapatite column from the culture medium. Western blot analysis indicated that the cells synthesized large quantities of the recombinant human protein. The molecular mass of the recombinant AHSG was the same as that of the protein in human plasma but was slightly lower than that of AHSG in the cell culture supernatants of HepG2 and higher than that of AHSG from Hep3B cell cultures, respectively.

AHSG is a 49 kd molecular mass glycoprotein produced by hepatocytes [1]. The protein consists of a heavy (A) chain (282 aa residues) to which a connecting peptide is attached (40 aa residues). These are linked to the light (B) chain (27 aa residues) by a disulphide bridge [2–4]. The 12 half-cysteine residues (11 on the A and 1 on the B chain,

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respectively), determine 5 disulphide loops, whose arrangement is very similar to that found in members of the cystatin superfamily [4].

AHSG has been supposed to have a role in various biological processes including enhancement of phagocytosis [5], binding to lymphocytes transformed by EBV [6] and decreasing the lymphocyte mitogenic response [7]. It behaves as a negative acute phase reactant [8]. AHSG accumulates in bone tissue and presumably is also involved in bone metabolism [9]. The parallel distribution of AHSG and its bovine homologue fetuin has led to the hypothesis that AHSG could play an important role in fetal tissue development [10, 11]. The protein pp63, the rat analogue of AHSG, has been shown to inhibit the insulin receptor tyrosine kinase [12]. According to recent observations AHSG is a natural antagonist of growth factors like insulin, EGF, and TGFβ and enhances the synthesis of hormone HGF/SF, a hormone which promotes cell proliferation [13–16]. It has been suggested that AHSG may have a regulatory role in tissue regeneration [16].

The AHSG molecule is very fragile and is difficult to purify it from human serum [17]. Therefore, we used recombinant DNA techniques to produce the protein. Here we report that we successfully produced a large amount of human recombinant AHSG in the baculovirus expression vector system. The recombinant AHSG has been purified and analyzed.

Materials and methods

Production and isolation of the recombinant baculovirus clone. The production of the recombinant baculovirus clone harboring the message for human AHSG was carried out as described previously [18]. In brief, human liver cDNA library, which had been constructed in λgt11 phages had been screened with specific polyclonal antibodies and the cDNA coding for AHSG was identified and isolated. This cDNA was subcloned into the p-Bluescript plasmid. Sequence analysis proved that it contained the whole message for AHSG. Thereafter the cDNA of AHSG was cloned into the baculovirus expression vector system by cutting out the insert from the p-Bluescript with EcoRV and BalI followed by subcloning it into the SmaI site of the pVL 1392 transfer vector (Invitrogen, CA). We co-transfected Sf9 cells with the plasmid and the wild type AcMNPV DNA using the calcium phosphate method. The recombinant clones were identified and isolated by limiting dilution and DNA dot-blot hybridization [18, 19].

Expression of the recombinant human AHSG. Sf9 cells and wild type AcMNPV DNA were provided by Max Summers (A&M University, USA). Cells were grown at 27 °C in Grace's insect medium with 10% FCS (Serva, Heidelberg, Germany), 3.3 g/l lactalbumin hydrolysate (Sigma) and 3.3 g/l yeastolate (Oxoid, Basingstoke, England). Twice 10^7 Sf9 cells were infected in a 175 cm^2 flask at 10 MOI. After incubating at 27 °C for 1 hour the medium was changed to 50 ml Sf900 serum free medium. Cell culture supernatant was harvested 72 hours later.

Protein purification. Three hundred ml of Sf9 culture supernatant was concentrated 10-fold on a PM10 ultrafiltration membrane (AMICON, Beverly, USA). This concentrated supernatant was applied onto a 2 × 6 cm hydroxiapatite (Sigma) column equilibrated with 20 mM phosphate buffer (pH 7.2) previously. The AHSG was eluted with 0.4 M phosphate buffer (pH 7.2) at the flow rate of 0.02 ml/sec. The identification of AHSG in the fractions collected was done by Western blot analysis. Fractions containing the AHSG were combined and dialyzed overnight against PBS (pH 7.2).

Western blot analysis. Ten % PAG was prepared as described by Laemmli [19]. For immunoblotting the method of Towbin was followed [20]. Proteins were transferred to a nitrocellulose sheet in a semi-dry apparatus (LKB Pharmacia). Nitrocellulose strips were incubated with goat anti-AHSG antibody (1:2000, overnight) and later with alkaline phosphatase-labelled rabbit anti-goat IgG conjugate as secondary antibody (1:3000, for 1 hour). Visualization was performed by the commercially available substrate of the alkaline phosphatase BCIP and with NBT.

Results

The concentration of the purified AHSG was 1.5 μg/ml, as calculated from the immunoblot preparation. The recombinant protein consisted 12.5% of the total protein content in the media of the Sf9 cell line. The immunoblot of the recombinant AHSG and the protein from various sources is shown in Figure 1. The recombinant protein had the same molecular mass as AHSG in NHS (either native or purified). However, the molecular mass of the recombinant protein was somewhat higher than that of AHSG in Hep3B and lower than in HepG2 cell culture supernatants, respectively. We have observed two bands for AHSG in the supernatant of the Hep3B cells. The band with the higher molecular mass represents the whole molecule, whereas the one with the lower molecular mass corresponds to the heavy chain of the molecule released in the cell medium.

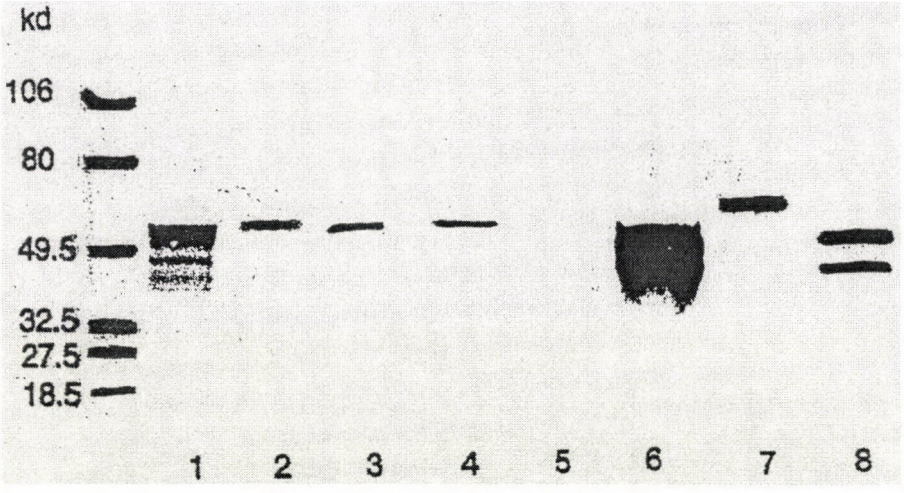

Fig. 1. The immunoblot of the AHSG from various sources. Lanes 1, and 2, NHS under non-reducing and reducing conditions, respectively; lanes 3, and 4, recombinant human AHSG; lanes 5, medium from an uninfected cell line; lane 6, AHSG purified from NHS; lanes 7, and 8, supernatants of HepG2, and Hep3B cell cultures, respectively

Discussion

Although AHSG has been described more than 30 years ago its exact biological role has not been established [17]. In order to study its functional effects, the protein should be present in large amounts in its active form. In case of AHSG, the purification of this fragile molecule in a biological active form is difficult. These prompted us to use recombinant techniques to produce the human protein. Our results indicate that the transfected Sf9 cells produce large amounts of the protein.

The production of proteins in the baculovirus expression vector system has several advantages. The yield of the recombinant protein is usually high. Thus, the additional purification of the recombinant protein is simple. In the case of AHSG this is especially important, as the molecule is very fragile. The heavy chain of AHSG in the supernatant of the Hep3B cells is probably a result of degradation (Figure 1). The degradation of the molecule is directly related to the number of steps during its purification. The purification of the recombinant AHSG needed only a few steps. This method is much shorter and easier than many techniques used to isolate of AHSG from human serum [22–25] and compares to the effectiveness of the Red Sepharose column [17]. An other advantage of production of AHSG in the baculovirus system comes from the fact that AHSG is a human homologue of the fetal hormone fetuin [10, 11]. Fetuin, when present in a concentration required for cell cultures (5–10%), gives a false positive result in assays for AHSG. The use of serum-free medium circumvents this problem. An other advantage of the baculovirus system is that the Sf9 cells can easily be maintained in serum-free medium, avoiding the presence of fetal calf serum proteins.

In contrast to prokaryotic and yeast expression vector systems, proteins expressed in the baculovirus system are subject to posttranslational modifications. Signal peptides are cleaved, glycosylation occurs (although recent results suggest that it can be terminated at the stage of high mannose glucose for N-glycans), and, most importantly, phosphorylation takes place [26]. In contrast to its rat protein analogue pp63, the human AHSG possesses biological activities only when phosphorylated [12, 18].

During the analysis of the recombinant protein we found slight differences of AHSG molecules from supernatants of HepG2 and Hep3B cell cultures. The exact cause of these differences is not known presently. There may be, however, differences in either the protein and/or the sugar components of AHSG from different sources. The AHSG molecule isolated from plasma has been described as a molecule consisting of two separate polypeptide chains. These chains are formed when a 40 aa long connective peptide is cleaved out. However, AHSG molecules with 39 amino acids connected to the whole A chain have also been reported to be present in human plasma [4]. It is therefore possible that this connective peptide can be absent or present, in whole or in part, on the finally secreted molecule in various cell cultures. Our recombinant molecule was both phosphorylated and contained the connecting peptide [18].

The structure of the polysaccharide chains has been described [2, 27]. There are two high mannose branches in the N-glycosides on the A chain, where the glycosylation of the recombinant protein may stop. The early termination of the glycosylation may

result in altered charge of the molecule due to the lack of the strongly negative charged terminal sialic acid.

In conclusion, this approach is of general interest when one wishes to obtain large amounts of trace proteins to investigate their biological activities.

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Abbreviations: aa, amino acid; (AcMNPV), *Autographa californica nuclear polyhedrosis virus*; AHSG, α2HS-glycoprotein; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; cDNA, complementary DNA; EBV, Epstein–Barr virus; EGF, epidermal growth factor; FCS, fetal calf serum; HGF/SF, hepatocyte growth factor/scatter factor; NBT, nitroblue tetrazolium; NHS, normal human serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecylsulphate polyacrylamide gel electrophoresis; TGFβ, transforming growth factor β.

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FIRST ISOLATION OF ENTERIC ADENOVIRUSES TYPE 41 FROM CHILDREN WITH ACUTE GASTROENTERITIS IN HUNGARY*

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In a retrospective study, 122 stool specimens, collected 1989–1990, from children with gastroenteritis were found to contain adenoviruses. Twenty-nine adenoviruses of 50 randomly selected adenovirus-positive fecal samples were successfully propagated in Graham 293 cells and were typed by DNA restriction enzyme analysis with *Sma*I. Six strains were typed as adenovirus 41 (Ad41), and 23 strains as non-enteric adenovirus. All six adenoviruses type 41 had the same DNA pattern identical with that of the variant Ad41/D12. Ad40 viruses were not detected. Enteric Ad41 infections were observed only in males and were found throughout the year. This is the first report on isolation and typing of enteric adenoviruses 41 in Hungary.

Adenoviruses (Ads) from stools of infants with gastroenteritis have been isolated for over four decades. However, epidemiological studies to date suggest that only Ad types 40 and 41, belonging to subgenus F, have been associated with pediatric diarrhea [1–5]. These viruses designated as enteric adenoviruses (EAds) cause an acute infection of short duration with very little seasonal variation under temperate climate [6, 7].

In contrast to the high particle numbers in stool specimens EAds are fastidious and grow poorly [8]. Even in Graham 293 cells which is now considered to be the most permissive cell line for their growth not all EAds multiply equally well [9, 10].

In cases where no or only a low level, transient cytopathic effect is seen, however, Ad specific antigen(s) can be detected by immunofluorescence (IF) [11]. Recently, monoclonal antibody-based enzyme-linked immunosorbent assays (ELISA) [5, 12, 13], dot blot hybridization [14], and genom amplification by polymerase chain reaction (PCR)

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[15, 16] provide rapid and definitive non-culture means for EAd diagnosis. Furthermore, if proper quantity of viral DNA is available, restriction enzyme analysis of viral DNA appears to be one of the simplest and generally used method to diagnose diarrheal diseases caused by enteric or nonenteric Ads and distinguish between them [17, 18].

During our study several adenoviruses were detected by IF after culturing stool samples collected from children with acute gastroenteritis in 1989–1990. Many strains were isolated and passaged in Graham 293 cells making possible to isolate sufficient quantity of viral DNA for restriction enzyme analysis. Here we report the first successful isolations and DNA restriction profiles of EAds, type 41 in Hungary.

Materials and methods

Fecal samples. For virus isolation, rotavirus-negative fecal samples taken from infants and small children less than 5 years of age, with diarrhea were collected from our routine samples submitted to the Regional Laboratory of Virology in 1989–1990. Stools previously screened for Ad content by latex agglutination (Adenolex; Orion Diagnostica, Helsinki, Finland) or IF on infected coverslip-cultures of Graham 293 cells using polyclonal anti-hexon rabbit serum and found to be positive, were selected and stored at –20 °C prior to inoculation into cell cultures for EAd isolation.

Cell cultures. Graham 293 cells, a continuous cell line of human embryonic kidney cells transformed with Ad 5 early region 1 DNA [19], were grown in Eagle's minimum essential medium (MEM; National Institute of Hygiene, Budapest, Hungary) with 5% foetal calf serum (FCS; Human, Gödöllő, Hungary) and 4% lactalbumin hydrolysate (Sigma Chemical Co., St. Louis, MO, U.S.A.). They were cultured in 25 cm^2 tissue culture flasks (Greiner GmbH, Nürtingen, FRG) and on coverslips and used between passages 76 and 105 in this study. Cells were mycoplasma-free by bisbensimide staining [20]. After inoculation, culture vessels were incubated at 37 °C with Eagle's MEM containing 2% FCS as maintenance medium.

Reference Ad40 and 41 strains. Ad40/D1 strain Hovi-X from Finland and Ad41/D12 strain 83-17952 from the Netherlands were used. They were passaged three times in Graham 293 cells. Infected cells at 7 day postinfection were aliquoted from each passages as controls for genome analysis.

Virus isolation. About 10% stool suspension in Hanks of Ad-positive samples were clarified by low speed centrifugation and inoculated directly into 2-day-old cultures of Graham 293 cells previously washed with serum-free Eagle's MEM. When cytopathic effects (CPE) were well developed, i.e., within 3 to 9 days postinoculation, cells were scraped into the culture fluid, transferred to a centrifuge tube, and pelleted at 100 × g for 10 min at 4 °C. The cell pellet from a single flask was suspended in 1 ml of ice-cold 20 mM Tris-HCl, pH 7.6 and transferred to microcentrifuge tube. After centrifugation again for 5 sec in Microfuge E (Beckman Instruments, Inc., Brea, CA, U.S.A.), the pellet was processed for viral DNA extraction and restriction endonuclease analysis.

Viral DNA extraction and analysis. Intracellular viral DNA was extracted from infected cells by a combination of the modification of Hirt's method [21] and the procedure described by Kang et al. [22] as follows: Cell pellet was disrupted with lysis buffer (20 mM Tris-HCl, pH 8.0, 0.45 M NaCl and 5 mg/ml Triton X-100). After centrifugation for 10 min at 15,900 × g the supernatant was treated with SDS and Proteinase K (Boehringer GmbH, Mannheim, FRG; final concentrations, 0.6% and 250 µg/ml, respectively) for 1 h at 37 °C. The sample was extracted with buffer-saturated phenol and chloroform : isoamyl alcohol (24:1, v/v) and, subsequently, nucleic acids were precipitated using absolute ethanol in the presence of 1 M NaCl, at –20 °C overnight. DNA was collected by centrifugation at 15,900 × g, and washed with 70% ethanol. After drying, the DNA pellet was dissolved in TE buffer (10 mM Tris-HCl,

pH 7.4, 1 mM EDTA), and stored at –20 °C. RNase treatment (Boehringer; 25 µg/ml) was also done at 37 °C for 60 min.

Restriction enzymes *Sma*I and *Hind*III were purchased from Amersham (Amersham International plc., Amersham, England). The digestion conditions were those described by the manufacturer. Samples were electrophoresed through 1% SeaKem agarose gel (FMC Bioproducts, Rockland, ME, U.S.A.) in submerged minigel apparatus, using TAE buffer (40 mM Tris-acetate, pH 7.6, 2 mM EDTA) and ethidium bromide (10 µg/ml). The electrophoresis was run for 2 h at 70 V with buffer circulation and the gels were photographed under ultraviolet light at 254 nm.

Results

Adenovirus isolation from clinical specimens. Fifty samples of a total of 122 stools previously screened and found to be Ad-positive by IF and/or the Adenolex test were randomly selected for isolation experiments. For the preparation of viral DNA, they were first inoculated into Graham 293 cells as a routine procedure in diagnostic work. Twenty-nine specimens displayed, usually 2 to 4 days postinfection, extensive CPE defined either as grape-like clusters of rounding of cells or as small clusters of single enlarged round-cells against a healthy background monolayer. The remaining 21 infected cell cultures did not show any CPE in 9 days of incubation when they were processed for DNA extraction.

Preparation and analysis of viral DNA. After 9 days or earlier when CPE was well developed, cells were harvested and intracellular viral DNA was extracted. Ten samples did not contain viral DNA and the amount of Ad nucleic acid was also insufficient for reliable DNA analysis from additional 11 samples. The use of restriction enzyme digestion with *Sma*I on 29 DNA extracts confirmed the isolation of enteric and nonenteric adenoviruses (Fig. 1). Six samples with the characteristic profile of Ad type 41 were identified. Examination of the profiles showed that all the type 41 strains gave the same pattern. The predominant profile seen for Ad41 viruses was similar to that of reference Ad41/D12 Dutch strain. Figure 1 also shows that *Sma*I digestion produced 10 fragments at least. However, the exact number of fragments was not visible in all cases, particularly in samples containing Ad41 strains (e.g. in line 4). When DNA samples were digested with *Hind*III, patterns were identical with that of the prototype strain (not shown). Adenovirus type 40 isolates were not revealed by either *Sma*I or *Hind*III digestions in the 29 samples with sufficient DNA. Restriction enzyme analysis with both enzymes of 23 samples, however, produced recognizable bands, which allowed them to be identified as non-EAds.

Age, sex and seasonal distribution of EAd type 41-positive cases. Of the six Ad41-positive children, 1 was in group ≤ 6 months of age, 1 was less than 12 months of age, 1 was 2 years old, and the 3 older children were 3.5 to 4 years of age. All of them were male. Of the total 29 cases where DNA analysis was successful, in groups ≤ 6, 7–12, 13–24, and >24 months of age, 5, 3, 6, 3 boys and 5, 4, 1, 2 girls were found, respectively. Detection of type 41 Ads throughout the study period did not show seasonal variation in the prevalence of subgenus F Ad gastroenteritis cases. Ad41 strains were detected in fecal samples of children with gastroenteritis in January, May, July, October, and November.

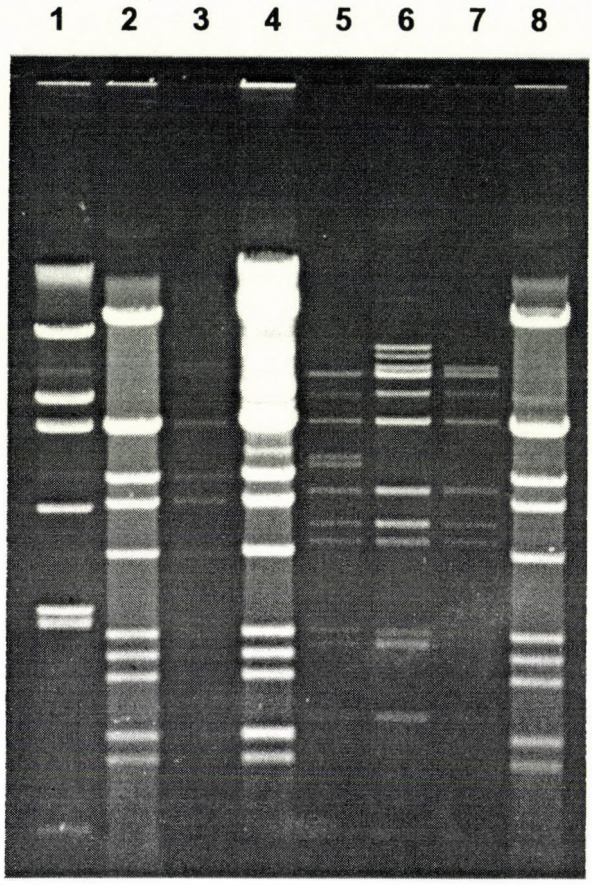

Fig. 1. Representative gel showing DNA restriction fragment patterns obtained after digestion with *Sma*I of viral DNA from adenovirus isolates and reference enteric adenoviruses type 40 and 41.
Lanes 1–2: Reference Ad40 Hovi-X and Ad41 83-17952 strains. Lanes 3, 5–7: untyped nonenteric adenovirus isolates. Lanes 4 and 8: Adenovirus type 41 isolates.

The seasonal distribution of the untyped non-EAds did not differ from that of Ad41 strains.

Discussion

This study was designated to isolate and propagate enteric adenoviruses in tissue culture and to determine whether serotypes 40 and 41 exist in the country. We used, therefore, Graham 293 cells which had been considered to be particularly useful for growth of EAds and applied the restriction enzyme cleavage analysis as a simple and

reliable method for Ad identification.Although all the 50 randomly selected samples contained Ads by the prescreening methods, only 29 of them produced enough DNA in Graham 293 cells for typing. Six enteric adenoviruses were successfully isolated with extensive CPE of numerous enlarged round-cells, and subsequently identified as Ad41 by their characteristic *Sma*I restriction pattern. Adenoviruses type 40 were not found. These results can be explained by the fact that not all EAds multiply equally well even in Graham 293 cells [23, 24]. Ad41 has been reported to grow better than Ad40 in this cell line [24]. Brown [23] observed that while the production of Ad40 virions in Graham 293 cells was 3- to 10-fold lower than that observed for other Ads, the yield of infectious virus was 100- to 1000-fold less. Moreover, differences in the susceptibility to virus growth in different batches of the same cell line, variations in the growth of different strains and differences in the growth of identical strains between laboratories have been reported [8]. Also, it cannot be excluded as an explanation for missing Ad40 strains that after 1986 a decline in the proportion of Ad type 40 has been observed and Ad41 became predominant in England [5], Japan [4], the Netherlands [25], and Canada [26]. It could be also taken into consideration that non-EAds which were present in the samples could grow to high titers and masked the presence of EAd.

There has been increasing evidence that Ad12, Ad18, and particularly Ad31 can cause cryptic enteric infections [5, 26–28]. We do not know the number of these serotypes among our 23 non-EAds because characterization and typing of the isolates have not been carried out in the lack of corresponding reference Ad serotypes.

The use of restriction enzyme analysis with *Sma*I on DNA extracts showed a characteristic profile of Ad41. All our six isolates gave the same pattern identical with that of the reference Ad41 strain provided by J. de Jong. At least 10 fragments were observed, some fragments however, could not be recognized in the upper part of the gel due to the high quantity and incomplete digestion of viral DNA (see in Fig. 1 lane 4). Restriction enzyme analysis has revealed the existence of 24 Ad41 DNA variants (D1-D24) [18]. In the present study, we found a single DNA variant with pattern of D12. Fragments 2 and 3, likely to the published pattern of variant D12, run together. This way 10 bands, instead of 11 were only recognizable in the gel. This variant was first detected by van der Avoort et al. [18] in 1982 and subsequently became the predominant Ad41 variant in many European countries [5, 29], now including Hungary.

Contrary to other studies [1, 5], we were unable to confirm that subgenus F adenovirus disease is prevalent in the less than 6 month age group [29] or occur most commonly in children less than 2 years of age [1, 2, 4, 5, 30]. Although only six children with EAd were found, one was only younger than six months but 3 were aged 3.5 to 4 years. For the 11 males and 12 females with non-EAds, these figures were 4 and 5, respectively, in the ≤ 6-month age group, and two boys and girls were older than two years of age.

All six infections of EAd type 41 occurred in males. Our finding of EAd infection being more common among males has also been observed elsewhere [1, 30]. In this study, we confirmed the absence of seasonal variation in the prevalence of EAd gastroenteritis in accordance with observations by others [1, 4, 5]. Our EAd strains were isolated throughout the year from January to November. Even though our limited data above are in good agreement with the majority of the published observations, they may yield

misleading information on the distribution of infections with EAds due to the small number of samples and short duration of investigation.

In conclusion, the present study documents first that adenoviruses, especially EAds type 41 variant D12, are important etiological agents in the childhood diarrhea in Hungary. Further studies, using PCR and sufficient number of samples are underway to detect more Ad41 strains, and probably unculturable Ad40 viruses.

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INVESTIGATION OF THE PRESENCE OF DIFFERENT BROAD-SPECTRUM BETA-LACTAMASES AMONG CLINICAL ISOLATES OF *ENTEROBACTERIACAE**

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Chromosomal or plasmid-encoded beta-lactamases are the most frequent causes of resistance to broad-spectrum beta-lactam antibiotics in clinical isolates of Gram-negative bacteria. Different screening methods can be used for their detection during routine laboratory work, while molecular biological methods may help in the detection of the genetic background of the phenotypic resistance. Clinical isolates of *Klebsiella pneumoniae* (170) and *Enterobacter cloaceae* (82) were obtained from different parts of Hungary, whereas those of *Serratia marcescens* (15) were isolated in our Department from a nosocomial outbreak. Disk diffusion and the Etest were used to screen inducible Class C beta-lactamase and plasmid-mediated extended spectrum beta-lactamases (ESBLs) among clinical isolates of *Enterobacteriaceae*. Single-strand conformation polymorphism (SSCP) analysis of the PCR products obtained after using SHV-specific primers revealed the presence of SHV-2 and SHV-5 ESBL among 170 *K. pneumoniae* strains in 12 and 3 cases, respectively. The results of the screening methods and the PCR-SSCP analysis suggested that 14 of the 15 *S. marcescens* strains not only produced the Class C, inducible chromosomal beta-lactamase, but also acquired a plasmid-mediated SHV-2-type ESBL. One strain isolated from the environment during the outbreak was genetically related to the other isolates, as demonstrated by the different typing methods, but it did not produce ESBL. The *in vivo* transfer of SHV-2 gene was assumed from an SHV-2 positive *K. pneumoniae* strain present in the same ward, in the same patient and at the same time. A very high prevalence of the stable derepressed mutants of *E. cloaceae* was confirmed among the Hungarian isolates. Seventy seven per cent of the strains produced high amounts of beta-lactamase without induction being responsible for their resistance to third-generation cephalosporins. Nineteen per cent of the strains were inducible when cefoxitin or imipenem was used, as confirmed by direct measurement of the MICs with the Etest.

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Concerning the different mechanisms of resistance of Gram-negative bacteria to beta-lactam antibiotics, the most important role in the development of such resistance is played by the beta-lactamases produced. Among the different species of the family *Enterobacteriaceae* resistance to broad-spectrum beta-lactams can arise via the production of different types of beta-lactamases encoded on distinct genetic locations. Therapeutic failures associated with the *in vivo* selection of stably derepressed mutants have been described in a variety of serious infections caused by Gram-negative bacteria possessing inducible chromosomal Class C beta-lactamases (according to Ambler's classification) (*Enterobacter spp, Citrobacter freundii, Serratia marcescens, Pseudomonas aeruginosa* and indole-positive *Proteus spp*) [1, 2]. Extended-spectrum beta-lactamases (ESBLs) confer resistance on newer cephalosporins, monobactams and also, depending on the amount of enzyme produced, beta-lactam-beta-lactamase inhibitor combinations [3]. The majority of these enzymes are located on transferable plasmids or transposons and are related to the TEM- or SHV-type beta-lactamases. The substrate spectrum has been broadened in consequence of point mutations of genes of the original TEM-1, TEM-2 or SHV-1 enzymes, which lead to alterations in the amino acid sequences close to the active sites of the enzymes [4]. Reviews have recently been published on the various types of ESBLs that occur world-wide [5–7].

The present paper gives a summary of our findings with regard to the occurrence and prevalence of beta-lactamase-based resistance to broad-spectrum beta-lactam antibiotics in clinical isolates of *Enterobacteriaceae* through the use of screening methods and molecular biological procedures.

Materials and methods

Bacterial strains. Clinical isolates of *Enterobacteriaceae* were screened during routine resistance testing for the presence of the inducible or stable derepressed form of Class C beta-lactamase and for ESBLs. 82 *Enterobacter cloaceae* and 170 *Klebsiella pneumoniae* strains originated from different parts of Hungary, whereas 15 *Serratia marcescens* strains were isolated in our Department from a nosocomial outbreak at an intensive care unit. To control the antibiotic susceptibility testing, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used. Control strains of *Escherichia coli* carrying known TEM and SHV-type genes were kindly provided by AB Biodisk, Solna, Sweden.

Antibiotic susceptibility testing. The susceptibility of the organisms to antimicrobial agents was first studied by the disk diffusion method, according to the guidelines of NCCLS [8]. The MICs of the different antibiotics for selected clinical isolates were tested by the Etest method (AB Biodisk, Solna, Sweden), using Mueller-Hinton agar [9].

Phenotypic identification of beta-lactamases. The ESBLs were screened by the synergistic effect of the combinations of amoxicillin-clavulanic acid with cefotaxime or ceftazidime in the double disk diffusion test, where cefotaxime and ceftazidime disks were placed 20 mm apart from the beta-lactamase inhibitor [10]. ESBL production was confirmed by the Etest ESBL strip.

Inducible beta-lactamase was screened by the disk approximation test [11]. The inducer disks, containing cefoxitin or imipenem, were placed on the surface of inoculated agar plates and the indicator disks (cefuroxime, cefoperazone, cefotaxime, ceftriaxone and ceftazidime) were applied approximately 14–16 mm from the centre of the inducer disks. If the inhibition zone was flattened by >1 mm by the

adjacent inducer disk after an overnight incubation at 37 °C, the organism was considered to exhibit inducible beta-lactamase. Direct measurement of the MICs of the indicator antibiotics was carried out without induction and after induction for those strains, which were resistant for the inducer, using the Etest strips. Cefoxitin strip as inducer was placed on one half of a Mueller-Hinton agar plate and incubated for 30 minutes at room temperature. After removal of the cefoxitin strip cefuroxime or ceftazidime strips were placed at the same site and on the other part of the plate. The MICs of the indicator antibiotics with and without prior induction were read off after incubation for 24 hours at 37 °C. The inducibility of the beta-lactamase production was also assessed by treating brain-heart infusion broth cultures of the bacteria with 50 µg/ml cefoxitin for *E. cloaceae* and 10 µg/ml cefoxitin for *S. marcescens* or with 2 µg/ml imipenem and incubating for 18 hours at 37 °C. The amounts of the beta-lactamase without and with induction were measured [12].

Beta-lactamase assay. Beta-lactamase activity was assayed spectrophotometrically with nitrocefin (100 µM) in 50 mM sodium phosphate buffer (pH 7.0 at 30 °C) as substrate in the ultrasound-treated supernatants of the centrifuged cells [13]. One unit of beta-lactamase was defined as the amount which formed 1.0 µmol of product per minute under these conditions. The protein content of the crude enzyme extract was measured by the Lowry method, with bovine serum albumin as standard [14].

DNA analysis. Plasmid DNA was extracted from clinical isolates by using the Magic Miniprep DNA Purification Kit (Promega, Madison, WI). Electrophoresis was performed on 0.7% agarose and the gels were stained with ethidium bromide. The plasmid DNA was extracted and used as template for the amplification of the TEM or SHV genes with TEM and SHV-specific primers [15]. For exact determination of the detected SHV genes, single-stranded conformation polymorphism (SSCP) analysis was performed. The amplification products were digested with *Pst* I and separated on a polyacrylamide gel in parallel with known SHV gene standards and the single-stranded DNA fragments were stained with silver nitrate [15].

Results

Evaluation of the presence of Class C beta-lactamase and its stable derepressed mutants among E. cloaceae isolates.

A total of 82 *E. cloaceae* isolates were evaluated for their resistance patterns (Figure 1). Although the strains were isolated in two centres in Hungary, considerable differences in resistance were not observed.

Forty-five isolates (55%) proved to be highly resistant to practically all beta-lactam antibiotics except imipenem. The MICs for these strains to all 4 third-generation cephalosporins tested were ≥128 µg/ml. Eighteen strains (22%) were resistant to second-generation cephalosporins (the MICs of cefoxitin, cefuroxime and cefamandol were ≥256 µg/ml) and displayed slightly lower MICs for the third-generation cephalosporins (the MICs of cefoperazone, cefotaxime, ceftriaxone and ceftazidime varied between 32 and 256 µg/ml). According to the resistance patterns these 63 *E. cloaceae* strains were considered to be stable derepressed mutants of the Class C chromosomal beta-lactamase gene (Table I). Sixteen strains (19%) were sensitive or moderately sensitive to most beta-lactam antibiotics, excluding cefoxitin. These strains were considered to be the classical inducible phenotype, as they exhibited susceptibility to third-generation cephalosporins (MICs in the range 0.25–1 µg/ml) and intermediate susceptibility to cefuroxime and

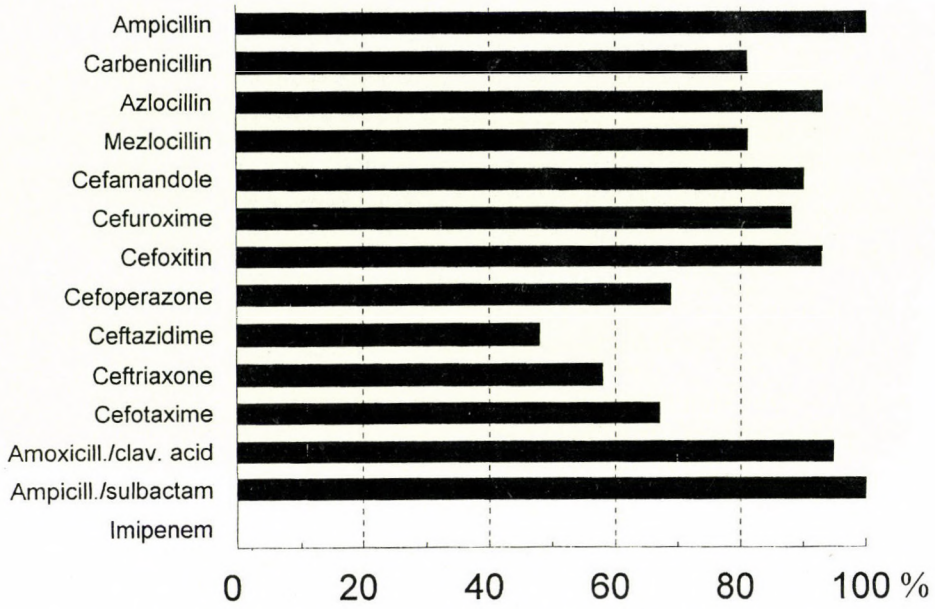

Fig. 1. Percentage of resistance of *Enterobacter cloacae* strains (82) to beta-lactam antibiotics

cefamandole (the MICs were 8–32 μg/ml). The MIC of cefoxitin for these strains was ≥256 μg/ml. Three further strains (4%) with the same sensitivity to second- and third-generation cephalosporins were found to be moderately sensitive to cefoxitin (MIC=16 μg/ml), representing the original basal beta-lactamase-producing strains. In the disk approximation test, cefuroxime proved to be the best indicator of the presence of inducible beta-lactamase for those strains which were susceptible or moderately susceptible to this antibiotic, but it could be used with only a limited number of strains as most of our isolates displayed resistance to this antibiotic. For most strains the presence of inducible beta-lactamase could be detected by the use of third-generation cephalosporins, as indicator antibiotics, in the disk approximation test. The direct measurement of the MICs of cefuroxime or ceftazidime before and after induction with cefoxitin by the Etest was also used to evaluate the presence of inducible beta-lactamase (Table II, Figure 2). This was possible only for those strains which were fully resistant to cefoxitin. Table II lists the MICs of cefuroxime and cefotaxime alone as read by the Etest, and the corresponding values obtained after a 30-minute induction with the cefoxitin Etest strip. For those strains which were susceptible or moderately susceptible to cefuroxime, but sensitive to ceftazidime (strains 10, 14, 2 and 29), marked increases in the MICs were observed for cefuroxime, while the MICs of ceftazidime were unchanged. For those strains which were highly resistant to cefuroxime, but moderately resistant to ceftazidime (strains 19, 58, 80 and 86), a 4-fold or greater increase in ceftazidime MICs was observed after induction.

Table I

The MICs of beta-lactam antibiotics and the beta-lactamase activity of three representative clinical isolates of E. cloacae with and without induction

Strains	CXM	CFX	CTX	CRO	CAZ	CFP	Disk approximation test*		Quantitative ß-lactamase assay		
							CFX	IMP	not induced	CFX 50 µg/ml	IPM 2 µg/ml
7	8	16	0.25	0.25	0.25	0.5	–	–	0	36	7
29	32	>256	0.5	0.5	0.5	1	+	+	19	1974	2268
58	>256	>256	256	256	32	256	+	+	1986	4657	3673

CXM: cefuroxime; CFX: cefoxitin; CTX: cefotaxime; CRO: ceftriaxone; CAZ: ceftazidime; CFP: cefoperazone

* As indicator antibiotics, cefuroxime, cefoperazone, ceftazidime, cefotaxime and ceftriaxone were used

Table II

Detection of inducible beta-lactamase of 8 cefoxitin-resistant Enterobacter cloacae strains by measuring MICs of indicator antibiotics with the E test

Strain	Cefuroxime alone	After induction with cefoxitin	MIC (μg/ml)	
			Ceftazidime alone	After induction with cefoxitin
10	16	128	0.5	0.5
14	16	256	1	1
21	8	256	1	4
29	32	2S6	0.5	0.5
19	256	>256	16	64
58	256	>256	16	128
80	256	>256	16	64
86	256	>256	32	128

Prevalence of ESBLs among K. pneumoniae isolates.

One-hundred and seventy strains of *K. pneumoniae*, originating from different parts of Hungary, were tested with regard to the presence of ESBLs. On the basis of the double disk synergy test, 15 strains (9%) were suspected of being ESBL-positive. The majority of the ESBL-producing strains were isolated from urine or sputum (4 and 7, respectively). Two ESBL-positive strains were isolated from blood and CSF samples, respectively. The Etest strip developed for the detection of ESBL in clinical isolates was used for all 15 isolates and the MICs of ceftazidime and ceftazidime/clavulanic acid were determined (Table III). If the ratio of the two MICs was higher than 4, the strain was considered to produce ESBL. All isolates which gave positive reaction in the double disk synergy test were also positive with the ESBL Etest strip (ratio of ceftazidime/ceftazidime-clavulanic acid MICs: ≥8). The 15 ESBL-producing *K. pneumoniae* strains harboured the SHV-type gene in a large plasmid (>80 kb, not shown). SSCP analysis of the PCR product revealed that 12 of the 15 ESBL-positive strains were SHV-2 producers and the remaining 3 expressed SHV-5 beta-lactamase (Table III, Figure 3). The SHV-5 beta-lactamase-producer strains gave high MICs for ceftazidime (32–256 μg/ml) and aztreonam (16–32 μg/ml), while in the cases of the SHV-2-type ESBL-producing strains the MICs of ceftazidime and aztreonam varied considerably (1–256 μg/ml). The MIC of cefotaxime for the ESBL-producing strains was 4–32 μg/ml. The fourth-generation cephalosporins (cefepime and cefpirome) are considered to be active against ESBL-producing strains. In our study, cefepime and cefpirome were observed to be active against all ESBLs expressing *K. pneumoniae* strains, with MICs varying between 0.5 and 8 μg/ml. However, one of the SHV-2-producing strains displayed a decreased sensitivity to cefpirome (MIC=16 μg/ml).

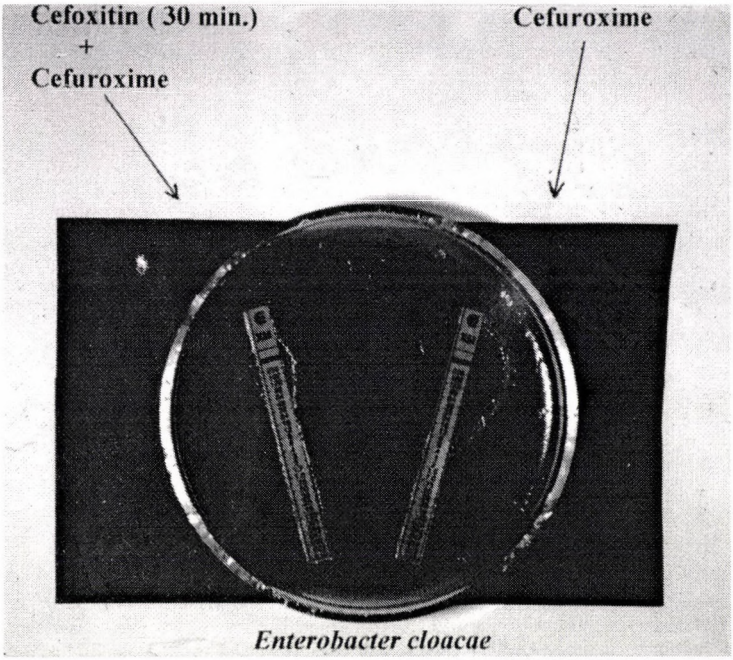

Fig. 2. Detection of inducible beta-lactamase by means of the E test in the case of cefoxitin-resistant *E. cloacae* strain No. 21. The inducer cefoxitin strip was placed on the left side of the plate and incubated for 30 minutes at room temperature. After removal of the cefoxitin strip, cefuroxime strips were placed at the same site and on the right part of the plate. XM: cefuroxime

Survey of the resistance mechanisms of outbreak strains of S. marcescens.

Cephalosporin and beta-lactam resistance profiles were determined for 15 strains of *S. marcescens* isolated from an epidemic in a neonatal intensive care unit. Of these strains, 12 were obtained from neonates (colonized or infected by the strain) and 3 were obtained from environmental samples. All isolates were found to be identical as concerns biotyping, phage typing and pyrolysis mass spectrometry (data under publication). The presence of an inducible chromosomal Class C cephalosporinase in all isolates was suggested by the presence of elevated levels of beta-lactamase activity after induction with cefoxitin (Table IV). In addition, the parallel presence of a plasmid-encoded ESBL was suggested in all but one of these strains (12 patient isolates and 2 environmental isolates) on the basis of the elevated MIC values of cefotaxime, ceftriaxone and ceftazidime, and the ability of beta-lactamase inhibitors to restore the activity of third-generation cephalosporins (Table IV). Consistent with this resistance profile, a large plasmid was isolated from the 14 ESBL-producing strains. Investigation of plasmid DNA by PCR, using SHV and TEM specific primers, confirmed that the ESBL production is due to the presence of SHV-type enzyme (Figure 4). The following SSCP analysis of the

Table III

MICs of different beta-lactam antibiotics against ESBL-producing K. pneumoniae strains

Strain	Beta-lactamase	AZT	CXM	CTX	CPM	CPO	CAZ	CAZ/CLA	Ratio
1	SHV-2	2	64	16	2	4	8	1	8
2	SHV-2	2	32	8	2	2	8	0.25	32
3	SHV-2	1	128	32	2	8	16	1	16
4	SHV-2	8	64	16	2	4	16	1	16
S	SHV-2	1	32	4	1	2	4	0.25	16
6	SHV-2	4	64	8	1	4	8	0.5	16
7	SHV-2	1	64	4	1	4	4	0.25	16
8	SHV-2	2	256	8	2	4	8	0.12	64
9	SHV-2	4	256	32	4	16	16	2	8
10	SHV-2	4	2.56	8	2	4	32	0.12	256
11	SHV-2	4	64	4	1	4	4	0.25	16
12	SHV-2	16	128	32	4	8	16	1	16
13	SHV-5	16	64	8	1	4	32	1	32
14	SHV-5	32	256	4	1	2	64	1	64
15	SHV-5	32	32	2	0.5	2	64	1	64

AZT: aztreonam; CXM: cefuroxime; CTX: cefotaxime; CPM: cefepime; CPO: cefpirome; CAZ: ceftazidime; CAZ/CLA: ceftazidime/clavulanic acid

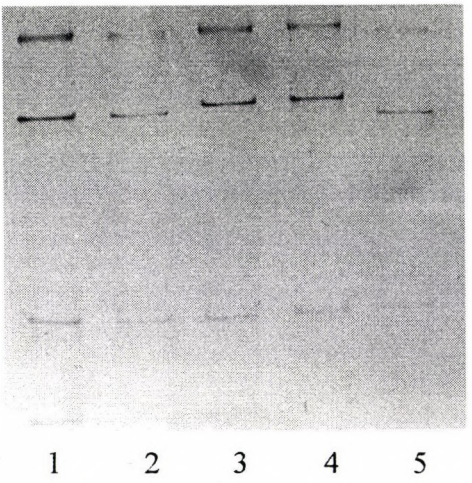

Fig. 3. Differentiation of the SHV type genes by means of the PCR-SSCP method originated from clinical strains of *K. pneumoniae.* (Lane 1, SHV-5 (control); lane 2, SHV-5 from clinical isolate; lane 3, SHV-2 (control); lane 4, SHV-2 from clinical isolate; lane 5, SHV-4 (control)

PCR product revealed the presence of the SHV-2 beta-lactamase gene (Figure 5). These data confirmed that 14 of the 15 strains (very probably originating from the same clone) involved in this nosocomial epidemic produced both a chromosomal cephalosporinase and a plasmid-encoded SHV-2 beta-lactamase.

Table IV

MICs of antimicrobial agents for S. marcescens and their beta-lactamase activity

Antibiotics	MIC (μg/ml)	
	E/3[a]	Sml[b]
Cefuroxime	256	256
Cefoxitin	16	16
Ceftazidime	1	8
Cefotaxime	1	32
Ceftriaxone	1	32
Cefoperazone	1	64
Aztreonam	0.12	2
Imipenem	0.5	0.5
Amoxicillin/clav. (2:1)	256	256
Ceftazidime/clav (2 μg/ml)	1	0.5
Ceftazidime/sulbactam (4 μg/ml)	1	2
	Beta-lactamase activity (U/mg of protein)	
Without induction	210	228
After induction[c]	2134	1875

[a] The third-generation cephalosporin susceptible, environmental isolate of S. *marcescens*

[b] *S. marcescens,* isolated from blood culture, representing all 14 isolates with ESBL

[c] Induction was carried out in the presence of 10 μg/ml cefoxitin

Discussion

Chromosomal Class C enzymes.

Among *E. cloaceae* and *P. aeruginosa* strains there is a close connection between the inducible expression of beta-lactamase and a high frequency of spontaneous mutation to a stably derepressed state. The genetic background of this phenomenon is a mutation which occurs in the *ampD* gene, which is an N-acetylmuramyl-L-alanine amidase, and

results in an increased transcription of *ampC* [16]. This mechanism provides a very efficient protection of the bacteria against beta-lactams, except for the carbapenems, and to a lesser extent cefepime (fourth-generation cephalosporin) [17]. The strains producing inducible beta-lactamase proved to be susceptible to carbenicillin and third-generation cephalosporins during routine *in vitro* testing of resistance. However, during the *in vivo* therapy, broad-spectrum cephalosporins can easily select the stably derepressed mutants the treatment of severe infections caused by *E. cloaceae* or *P. aeruginosa* should therefore be avoided [18, 19]. In strains of *Serratia*, *Providencia* and *Morganella*, also producing the Class C-type chromosomal beta-lactamase, the levels of enzyme and resistance of the derepressed mutants to third-generation cephalosporins are about 10 times less than those observed in the case of the derepressed mutants of *E. cloacae* or *P. aeruginosa* [20]. The rates of resistance to ceftazidime, which may be a good indicator of the presence of the stable derepressed mutants of these strains, were found to be different in various countries: 20-30% among *Enterobacter spp* in France [21], 38% in *E. cloacae* in The Netherlands [22] and 26% in Canada [21–23]. In our study, the rate of resistance of ceftazidime in *E. cloacae* strains originating from two centres was found to be as high as 77%, which was very similar to that demonstrated in Greece for the same species [12].

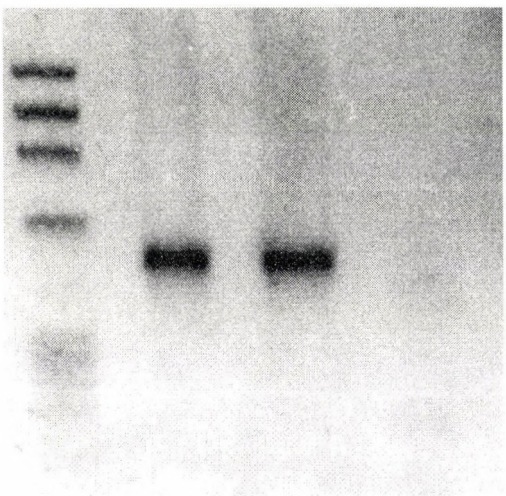

Fig. 4. Ethidium bromide-containing agarose gel showing specificity of PCR for the detection of SHV type extended-spectrum beta-lactamase. Lane 1, *ΦX*174 *Hae* III; lane 2, clinical isolate of *S. marcescens;* lane 3, SHV-2 (control); lane 4, TEM-2 (control)

The primary cause of multiresistance to beta-lactam antibiotics was the extremely high level of beta-lactamase produced by our isolates without any induction. This very high prevalence of stable derepressed mutants of *E. cloaceae* is probably to be attributed to the abuse of third-generation cephalosporins and suggests the need to create effective antibiotic policies in Hungary.

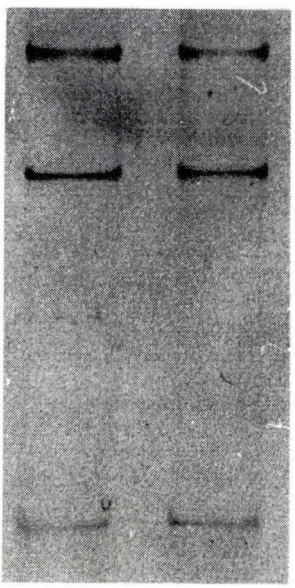

Fig. 5. SSCP analysis of the amplified SHV type gene from *S. marcescens.* Lane 1, SHV-2 (control); lane 2, S. *marcescens* (Sm1)

Extended-spectrum beta-lactamases.

After third-generation cephalosporins were introduced at the beginning of the 1980s, a report of transferable resistance to cefotaxime in clinical isolates of *K. pneumoniae* and *S. marcescens* was published in1983 from Germany [24]. Subsequently, plasmid-mediated beta-lactamases with cefotaximase or ceftazidimase activity have been reported from five continents. A great number of enzymes belonging in the plasmid-mediated TEM or SHV group have been detected so far [7]. In addition, the chromosomal *ampC* type genes from *E. cloacae*, *C. freundii* and *P. aeruginosa* have escaped onto plasmids [25, 26] and inhibitor-resistant mutants of both TEM and SHV enzymes have evolved [27]. In general, most of the TEM enzymes confer higher resistance on ceftazidime than on cefotaxime, while all SHV enzymes hydrolyse cefotaxime better than ceftazidime. As most bacteria, exhibiting ESBLs produce a low level of enzyme, they

could remain susceptible *in vitro* to cefotaxime or ceftazidime showing low MICs. The detection of ESBLs is important, and once one of these enzymes is found, the use of broad-spectrum cephalosporins should be avoided, as these agents are efficiently hydrolysed *in vivo*, at the site of infection due to the inoculum effect. For the treatment of bacteria possessing this mechanism of resistance, three alternatives exist: cephamycins (cefotetan, cefmetazole), which are not hydrolised by the ESBLs, carbapenems, which are highly stable against these enzymes, and the beta-lactam-beta-lactamase inhibitor combinations, preferentially amoxicillin/clavulanate or piperacillin/tazobactam, whereas sulbactam is a very inefficient inhibitor of the ESBLs, and especially of the SHV enzymes. However, resistance to these combinations can arise from the production of an elevated amount of ESBL by bacteria. It is also proven that the overuse of these combinations can select inhibitor-resistant (IR) variants of TEM- or SHV-type enzymes. The relatively low prevalence (5%) of IR enzymes among Gram-negative bacteria is due to the increased sensitivity of these bacteria to cephalosporins (even to cephalexin) as compared with the ESBL producers.

The prevalence of ESBL producing *K. pneumoniae* strains varies from hospital to hospital and country to country, being 9.6% on average in France, 14% in the UK and 24% in Greece [21, 28, 29]. In our study, the prevalence of ESBL-producing *K. pneumoniae* strains was found to be 9% among isolates originating from different parts of the country. Interestingly, in spite of a great number of different ESBLs, only a few of them have spread world-wide. SHV-2 has been detected in more than 10 different countries [30]. Medeiros [31] reports that SHV-5 is the most prevalent ESBL in the world. In Hungary, these two ESBL types were found among the *K. pneumoniae* strains, but SHV-2 was more prevalent than SHV-5. Marked differences were detected among the ESBL-producing strains as concerns their susceptibility to beta-lactam-beta-lactamase inhibitor combinations. Similarly as observed by others, sulbactam did not inhibit the SHV-2 and SHV-5 enzymes. Of the 15 strains 12 were susceptible to amoxicillin-clavulanic acid, but only 6 to the piperacillin-tazobactam combination.

Survey of the resistance mechanisms of outbreak strains of S. marcescens.

S. marcescens is a common nosocomial pathogen in ICUs. The strains may be susceptible to most potent antibiotics, but multiresistant clones can also be selected. The occurrence of SHV-type ESBLs among *S. marcescens* strains is rather rare. They frequently produce an inducible chromosomal Class C cephalosporinase. The amount of the enzyme encoded by the *ampC* gene and produced by the inducible strains and the stable derepressed mutants is less than in the case of *E. cloaceae* [1]. The selection of the stable derepressed mutation also takes place less frequently. During an outbreak caused by *S. marcescens* in one of our ICUs, 15 isolates (12 from patients and 3 from the environment) proved to be originated from the same clone, as confirmed by different typing methods. The only difference was that one isolate, obtained from the environment in the same period as when the outbreak was observed, was more susceptible to third-generation cephalosporins and aztreonam and did not harbour the high molecular weight plasmid. Two factors lead us to consider that SHV-2 enzyme was acquired by *S. marcescens* from *K. pneumoniae* during this outbreak: (i) the SHV-2 enzyme had been detected earlier in *K. pneumoniae* originating from the same ward as where the outbreak

was observed; (ii) one of the environmental *S. marcescens* isolates lacked the resistance plasmid, but proved to be genetically related to the other isolates, as demonstrated by the different typing methods.

During routine testing of the antibiotic susceptibilities of clinical isolates to beta-lactam antibiotics, it is extremely important to detect special resistance mechanisms, and especially those which could contribute resistance to broad-spectrum cephalosporins. Screening methods are often not enough; exact determination of the MIC values (revealing slight increases in the MICs of broad-spectrum cephalosporins) and the use of molecular biological methods to detect the genetic background of phenotypical resistance may be important for the planning of a better local antibiotic policy and for an explanation of the epidemiology of the spread of resistance.

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