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MACROETHICAL RESPONSIBILITIES OF SOCIETIES OF GYNAECOLOGISTS AND OBSTETRICIANS

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(Received: January 6, 1992)

The theme of maternal mortality and morbidity is of transcending macroethical importance. High rates of maternal mortality and morbidity can be significantly reduced by cost-effective means that are not dependent on advanced biotechnology. It has been shown that a major cause of maternal mortality comes from women (i) bearing children too early or too late in their reproductive lives, (ii) too frequently or at insufficiently spaced intervals. If women were able to control their fertility in order not to have children at unwanted times in periods of their life when pregnancy is inimical to their health, the incidence of maternal mortality and morbidity would drop. Improved standards of women's education, both in general and in particular regarding women's reproductive health, would reinforce the understanding of how to protect and improve one's reproductive health, and would accelerate the decline of maternal mortality and chronic morbidities. Accordingly, the macroethical demands of respect for autonomy, beneficence and justice would coincide. The value of justice would he served not only regarding women themselves, particularly those who have traditionally been vulnerable by virtue of their growing age, or dependent status in their communities, but also regarding the children dependent on such women, and families also dependent on the services of such women, like mothers, wives, daughters and grand-daughters.

Keywords: Macroethics, ethical principles, societics of gynaecologists and obstetricians, reproductive health

Introduction

Transcending the claims to ethical care that individual patients may properly make on their doctors are the claims that communities and nations may make on the professional societies of gynaecologists and obstetricians that practice among them. Individual practitioners who behave ethically

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towards each patient may nevertheless be collectively in breach of wider ethical duties to communities if their services are inequitably available to members of wider populations of potential patients among whom the physicians live and earn their livelihood. For instance, where particular specialists conscientiously serve the patients they accept, but decline to accept those with family income a given level, or who exceed a given parity or age, or who are under the scrutiny of third parties (such as parents), the professional societies of which such practitioners are members will be in default of meeting the ethical claims that properly may be made on them.

The ethical duties to ethical care that individual patients may properly make on their doctors are the claims that communities and nations may make on the professional societies of gynaecologists and obstetricians that practice among them. Individual practitioners who behave ethically towards each patient may nevertheless be collectively in breach of wider ethical duties to communities if their services are inequitably available to members of wider populations of potential patients among whom the physicians live and earn their livelihood. For instance, where particular specialists conscientiously serve the patients they accept, but decline to accept those with family income a given level, or those who exceed a given parity or age, or who are under the scrutiny of third parties (such as parents) the professional societies of which such practitioners are members will be in default of meeting the ethical claims that properly may be made on them.

The ethical duties to individuals are known as microethics, and are generally distinguishable from macroethics the ethical duties to communities. Microethics apply in relations between individuals, such as patients and physicians, while macroethics addresses relations between collective agents such as government departments or population groups and individuals. Macroethics accordingly raises questions of services made available by governments and of individuals' rights arising by virtue of membership of a socially classifiable category of persons. Such categories may be drawn by reference to inherent physiological features such as gender and ethnicity, while others may be constructed, such as of handicap by reference to a specific function, e.g. childbearing and status, such as being unmarried.

There is an inherent tension between micro- and macroethics, between the individual and the common good. There are no easy answers to resolve this tension. The purpose of this paper is to explain some of the macroethical challenges that face societies of gynaecologists and obstetricians

around the world in order to find an appropriate balance in the context of their communities between micro- and macroethical responsibilities.

BIOETHICAL PRINCIPLES

Three central bioethical principles, derived from the frequently invoked Belmont Report /11/, may be applied to access to reproductive health services at the macroethical level of analysis. These three principles are respect to persons, beneficence and justice.

Respect to Persons

The principle of respect to persons has at least two components, viz. autonomy, and protection of persons incapable of autonomy. Autonomy at the microethical level is reinforced by ensuring that voluntary and informed consent is obtained before a patient is treated. Disrespect of autonomy occurs when an individual chooses a method of family planning, not having been informed of its failure rate, and it fails and unwanted pregnancy results. The ethical responsibility at the programmatic level of Societies might be to ensure that accurate information on failure rates of the range of family planning methods is made widely available. For example, the rates of contraceptive failure range from 2% of women using the pill with failure during the first 12 months of use to 10% to 16% for the use of condom, diaphragm, and rhythm method and 26% for the use of spermicides /6/. Moreover, information on how failure rates vary by groups also needs to be made available. For example, the likelihood of contraceptive failure generally declines as individuals get older /4/. Fact sheets on failure rates might be developed and printed by Societies for distribution by their members and family-planning clinics.

Ensuring respect for autonomy can be disputable. Marriage is generally not a condition of personal autonomy. Some health or family planning clinics make marriage a condition of distribution of family-planning services. Accordingly, the offer of means both of birth control and birth promotion to individuals not as individuals but only as partners in marriage can be said to offend the principle of autonomy. Moreover, the law in many jurisdictions provides for elimination of discrimination on grounds, among others, of

marital status. Accordingly, the ethical principle of respect for the reproductive preferences of autonomous people should prevail, without regard to marital status.

Reinforcing respect for autonomy is the principle that autonomy should be at the maximum degree. Societies should ensure adolescents not be generically classified as incapable of autonomy, but be assessed on a case-by-case basis with regard to functioning independently in their communities.

The second component of respect for persons is protection of the vulnerable. Vulnerability can be classified by reference to a variety of criteria, such as capacity for autonomous decision-making. Traditionally, the principle of protection of the vulnerable has been applied to mentally handicapped persons to ensure that their reproductive autonomy is not violated by, for example, contraceptive sterilization. Societies can also classify vulnerable groups by reference to risk factors, such as high risk of unintended pregnancy. The sheer number of unintended pregnancies among adolescents suggests that this age group is vulnerable and in need of protection against unintended pregnancies, abortions, and sexually transmitted diseases. In the United States, for example, "1 031 000 teenagers became pregnant and of these 31 000 were younger than 15. The pregnancy outcomes included 477 710 live births and 412 617 induced abortions, the remainder were stillbirths /2/. On a world scale, yearly 15 million teenagers give birth 80% of them in the developing countries /5/.

Given the vulnerability of this high-risk group, Societies might usefully play a leadership role in developing strategies to prevent premature pregnancy and childbearing. Such strategies include: 1) developing responsible sexual behaviour, including abstinence among girls and boys /9/; 2) effective use of contraception by girls and boys /14/; 3) use of abortion to prevent untimely and irresponsible parenting /13/; 4) prevention of morbidity and mortality among young women and premature births through adequate prenatal and follow-up care of mothers /12/; 5) encouragement and support for responsible parenting among both parents /1/ and 6) sex or family life education /7/.

Beneficence

This principle goes beyond the negative medical ethic "Do No Harm", by imposing a positive duty to do good, if necessary by initiating action that will advance the welfare of individuals and communities. The ethical

principle of beneficence is sometimes expressed as the principle of utility or of utilitarianism /8/. Utility requires that actions be taken that prove useful rather than useless or dysfunctional. The concept of utilitarianism, often expressed as an obligation to seek the greatest happiness of the greatest number, reflects a major philosophical tradition of that name.

The macroethical duty of beneficence is not only to seek good, but also to actually do good. An ethical duty exists to monitor the effects of well-meaning policies to see whether they achieve the good they intend or whether they are unsuccessful or do actual harm in their consequences. A programme to assess the incidence and causes of infertility would not be sufficient to meet the ethical duty of beneficence; it would have to be followed by services to prevent and treat infertility. Moreover, the principle of beneficence compels proponents of reproductive health programme to build into their programme a capacity for continuous self-evaluation lest a programme designed to do good may inadvertently do harm to vulnerable individuals and to the community itself.

Justice

The most obvious ethical issue implicated in the principle of justice is that of access to health services. Macroethically, justice requires similar treatment for similarly situated groups. It may require, for instance, that each group collectively receives an identical allocation of resources, and be identically taxed or otherwise burdened. It may also mean, however, that each group receives services proportionate to its needs, and contribute proportionately from their means. Variants of application of the principle of justice include equal entitlements for groups of equal social merit or worth, and that groups be rewarded proportionately to the contribution they make to society.

Underlying doctrine on collective justice is a need to identify appropriate collectivities of persons. Beyond obvious collectivities identified by sex, race, religion, ethnic or national origin and, for instance, age, collectivities may be established by reference to other objective criteria. The infertile of a community are identified by a reference to a definition of infertility that may be derived from medical, social or, for instance, demographic sources. Those so found to be infertile may seek clinical care, but the collective interests of an infertile population will

require preventive services, therapeutic services, access to assisted reproduction and access to alternative legal possibilities, such as adoption, to rear children in their families.

The right not to be victimized by discrimination on account of the common feature may find expression in legal as well as macroethical practice. The ethic of justice that condemns discrimination on grounds of sex would require that both women and men have equal access to family planning. As a result, family planning services that do not provide services for men would violate the principle of justice. Similarly women who are denied access to family planning services because of a requirement that thely first obtain their husbands' authorization would not meet the principle of justice if husbands enjoy autonomous access.

In addition to the ethic of sexual nondiscrimination there is a widely recognized principle in international and national human rights codes of nondiscrimination on grounds of handicap or disability. In the same way that health services are available to assist reproductively functional people to achieve their reproductive goals, services may be claimed by the infertile; not simply clinical services to overcome their disability if possible, but also to services that offer alternative options to being equipped for natural reproduction.

ETHICAL ALLOCATION IN PRIVATIZED AND SOCIALIZED SYSTEMS

The growing sensitivity to bioethical issues and concerns that developed in the 1960s and 1970s was conditioned essentially by technological developments. The development of machines, drugs and vaccines affecting the health of patient populations and the delivery of health services to communities was recognized to have a social as well as clinical dimension. More recently, however, bioethical concerns are being driven by economic scarcity. To the question of who gets health care is being added the question of who pays, and who bears the sacrifice of foregoing care in order to make scarce resources available to others. The distribution of inadequate resources to unlimited demands is a central issue in the science of economics, and increasingly the question of who should pay for services is being related to the question of who benefits from them.

The professional duty to allocate scarce resources equitably, including limited resources of time, space, beds and equipment, is particular-

ly demanding where professionals operate under legally created and protected monopolies. While claiming to serve the public interest in the provision of specialist services through appropriately trained and conscientious practitioners, professional licensing agencies define who may undertake professional services, and may function with the effect of precluding from practice alternative sources of services that potential patients would find satisfactory. Preservation of excellence of services to patients may be at the cost of convenient and affordable access to services. Accordingly, licensing authorities and professional societies of medical specialists committed to the provision of good-quality health care must take into account not only standards of clinical care but also standards of availability of, and access to, services.

Countries respond to issues of the cost, benefit and distribution of services differently. Socialized health-care systems are conditioned by the belief that it is the society at large that benefits from an improved health care among its members, and that it is again the society at large that should pay the costs. Services are distributed according to medically determined need, and are funded according to economically determined means, economic policies aimed at preventive misuse of public health resources and rewards for economic use. Private health care systems make services available for those who wish to receive them and have the means to pay for them. Some participants in societies with such systems who lack personal means such as private insurance and lack provision of services through a third party pay or such as an employer or specialist public service (for instance for military veterans) may forego services they wish to receive which are medically indicated for them.

Both socialized and privatized health care systems present macroethical challenges to societies of gynaecologists and obstetricians. In the analogy of the medical lifeboat, privatized systems show some people safely aboard, but others struggling for admission and at risk of drowning before help comes. In socialized systems, everyone is aboard, but provisions may be low and some in the lifeboat may perish. In both systems therefore critical questions of allocation arise, in privatized systems to make some level of service available to the otherwise unserved, and in socialized systems to make an equitable level of service available to claimants who in principle rank as equal.

Socialized systems face the question of which of the governing principles should determine the allocation of public resources. The goal of

awarding priority to the more sick over the less sick may seem equitable in that in matches resources to needs. In terms of effectiveness of use of resources, however, this may be dysfunctional, in that resources are allocated to those less likely to recover and resume lives as productive members of society. As a result, resources are denied to those more likely to recover until their conditions and prognoses deteriorate to the point where they join the more sick who will be less likely to recover. Resources may accordingly be allocated to serve the outcome of maximum recovery, but at the sacrifice of more seriously ill patients who may be afforded comfort measures but minimal medical care.

Another basis of resource allocation in socialized systems is to prioritize less costly treatments over more costly, and to embloy resources in preventive rather than curative programmes. The result may be to invest in high-cost technologies, sparingly and to use specialists to plan and execute mass educations and training programmes, difficult to achieve where the levels of literacy are low. Indeed, promotion of literacy may be considered a proper charge on the health budget either to facilitate individual self-help of to train auxiliary health professionals to deliver basic services throughout the population.

The allocation of resources according to the principle of achieving maximum reduction of health risk factors includes both preventive and curative aspects of health care. The principle depends, of course, on reliable identification of the factors constituting risks to the relevant health status (for instance, affecting reproductive health) of target populations, and initial allocation of resources might be devoted to this end. Epidemiological studies might be required, reinforced perhaps by acquisition of anecdotal case studies, from health professionals and self-report studies, from patients who would be subject to interpretation by professionals in appropriate disciplines and specialists in cultures of study populations.

It may be anticipated that a major part of resources devoted to risk reduction would be given to programmes of preventive care, including education through appropriately communicated information and explanation, and removal of physical and, for instance, iatrogenic and even nosocomial sources of risk. In addition, however, resources might have to be given to the cure of medical disorders which caused or preconditioned secondary risk to the health of affected patients. For instance, the cure of venereal disease should both reduce the spread of infection and protect the affected patient against the risk of consequent infertility.

Privatized health systems raise the question of whether the scarce resource of professional time should be given to meeting patients' wishes to indulge themselves with costly treatments unneccessary for their basic health care and that are unrelated to risk factors and to obtaining and using costly equipment the beneficial effects of which are unproven or in the context of communal health standards marginal.

An equally pressing, often more pressing, question is how members of a medical specialty can render services to those who cannot pay. An equilibrium may arise if practitioners supply services to fee paying patients and therapy obtain resources to subsidize services to those who need them but cannot pay. Professional societies may, for instance, make it a condition of membership that members render a proportion of their time and facilities available to meet the needs of indigent patients. Similarly, private hospitals or clinics may give a proportion of their services and facilities to needy patients unable to pay for them. Such services may be considered to be given as charity rather than in fulfilment of professional macroethical duties, but microethics require that the autonomy of patients be given maximum respect, and that patients' dignity never be compromised.

A restriction of privatized health systems is that in themselves they do not equip health professionals (particularly when paid on a fee-for-service basis) to undertake preventive communal health care, although individual preventive care may be undertaken. Under such systems, however, governments may be willing to support preventive health programmes as part of public health care or as a contribution, for instance, to maintaining employment, educational, and maternal and child health standards.

Experience indicates that many health systems are neither purely socialized nor purely privatized. Socialized systems often accomodate private-practice-supplying additional or competing services for patients who wish to use them and can afford to with privatized systems often include public provision of services for such populations as the needy elderly and the deprived young. Accordingly, the macroethical challenges associated with socialized and privatized systems may both be faced in mixed systems, and the interaction of, and balance between, socialized and privatized elements of a mixed system may generate macroethical problems of their own. For instance, private beds in public facilities may subsidize the rich and diminish resources available for those who depend on public provision, and inaequate public provision of services may be protected or concealed where people of modest means feel compelled to strain their resources to purchase

services in the private sector rather than incur delays in access to public facilities.

An additonal problem, experienced by individual physicians but affecting public investment in health services, is whether they are willing and able to prescribe treatments for individual patients according to their availability, or whether they will prescribe unavailable resources and require patients to press the political system to increase the supply of resources. Physicians may conceal macroallocation judgements whithin individual prescriptions and prognoses, maintaining the status quo of perhaps inadequate public provision of resources, or prescribe and make prognoses without regard to public provision of services, thereby forcing some patients into the private sector or provoking medical professional conflict with the government.

REPRODUCTIVE HEALTH CARE

The preconditions to macroethical planning and pursuit of reproductive health care are the development and the maintenance of epidemiological and related information relevant to the reproductive health status and risk factors of a target population. The sponsorship of studies that generate such information might be the responsibility of governments, but alternative sources of support and leadership might be available through universities, university-based medical centres and independent hospitals, and clinics. The latter may be unable to support more than modest studies of the population within their own catchment areas. Societies of gynaecologists and obstetricians could offer encouragement and leadership at many levels, for instance to press for and facilitate national or regional studies, to recommend hospital-based and clinic-based studies by their members, and collaboration by hospitals and/or clinics within a region pooling their data, and, for instance, providing standardized means and expert assistance that may be applied to the task of completing relevant epidemiological and associated studies.

Many factors contribute to maternal mortality and chronic morbidity. Some factors combine with others to compound the risk of death or disability faced by pregnant women. Obstetric, health service and reproductive factors may be isolated and treated in sequence for purposes of presentation, but in practice such factors my interact, coincide, or arise from common origins.

Moreover, these factors are only the medical factors and do not necessarily include social factors, such as low-status of women, that can compound the medical risk factors, leading to higher rates of maternal mortality and morbidity.

Obstetric Factors

Maternal death and injuries are usually divided into three categories: (i) "direct" obstetric deaths and injuries — those resulting from complications of pregnancy, delivery or their management; (ii) "indirect" obstetric deaths and injuries — those due to other medical factors that were aggravated by pregnancy, for example, heart disease or anaemia; and (iii) unrelated deaths and injuries — fortuitous deaths and injuries suffered by a woman while pregnant, for example, from accidents.

The major causes of direct obstetric deaths and injuries, particularly in the developing world, are haemorrhage, infection and toxaemia (pregnancy-induced hypertension or high blood pressure of other origin. Other causes of obstetric death and injury include inskilled abortion, obstructed labour due, in part, to a deformed pelvis which can arise from chronic malnutrition, and a ruptured uterus. Some of these factors will contribute, more than others, to maternal mortality and morbidity.

Responses to these factors that may be undertaken by Societies include advancing the means to identify high-risk pregnancies and to reduce the rate of associating complications. Societies might develop programmes to educate their members in these areas and also to warn women and couples of indications of high risk and of the physical and life-style changes they might pursue that would reduce the risk. Similarly, the identification of medical factors that make pregnancy contraindicated or that are aggravated by pregnancy might be undertaken, and identified factors might form the basis of professional and popular education programmes. In addition, practitioners of other medical specialities might be offered means of education on the harmful effects of their studied pathology on pregnancy, and of pregnancy on the pathology in which they specialize. Moreover, Societies might institute training programmes to make treatment methods least damaging to the physical and mental health of the women popular.

Health Service Factors

Health services can be deficient in many respects. They can be unavailable or deficient in certain areas of a country. Women can lack access to services for a variety of reasons, including structural problems such as lack of roads or legal or cultural problems, such as the requirement of husbands' prior authorizations for distribution of contraceptive services to their wives. Health personnel can be inadequately trained or lack necessary supplies and equipment to provide adequate services. Particularly in developed countries, fear of liability to malpractice suits may cause gynaecologists and obstetricians to reduce their obstetric practice and limit themselves to the rendering of gynaecological services.

Societies might take leadership initiatives to assess the health service factors that are most directly related to excessively high rates of maternal mortality and morbidity, and advocate necessary changes to reduce the risk factors associated with inadequate health services.

Regarding reduction of obstetric services due to fears of litigation, for instance, Societies might provide expert witnesses to assist courts to render just decision. Moreover, Societies might participate in legal and judicial education programmes showing that advanced electronic monitoring techniques would not reduce the risk of maternal mortality or morbidity or of stillbirth or neonatal injury, and that the prevailing standards of professional practice do not require employment of theoretically, or expensively available high-technology-based care. Societies might establish that not everything that might be used must be used in order to satisfy legal and/or ethical standards, and that macroethically the excellent should not become an enemy of the good or adequate, or an enemy of wide access to sound care. That is, societies need to ensure that standards are developed for the health of their patients, not simply in order to defend their members in court.

Reproductive Factors

It is well known that the risks of morbidity and mortality associated with pregnancy are greater for women in the following categories:

+ women less than 18 years of age,
+ women 35 years or older,
+ women whose last births occurred in the preceding 24 months,

+ women with limited access to reproductive health services, such as those who live in rural areas, and

+ women with unwanted pregnancies liable to end in unskilled abortion.

Societies might assess which categories of women in their countries and regions of their countries are at greatest risk of maternal mortality and morbidity due to these conditions, and institute professional and popular programmes to reduce the number of high-risk pregnancies and, for instance, to advocate the introduction of programmes in areas where services are unavailable or inadequately available.

Conclusion

The above are only some examples of the ways in which societies of gynaecologists and obstetricians might begin to face macroethical challenges. The potential of Medical Societies as professional groups to utilise their positions of influence on improving reproductive health in their countries is significant. Medical societies can lead the way by proactively using their professional positions to explain and direct what programmes would maximile the reproductive health of their communities, rather than only be reactively responding to misconceived governmental and other initiatives. Medical Societies might ask themselves what more they can do to advocate and facilitate reform in services, laws and, for instance, cultural or religious practices that would reduce the risks associated with obstetrics, health service and reproductive factors that prejudice reproductive health.

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CLINICAL UTILITY OF QUANTITATIVE ASSESSMENT OF LIVER HAEMODYNAMICS IN CIRRHOSIS PROVIDED BY DYNAMIC HEPATOSCINTIGRAPHY

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Interrelationships between quantitative assessment of portal (%Qp) and arterial (%Qa) components of hepatic blood supply obtained by dynamic hepatoscintigraphy, and clinical variables characterizing the severity of liver cirrhosis and portal hypertension were studied in 25 cirrhotic patients. The variables, clinical state, size of oesophageal varices, ascites accumulation, sonographic stigmata of portal hypertension, liver mass and elimination rate of lidocaine and antipyrine were studied. The %Qa rose in proportion to the severity of liver injury estimated from the Child-Turcotte and McCormick grading scores. The mean %Qa for patients with Child A cirrhosis was significantly higher than that for 8 healthy subjects ($34.8 \pm 7.9\%$ vs 18.1 ± 4.0; $P < 0.01$). The %Qp values showed relationship with the size of esophageal varices, provided discriminatory data with respect to the ascitic fluid accumulation and the development of intraabdominal collateral circulation. The liver mass had no impact on hepatic dual blood supply pattern, but was linked with the rate of antipyrine clearance. Neither antipyrine clearance nor lidocaine elimination rate corresponded to alterations of hepatic dual blood supply. The %Qp showed a negative correlation with the initial half-life of lidocaine, which was referred to lowered hepatic uptake of the drug. It is concluded that the quantitative assessment of %Qp and %Qa reflect the advancement of portal hypertension better than liver function failure does.

Keywords: Hepatoscintigraphy dynamic, liver cirrhosis, portal hypertension, drug metabolizing capacity

Introduction

Hepatic blood flow is of importance for the function of the liver; it may have pathophysiological as well as clinical-pharmacological implications. The sinusoidal hepatic blood is a mixture of hepatic arterial and

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portal venous blood. About two-thirds of the blood is normally supplied by the portal vein while in cirrhosis the hepatic artery is the predominant source of blood supply /19/. The cirrhotic pattern of hepatic dual blood supply is attributed to augmented vascular resistance within regenerative nodules, increased hepatic artery flow and, chiefly, to decreased portal inflow referred to portal-systemic collaterals /11/.

The quantitative assessment of arterial/portal contribution to total hepatic blood flow (HBF) can be obtained by an analysis of first pass flow studies through the liver using radionuclides which are not trapped by this organ on its first pass 64/. This method was shown to be reproducible and to correlate well to the clinical state and splanchnic vascular abnormalities /18, 21/. Furthermore, the dynamic intravenous hepatoscintigraphy appeared to be a valuable tool in diagnosing portal thrombosis /18/ or qualifying patients to shunt surgery /1/. Nevertheless, the clinical importance of determining the portal/arterial HBF has received scant attention.

In chronic liver injury, a continuous loss of functional integrity and reduction of parenchymal mass is closely associated with vascular reconstruction within and beyond the liver. Hence, in the present study we investigated the relationships between haemodynamic parameters derived from dynamic hepatoscintigraphy and clinical parameters characterizing the liver function and portal hypertension.

Patients and Methods

The present study was approved by the local Research Ethical Committee.

We have studied 8 healthy male subjects and 25 patients (14 males, 11 females), aged between 28 and 70 years, suffering from liver cirrhosis of various aetiology, 7 alcoholic, 12 posthepatitic, 2 primary biliary and 4 cryptogenic cirrhosis. The diagnosis of cirrhosis was histologically confirmed in 14 cases, the remainder were clinically and biochemically evident. The clinical state of cirrhosis was assessed by the combined clinical and laboratory criteria of the Child-Turcotte /6/ and the McCormick /17/ classifications.

Radionuclide study

For radionuclide angiography, patients were positioned supine beneath the gamma camera Toshiba, GCA 202 interfaced to the digital computer Trinary HC-3200, including in the field of view of heart, abdominal aorta, liver, spleen and kidneys. The patients were allowed to relax for 15 min prior to scintigraphy. Sodium phytate (IEAiR Świeck, Poland) labelled with 10 mCi (370 MBq) of ^{99m}Tc pertechnetate was injected through a polyethylene catheter into the left antecubital vein as a 1 ml bolus, followed immediately by 20 ml of saline in order to ensure rapid transit of the radionuclide into the heart.

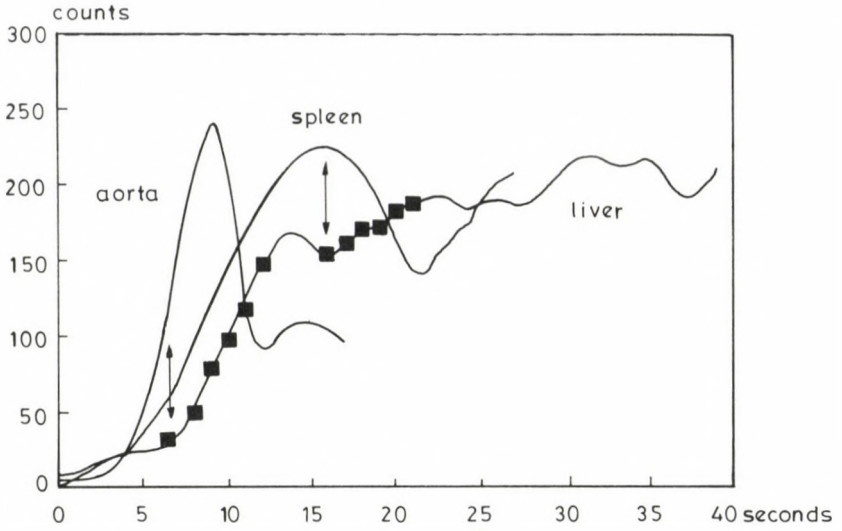

Fig. 1. Handling time/activity curves drawn for liver, spleen and abdominal aorta regions of interest. Double-head arrows mean the beginning points of arterial and venous influx to the liver. Solid squares mean the time intervals of hepatic curve taken to calculations

Images were acquired into a $64^{\times}64$ matrix at rate of 1 s image for 80 s. Computer acquisition was commenced with the onset of the injection. Time-activity flow curves were generated over the right hepatic lobe, excluding its medial and inferior part, and over the abdominal aorta, spleen and right kidney. Original curves were smoothed and divided by the number of pixels of its own region of interest. Hepatic curves were analyzed in arterial and portal compartments, each of 6 s duration (Fig. 1). The onset of the arterial hepatic inflow was defined as the first point on the hepatic curve following 25% peak radioactivity over the abdominal aorta. The onset of the portal venous inflow was set at the peak of the splenic curve, or in the kidney curve, if the splenic peak was blurred (5 cases). Percentage rates of arterial (%Qa) and portal (%Qp) flow to the liver were calculated according to Sarper /20/.

$$\%Qa = ka/(ka + kp)^{\times}100$$

$$\%Qp = 100 - \%Qa,$$

where ka and kp are respective constant rates of arterial and portal segments of hepatic time-acitivity flow curve fitted with linear function.

The liver mass was estimated from the right lateral hepatic area measured by computer according to Eikman's formula /8/.

The dynamic hepatoscintigraphy was tested for interobserver error i.e., a second observer unaware of the clinical data extracted all measurements independently. The coefficient of variation for the %Qa measurement was 7%. The reproducibility of the method expressed as relative difference between two successive %Qa measurements performed in the same subject (4 healthy probands) 7 days apart ranged from 4.5% to 12.4%.

Clinical studies

All patients with liver cirrhosis were examined by both sonography and oesophagogastroscopy. Oesophageal varices were graded according to classification set by the Terminology Committee of the World Society of Digestive Endoscopy-OMED, depending on the degree of protrusion of the varix into the esophageal lumen and noted 0 for none present to grade IV for the most severe /16/. Criteria for the diagnosis of variceal bleeding was preexisting haematemesis or melaena in patients with varices without other potential sources of blood loss.

Sonography examination of the portal vein and its related structures was carried out using a real time sonography equipment -Sigma 20, with a linear transducer of 3.5 MHz frequency. Patients were fasted overnight and the following measurements were taken during suspended inspiration: the largest diameter of the portal vein, and the transverse and longitudinal diameters of spleen. Scanning for collaterals around the spleen, pancreas, stomach, and gallbladder, and along the splenic and portal vein (cavernous transformation) was made. For purposes of this investigation a portal hypertension score was developed and designated the "Sonoscore". One point was scored for each of the following: (a) portal vein larger than 1.3 cm, (b) splenomegaly (the upper limits for longitudinal and transverse diameters were 12.8 and 4.0 cm), (c) clear presence of abdominal collaterals in at least one vascular bed. In case of the sonographic image being obscured by ascitic fluid, the examination was performed after peritoneal puncture. The degree of ascites was graded from 0 to 3+; 1+ indicates sonography detection of liquid exclusively, 2+ means clinically detectable ascites with protrusion of the umbiliculus, 3+: tense ascites.

Pharmacokinetic studies

Patients received a single 1000 mg dose of antipyrine (Phenazone, Sigma) orally in the fasting state. A venous sample of 5 ml was obtained in a heparinized tube 24 h after antipyrine ingestion. Antipyrine concentration was measured by gas chromatography /12/. The simplified one sample antipyrine clearance (Cl_A) was calculated from /7/.

$$Cl_A = [\ln (d/Vd) - \ln c]/t_{24} \times Vd,$$

where d is the dose of antipyrine given and c is the antipyrine concentration at a single time of sampling (t_{24}). The volume of distribution (Vd) was estimated on the basis of body weight, height, age and sex /3/.

Eleven patients were given lidocaine (Xylocaine 2%, Astra) intravenously in a dose of 50 mg. Venous blood was drawn from the antecubital vein through a polyethylene catheter 5, 15, 30, 60, 120 and 240 min after the administration of lidocaine. Serum lidocaine content was assayed by gas chromatography /14/. Lidocaine concentration-time data were plotted on semilogarithmic computer display graph for each subject. The elimination rate constants for the initial phase (k) and terminal phase (β) were calculated by means of least squares regression using the data points lying on the initial and terminal straight-line portions of the graph. The area under the curve (AUC) was calculated from time zero to the last sample time whereas half-lives (T2) for both phases were calculated by dividing ln 2 by k and β, respectively. The data are presented as mean $\pm$ S.D. The non-matched two sample Mann-Whitney test and linear regression were used in the statistical analysis.

Results

Figure 2 shows the %Qa results as a function of the patient's clinical state. The %Qa results for healthy subjects ranged from 12.8% to 23.3% and were significantly lower than the %Qa for mild (grade A) cirrhotics

Subjects	N	mean ± S.D.	% arterial flow 0 50 100
Normals	8	18.1 ± 4.0	
grade A	7	34.8 ± 7.9	
Cirrhotics			
grades B+C	18	57.4 ± 17.1	

Fig. 2. Arterial contribution to hepatic blood supply in healthy controls and cirrhotics, subgrouped according to the Child-Turcotte grading score

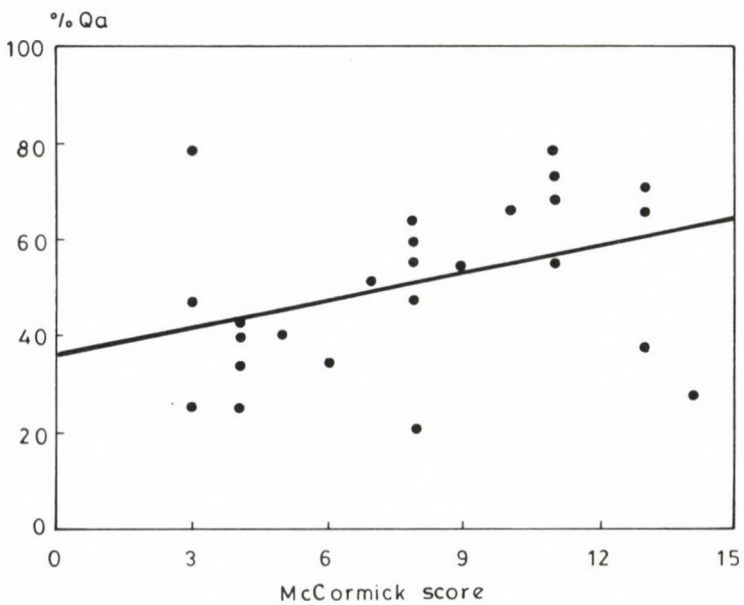

Fig. 3. Relationship between clinical evaluation of cirrhosis by McCormick grading score and arterial contribution to hepatic blood supply

(18.1 ± 4.0 vs 34.8 ± 7.9%; $P < 0.01$). The patients with severe cirrhosis (grade B+C) presented significantly lower %Qa ($t = 2.78$; $P < 0.02$) then those with mild cirrhosis. The measurements did not provide significant separation between grade B and C (56.9 ± 17.2% vs 58.5 ± 16.7%). Figure 3 correlates %Qa results with cirrhotic gradings according to McCormick, including in contrast to the Child-Turcotte classification portal hypertension stigmata like oesophageal varices, splenomegaly, collateral abdominal veins and hydrothorax. The comparison of %Qp due to ascites accumulation and "Sonoscore" result is presented in Tables I and II.

The mean %Qp results subgrouped by the endoscopical degree of oesophageal varices are depicted in Fig. 4a. The %Qp for patients with absent or

Table I

Portal venous contribution to HBF (mean ± S.D.) subgrouped according to the degree of ascites accumulation

Ascites[a]	n	%Qp
—	10	59.2 ± 12.9
+/++	9	44.0 ± 19.5
+++	6	39.3 ± 15.1‡

[a] for symbol see "Patients and Methods"
n = number of patients

‡ significantly lower than for patients without ascites ($t = 2.24$; $P < 0.05$)

Table II

Portal venous contribution to HBF (mean ± S.D.) subgrouped on the basis of "Sonoscore" result

Sonoscore[a]	n	%Qp
1	6	67.0 ± 10.6
2	7	59.3 ± 9.8
3	12	33.9 ± 11.2‡

[a] Sonoscore criteria see "Patients and Methods"
n = number of patients

‡ significantly lower than for Sonoscore 1 ($t = 3.18$; $P < 0.01$) and Sonoscore 2 ($t = 3.21$; $P < 0.01$) groups

Variceal grade	N	mean ± S.D.	%portal flow 0 50 100
0 1	9	66.8 ± 9.0	
II - III	13	43.1 ± 16.3	
IV	3	28.7 ± 8.5	

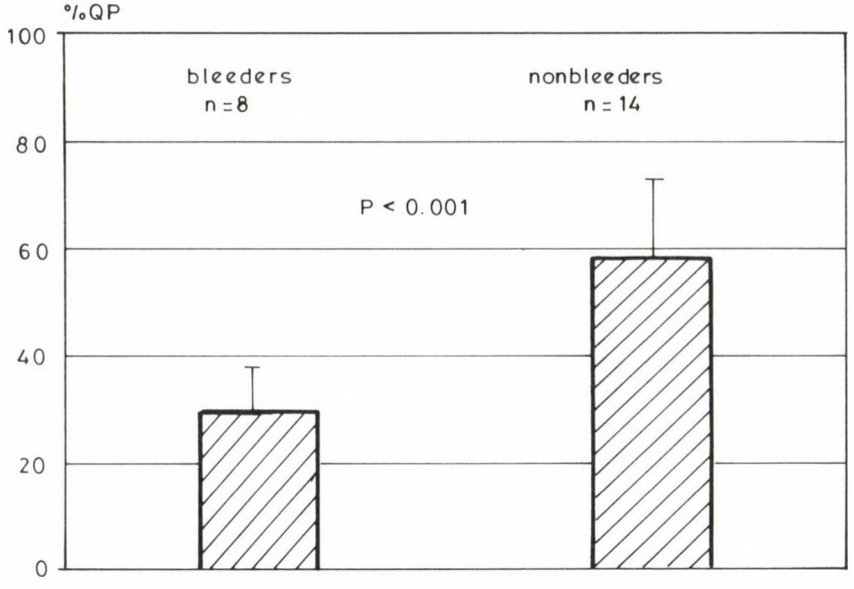

Fig. 4. Portal venous contribution to hepatic blood supply as a function of; (a) endoscopical grade of esophageal varices and (b) occurrence of gastrointestinal bleeding (mean + S.D.)

I-degree varices was 66.8 ± 9.0%; it was found to be significantly higher than the %Qp in groups of patients with larger varices. The comparison of %Qp between bleeders and nonbleeders is shown in Fig. 4b.

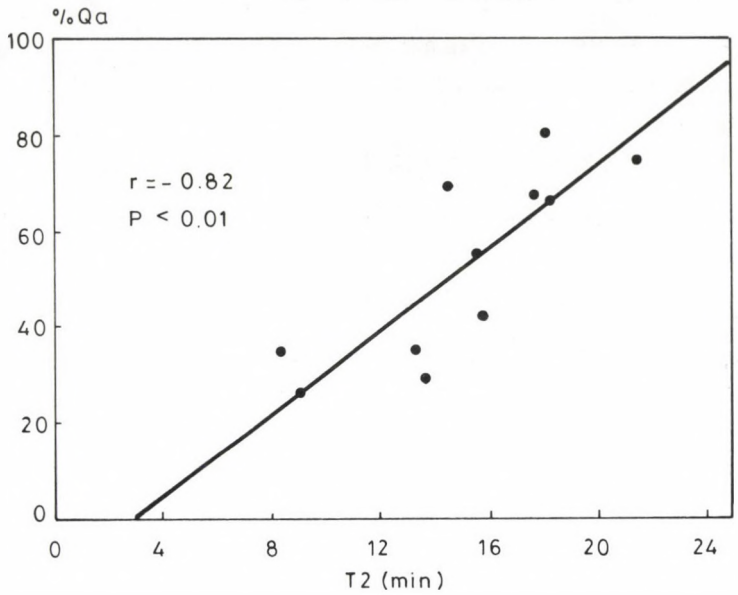

Fig. 5. Relationship between lidocaine half-time (T2) in the initial phase of systemic elimination and the arterial contribution to hepatic blood supply

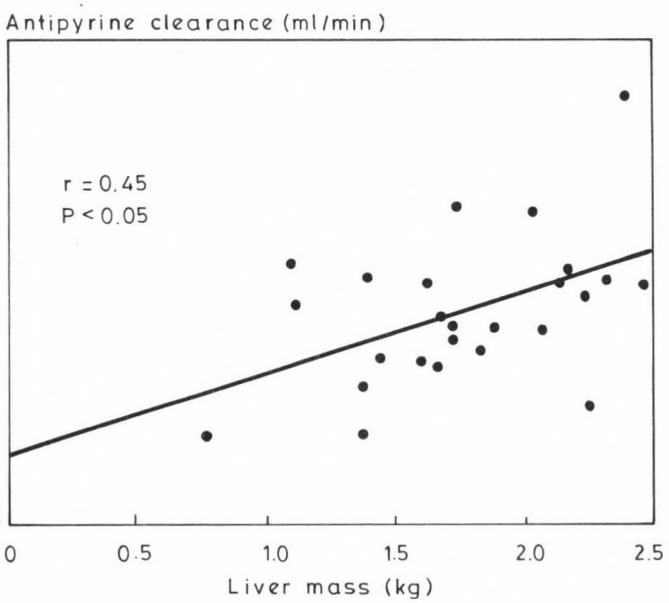

Fig. 6. Relationship between antipyrine clearance and calculated mass of the liver

The liver mass ranged from 0.80 to 2.46 kg (mean value: 1.76 ± 0.42 kg) and showed no relationship with blood-flow estimates. The mean initial and terminal-phase T2 of lidocaine elimination were 15.1 ± 3.8 min and 215.7 ± ± 135.8 min, respectively. Terminal-phase T2 of lidocaine elimination did not correspond to quantitative haemodynamic parameters, whereas initial-phase T2 of lidocaine showed positive correlation with %Qa (Fig. 5). Antipyrine clearance ranged from 7.8 $ml^{*}min^{-1}$ to 37.8 $ml^{*}min^{-1}$ (mean value was 18.7 ± 6.4 $ml^{*}min^{-1}$) and was related to the liver mass (Fig. 6) but did not correlate with quantitative haemodynamic estimates ($r = 0.10$, $P > 0.05$).

Discussion

It may be assumed from our study that the dynamic hepatoscintigraphy based upon measurement of the slopes of the hepatic flow curve provides certain differentiation between healthy subjects and advanced cirrhotics. We confirm that the contribution made by the hepatic artery is considerably increased in severe forms of liver cirrhosis (grades B and C), whereas in grade A patients the portal vein is the predominant source of hepatic blood supply. Nevertheless, not all patients with histologically proven cirrhosis showed a 30% elevation of the arterial component. This finding indicates that blood flow alterations may not occur in the early stage of this disease and precludes dynamic hepatoscintigraphy from being a diagnostic test in liver cirrhosis.

Apart from ascites, the Child-Turcotte classification does not include the criteria of portal hypertension. By contrast, among the nine criteria of McCormick classification five are directly attached to haemodynamic sequelae of portal hypertension. Most of the %Qa measurements exhibited clear correlation with McCormick score, however there were four values being far beyond 95% confidence limits of linear regression giving rise to a suggestion that in some still undefined conditions the clinical advancement of portal hypertension is not followed by parallel arterialization of hepatic blood supply. More detailed studies of this problem are warranted.

Both classifications of severity of liver injury derive from general clinical experience and are recommendable for prognostic purposes. Hitherto, the relative portal/arterial pattern may be viewed as a single clinical estimate of prognostic significance. In accord, the ascites accumulation which was recognized an independent prognostic factor /2/ correlated well with the portal contribution to HBF.

The portal/arterial HBF is clearly associated with unremittingly high portal pressure. The prevalence of extrahepatic collaterals was evaluated by combined sonography and oesophageal endoscopy examinations. The size of oesophageal varices was clearly related with the magnitude of %Qp. Furthermore, the lowest %Qp values were found in cirrhotics with a history of variceal bleeding. This finding is in agreement with the evidence that large varices remain the most likely to bleed /5/. Varices do not constitute the major route within collateral circulation, however they represent an indicator of generalized development of portal-systemic circuit. Similarly, the %Qp values effectively subdivided the patients according to extrahepatic collaterals detected by sonography. The patients with apparent portal hypertensive markers had the lowest %Qp, which on the average was the half of the %Qp found in patients with mild or absent collaterals. This findings points out that only large-size vascular channels, constituting significant pathways into systemic circulation are visualized in routine sonography.

Cirrhotic liver may be shrunken, normal sized or enlarged, hence, the total cross area of intrahepatic vasculature can differ from patient to patient. The influence of hepatic volume on early survival time after shunt surgery had been stressed /15/. In the current study the liver mass estimated from lateral hepatic scan image showed no relationship with liver haemodynamics. This finding seems surprising as liver volume in cirrhosis is also determined by the extent of fibrosis which promotes an increase in intrahepatic vascular resistance.

The liver plays the principal role in disposition of drugs and half-lives of these are generally prolonged in cirrhosis. The drug elimination capacity by the liver cannot be predicted on the basis of known laboratory tests. We attempted to ascertain whether a loss of hepatic vascular integrity is linked to a significant impairment of hepatic drug elimination. In a former study /9/ we found that propranolol bioavailability changed inversely to portal contribution to HBF. Now, we have studied elimination rates of two drugs extensively cleared by the liver, prototypes of low and high extracted compounds /13/, respectively.

Antipyrine clearance was related with the liver mass, but not with the Child-Turcotte score and hepatic dual blood supply. Similarly, lidocaine elimination rate did not correspond to the measurements of portal/arterial HBF. It cannot be answered on the basis of the present data whether lidocaine elimination followed the changes in total HBF, however it has recently been shown /10/ that in late cirrhosis the magnitude of HBF is of little

importance to systemic lidocaine clearance. Generally, it should be rather assumed that in cirrhosis the hepatic metabolizing capacity, and not vascular derangements is the rate-limiting factor to clearance rates of both drug categories.

Surprisingly, the %Qp corresponded to the half-life of the initial elimination curve of lidocaine. This time interval ranging in our study from 8.3 to 21.6 min, chiefly reflects compartmental distribution, although the calculated volume of distribution was unrelated to %Qp (data not shown). The liver may be considered a large compartmental space in lidocaine distribution, access to which depends on the efficiency of hepatic extraction. On the other hand, it is known that in cirrhosis hepatic uptake of drugs may be affected by sinusoidal capillarization /22/. So, it is tempting to postulate a linkage between the arterial blood supply and the efficiency of lidocaine hepatic extraction.

Up to now, there is no direct method measuring portal venous and hepatic arterial blood flow separately. Dynamic hepatoscintigraphy permits the quantitative separation of the portal from the arterial blood supply at the hepatic level. The results obtained by this method reflected the clinical state of patients with liver cirrhosis corresponding well to clinical classifications of the severity of liver injury, and, in particular, giving a considerable insight into the haemodynamic consequences of portal hypertension. In contrast, portal/arterial HBF assessment was not sensitive enought to diagnose liver cirrhosis and did not allow the evaluation of hepatic drug metabolizing capacity.

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DIPYRIDAMOLE THALLIUM-201 SCINTIGRAPHY IN PATIENTS WITH ARTERIOSCLEROSIS OBLITERANS. INCREASED ACCURACY IN IDENTIFYING CARDIAC RISK

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Forty-eight preselected patients (pts) with arteriosclerosis obliterans were investigated by dipyridamole thallium scintigraphy (DTS). No correlation was found between the distribution of positive or negative exercise ECG testing (ExECG) and isotopic risk-scores ($P > 0.1$ in the chi-square test). We assessed cardiac ischaemia in 12 pts with insufficient ExECG. Although only 2 pts had documented previous myocardial infarction, 20 pts exhibited irreversible perfusion defect. Silent reversible or irreversible ischaemia was identified in 12 pts (25%). Seven pts would not have been diagnosed to have coronary artery disease (CAD) even by ExECG. In conclusion, DTS was found very useful in these cases. We support a stepwise cardiac risk stratification before major vascular surgery.

Keywords: Arteriosclerosis obliterans, coronary artery disease, cardiac risk stratification, dipyridamole thallium scintigraphy, exercise stress ECG testing

Introduction

The cardiac risk of peripheral vascular surgery is increased because of the high incidence of ischaemic heart disease in this patient population /9/. Besides, the clamp of the aorta elevates the afterload that can cause an extreme burden on the heart. The release of compression usually results

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Abbreviations: AMI: Acute myocardial infarction, CAD: Coronary artery disease, ExECG: Exercise stress ECG testing, DTS: dipyridamole thallium scintigraphy, G: grade, PD: perfusion defect, Pts: patients, Rd: redistribution, Tl-201: Thallium-201 chloride

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in a drop of blood pressure /2/. There are two possibilities to decrease the risk of perioperative cardiac events (unstable angina, heart failure, myocardial infarction, sudden death): (i) previous coronary revascularization if necessary, (ii) and/or invasive haemodynamic monitoring during the vascular operation /6/.

Therefore, the diagnosis of ischaemic heart disease is an important task of the preoperative medical consultant /10/. However, the diagnosis is made difficult by the poor exercise tolerance of these patients (pts) /5/. Angina occurs less frequently than significant coronary stenoses, and the physical stress ECG (ExECG) is of less diagnostic value. For this reason, Hertzer et al. suggested routine coronarography before lower limb vascular surgery /9/. Recently dipyridamole Thallium-201 scintigaphy (DTS) seems to be a reliable heart screening test to establish the relative operative risk /3, 16/.

In this study, we made efforts to obtain additional information from DTS beside ExECG.

Patients and Methods

Forty-eight patients (pts) (40 men and 8 women) were studied. Mean age: 55.6 ± 8.5 (40—77) years. All of the pts had documented peripheral occlusive arteriosclerosis. The pts were examined by an experienced vascular surgeon and a cardiologist. Among others, leg Doppler index measurements, rest ECG and in 44 cases multistep graded, moderate bicycle exercise ECG testing (ExECG) were performed. The ExECG was considered to be (1) positive if 1 or more mm of ST segment (downsloping or flat) depression or elevation was induced; (2) inconclusive if the exercise was stopped due to claudication at a lower level than target heart rate, and no conclusive ECG finding culd be seen; (3) negative (if neither of these criteria were fulfilled).

For the radionuclide studies pts were chosen mainly on the basis of clinical and ECG findings: those with angina and/or positive or inconclusive ExECG were preferred. The scintigraphy was carried out after taking informed consent, generally in fasting state. We infused 0.56 mg/kg dipyridamole (Persantine; Thomae GmbH) over 4 minutes. Serious complication occurred in one occasion, in the form of transitory symptomatic drop of blood pressure.

After 3 min delay 65 MBq Tl-201 chloride was administered intravenously. Within ten min a 3-view acquisition had begun (LAO, A, L-70) on a gamma camera (Gamma MB 9200) equipped with an all-purpose, parallel-hole collimator. Besides X-ray film, 8 min-pictures were obtained on a computer (DIAG), too. We took redistribution pictures after 3 h in rest.

Images were interpreted by two observers unaware of clinical and ECG data. Visual analysis, modified interpolative background subtraction, digital display, circumferential profile curve generation and comparison with normal range were made. We considered an investigation to be positive for perfusion defect (PD) if at least 18 degrees of the profile curve were under the normal range (mean value ± 2 S.D.). We assorted the redistribution (Rd) as complete, partial or reverse (if the PD appeared or was more conspicuous on the rest images). We used a four-grade (G) score to characterize the severity of PDs: 0: no PD; I: fixed PD; II: reversible PD in one region; III: multiple PDs (in two or more coronary territories with Rd in at least one of them).

For statistical analysis the chi-square tests were applied.

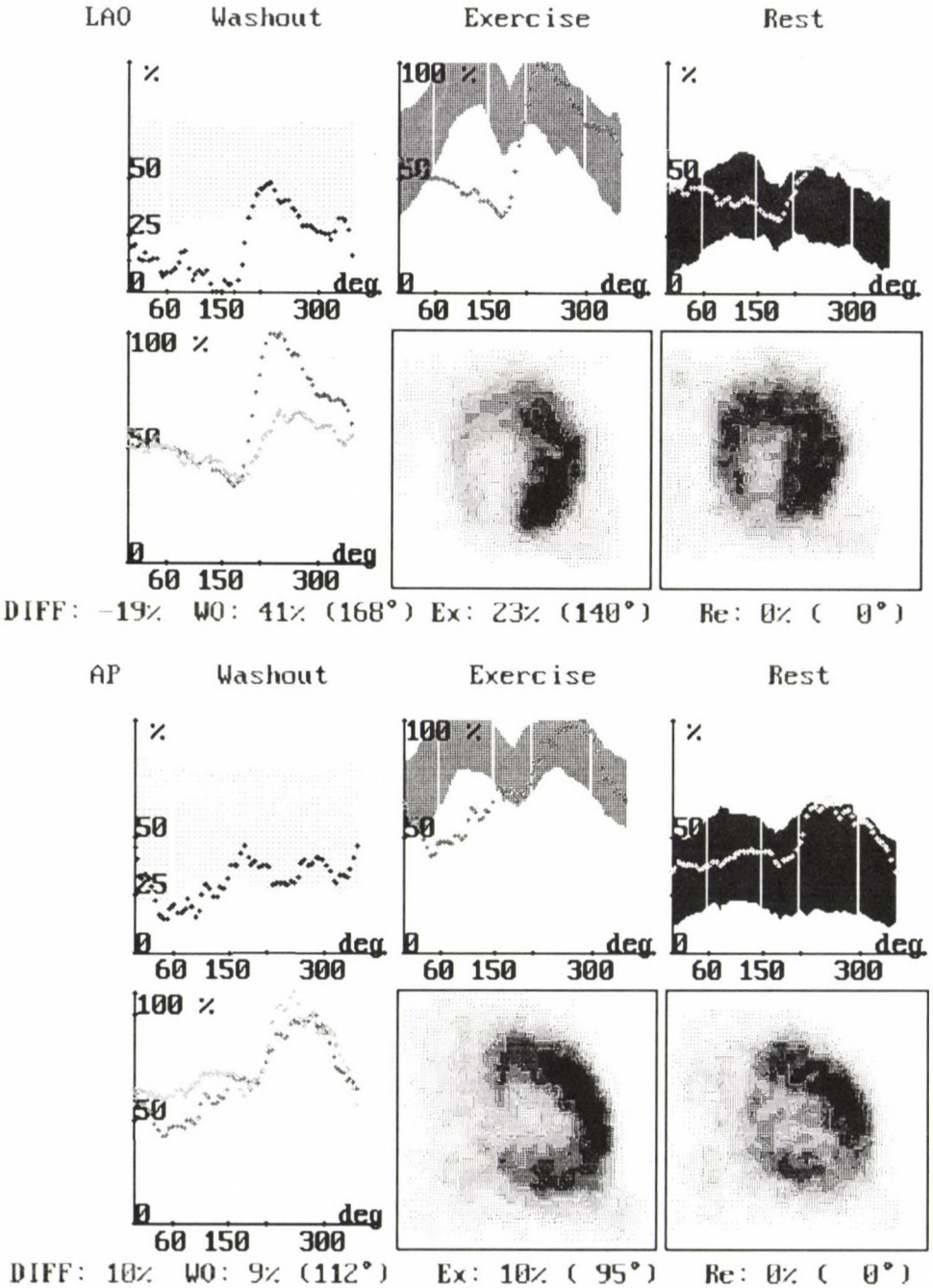

Fig. 1. A typical example of a high risk (G III) scintigraphy: septal and infero-apical reversible perfusion defects (PD) in LAO view (Fig. 1A). Inferior wall fixed PD in anterior view (Fig. 1B). (Beside the digital display, the corresponding circumferential and washout curves with normal ranges can also be seen)

Results

The distribution of the outcomes of DTS was the following: negative in 12 cases, G I, G II, and G III in 14, 11 and 11 pts, respectively (Fig. 1).

We compared these results with clinical and ExECG data. In three independence analyses DTS grade 0-III were set against (1) clinical variables (presence or absence of angina and/or rest ECG abnormality); (2) the total; and (3) the unequivocal ExECG results (n = 44 and 33, respectively). In Table I the former two respects; (1; 2) were compared with DTS, in a

Table I

Distribution of the results of two stress tests: The correlation between dipyridamole thallium scintigraphy and exercise ECG, depending on clinical variables (angina/rest ECG)

		Exercise ECG					
		-		+		?*	
Angina/Rest ECG		-	+	-	+	-	+
Dipyridamole	0	1	0	3	2	3	0
thallium	I	2	0	2	3	2	5
scintigraphy	II	1	3	1	4	0	1
grades	III	1	2	3	4	0	1

n = 44 $P > 0.05$ and 0.1 in the chi-square test (see in text)

*: Inconclusive testing
Grade 0: No perfusion defect (PD)
I: Fixed PD
II: Reversible PD
III: Multiple PDs

Table II

Correlation between dipyridamole thallium scintigraphy and unequivocal exercise ECG

		Exercise ECG	
		-	+
Dipyridamole	0	1	5
thallium	I	2	5
scintigraphy	II	4	5
grades	III	3	7

n = 32 $P > 0.10$

combined way, while in Table II the latter one (those pts with unequivocally positive or negative ExECGs). (From the second parallel the 12 pts with inconclusive ExECG were excluded.) No significant correlation was found between the severity of ischaemia assessed by scintigraphy and other (1; 2; 3) available parameters ($P > 0.05$; 0.1; 0.1, respectively).

We regarded a positive DTS as having clinical consequences if there was (1) PD after negative (5 pts) or inconclusive (7 pts) ExECG, or (2) a high risk pattern after a positive ExECG. So the isotope study offered special information in more than 50% of the pts (the 25 cases underlined in Table I). Besides significant PD, in 16 of these 25 pts we found Rd, too. Reversible or irreversible silent ischaemia was detected in 12 pts (25%).

Twenty-five pts showed persistent PD (20 pts had fixed, irreversible PD, with or without Rd in another territory, and 5 pts had partial Rd or "worsening" PD). The other clinical variables of these pts can be seen in Fig. 2: only slightly more than half of these pts had ever experienced angina, and only 1 suffered from a documented AMI. (The other post-infarction patient did not have a fixed PD.) Only one of the 11 angina-free pts had characteristic rest ECG abnormalities. In addition, 5 of them had negative or inconclusive ExECG.

Discussion

Dipyridamole is a potent coronary dilatator with acceptable side effects even during intravenous administration. It can be used for cardiac stress testing in case of pts with low exercise tolerance. If the effect of the drug is investigated with Thallium-201 isotope, the maximal reserve capacity of perfusion of the different coronary branches can be compared directly, and the sensitivity for significant stenosis is the highest among the noninvasive tests.

By the reversibility of PDs myocardial ischaemia can usually be differentiated from infarction which is manifested in a fixed decrease of activity of a coronary territory.

In order to diminish the coronary complication rate of peripheral vascular surgery, it is important to identify high-risk patients before the planned procedure. DTS is a method suitable for this purpose. Boucher et al. /3/ found postoperative cardiac events in 50% when reversible PD existed. However, there is no general consensus regarding the prognosis of fixed,

irreversible PDs /13/. McEnroe et al. /14/ claimed that the operative risk is significantly higher in these cases as compared to negative studies, while Boucher et al. could not find more frequent coronary events in these cases.

Some authors /1, 12/ showed a higher accuracy of DTS when applied semiquantitative evaluation. We used a four grade score to characterize the severity of myocardial ischaemia (from normal through intermediate risk of single territory PD with or without Rd to the multiple PDs).

Opposing to other clinical situations /4, 8, 11/, the outstanding superiority of myocardial scintigraphy in risk stratification of peripheral vascular pts was questioned by some authors /7, 15/, as the combined, multi-step diagnostic approach resulted in more accurate assessment. We used a similar stepwise algorithm in this work to optimize the cost/benefit ratio, and to decrease the probability of false positive results. This meant that pts with positive or inconclusive ExECG were preferred (but not exclusively chosen) in the recruitement. Although negative scintigraphic results were less frequent than in a general population with peripheral vascular disease, we could compare the ratios of positive/negative ExECGs in the different risk categories. There was no significant difference in the clinical and ExECG parameters between the low, intermediate and high risk groups defined by DTS ($P > 0.1$). Of the two stress testings, ExECG and DTS, the latter

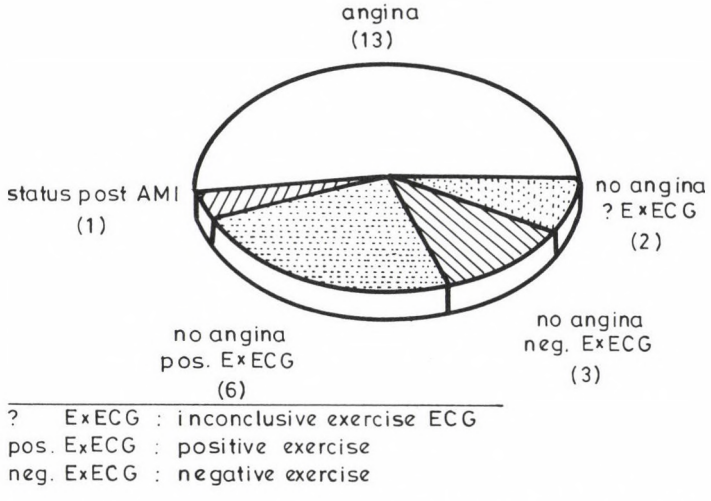

Fig. 2. The clinical and ECG variables of patients with persistent PDs (n = 25 patients)

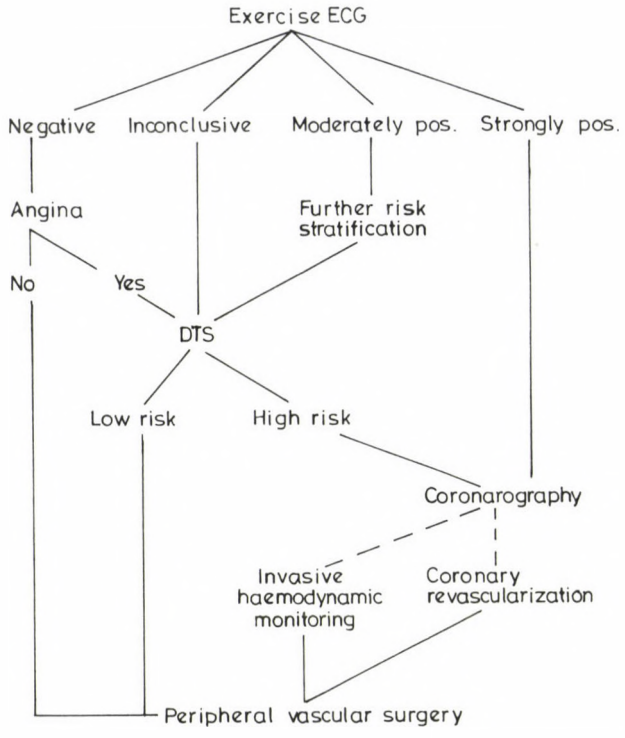

Fig. 3. A possible algorithm for cardiac investigation and treatment before and during major vascular surgery

proved to be more accurate in almost all clinical situations. We also found DTS more reliable in the special indication we studied. Seven of 36 pts with positive scintigraphy were not suspected to have ischaemic heart disease by clinical and ExECG findings. Although only two patients had had AMI, we found irreversible ischaemia in at least one coronary region in 20 pts. This fact, even if we are aware of the inevitable false positive rate of DTS, and despite the preselected population, may highlight the great prevalence of ischaemic heart disease and, with special importance, its silent form among these patients. We think that the rest ECG alone is clearly insufficient for the risk assessment before peripheral vascular surgery.

Taking into account the cost/benefit ratio, we suggest a diagnostic flowchart for preoperative medical consultation before planned vascular surgery (Fig. 3). The first step is an attempt to perform an ExECG that is rarely definitely negative in these cases. So we think that the DTS is neces-

sary in the majority of cases and the most jeopardized patients can be selected, for whom coronary revascularization may offer the most beneficial effect before leg arterial surgery.

There are limitations of this study. The bias in the inclusion criteria we mentioned above makes our claims partly limited to this special population. As in our patients with coronary artery disease coronarography had not been performed merely because of planned vascular surgery, we could not calculate the sensitivity and specificity of these tests exactly. On the other hand, as not all the patients included in this work underwent peripheral vascular surgery, we could not make a correlation between isotopic risk categories and operative complication ratios.

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METABOLISM

THE EFFECT OF SULPHONYLUREA THERAPY ON THE OUTCOME OF CORONARY HEART DISEASES IN DIABETIC PATIENTS

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A retrospective study was performed on 1040 diabetic patients. The survival time of those treated with first generation sulphonylureas (n = 227) was considerably ($P < 0.001$) shorter after the first attack of angina pectoris (5 ± 1 years, mean ± S.E.) or acute myocardial infarction (6 ± 1 years) than of those (9 ± 1 years) on glibenclamide treatment (n = 144), with regime alone (n = 282) or treated with insulin (n = 387). The systolic blood pressure of patients with first generation sulphonylureas (166 ± 1/91 ± 1 mmHg) proved to be higher ($P < 0.01$) than those treated with glibenclamide (159 ± 1/91 ± 1 mmHg) or being on regime alone (155 ± 1/89 ± 1 mmHg) or on insulin (156 ± 1/89 ± 1 mmHg) treatments. Serum sodium level was found to be lower ($P < 0.05$) in patients treated with any kind of sulphonylureas (138 ± 1 mmol/l) than in the other patients (143 ± 1 mmol/l). During an observation period, 576 of patients died, 412 of them due to cardiovascular or renal failures. Among the diabetic subjects suffering from coronary heart disease no difference could be detected in risk factors except for higher systolic blood pressure. The shorter survival time of patients treated with first-generation sulphonylureas might be explained by the arryhthmogenic activity of first-generation sulphonylureas. Improvement in therapy, metabolic and cardiovascular alterations during the survey can not be responsible for the shorter survival time of patients treated with first generation-sulphonylureas.

Keywords: Coronary heart diseases, survival time, hypoglycaemic sulphonylurea compounds diabetics

Introduction

Since insulin was discovered cardiac and vascular complications have become the most common causes of diabetes mortality /23/. According to the conventional theory this may be explained by severe arteriosclerosis of diabetic vessels. Epidemiologic studies involving large populations really

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demonstrated a higher frequency of macroangiopathy in diabetic vs metabolically healthy individuals /7/. On the other hand, autopsies /14, 17, 21, 29, 31, 34, 35/ drew attention to the fact that most of the diabetic cardiac disorders could not be explained simply by microangiopathy. Further, a discrepancy was demonstrated by coronarography in diabetic subjects suffering from angina pectoris between vascular narrowing and the intensity of pain /29/. However, mortality due to coronary heart disease is known to be twofold in diabetes than in metabolically healthy states /13, 36, 37/.

First-generation sulphonylurea compounds enhanced cumulative cardiovascular mortality /38/ and ventricular arrhythmias /27/ in human beings, cardiotoxicity of cardiac glycosides /4, 27/ and ischaemic arrhythmias /5/ in animals. Glibenclamide affected ventricular arrhythmias of diabetic patients /19, 27/, experimentally induced arrhythmias /4, 5, 27/ as well as haemodynamic /3, 26/ and electrocardiac parameters /6, 28/ oppositely. The cardiac effects of antidiabetic sulphonylurea drugs might be also involved, therefore, in the more severe outcome of coronary heart disease. To approach this question, we analyzed the relationship between the type of antidiabetic medication and the outcome of coronary heart disease in the Diabetic Outpatient Clinic of our Institute.

Patients and Methods

In a retrospective study 1040 diabetic patients (481 men, 559 women) were followed in the Diabetic Outpatient Clinic of our Institute between November 1, 1967 and April 30, 1991, 282 (163 male and 119 female) patients were controlled only by regime, 227 (105 male, 122 female) were treated with first generation sulphonylurea compounds in the following distribution: 93 (44 male, 49 female) were on carbutamide (Bucarban; Chinoin, Budapest); 95 (46 male, 49 female) on tolbutamide (Oterben; Chinoin); 39 (15 male, 24 female) on chlorpropamide (Diabinese; Pfizer); and 2 (1 male, 1 female) on gliclazide (Diamicron; Servier) therapies. While with the second-generation sulphonylurea compound, glibenclamide (Euglucon, Hoechst till 1975; Gilemal, Chinoin from 1975 on) 144 (58 male, 86 female) patients were treated, and 387 subjects (155 male, 232 female) were on insulin therapy (Novo-Nordisk products). The therapy was practically homogeneous in each groups: it occurred only in 6% (13 cases) and 7% (10 cases) of patients that first-generation sulphonylureas and glibenclamide, respectively was substituted for insulin. The percentile proportion of subjects treated with first-generation sulphonylurea compounds or glibenclamide were not changed between 1967 and 1991. No differences existed between the different kinds of sulphonylurea compounds during the therapy (12 ± 1 years). Insulin was applied for 16 ± 1 years and diet only for 7 ± 1 years.

The drop out of patients was an average of 6%; 1% (2 patients) on first-generation sulphonylurea, 2% (3 patients) on glibenclamide therapies, 9% (25 patients) on diet only and 9% (35 patients) on insulin stopped presenting.

The alcohol consumption did not exceed 300 ml spirits or 1.5 litre wine weekly except two patients.

The diabetic subjects were controlled on the average meanly once a month (most frequently weekly, rarely annually). On these occasions postprandial blood glucose /32/ values, or recently blood glucose profile (method of Petrányi et al. /25/ or glucometer) as well as glucose /32/ and acetone /30/ excretions of urine collected over 24 hours were measured. Other blood parameters, like serum creatinine /15/, uric acid /10, 11/, cholesterol /1, 8/, triglycerides /20/, sodium /24/, potassium /24/, from 1980 on HDL-cholesterol /22/ and from 1981 HbAlc /12/ levels were determined at least annually.

Patients were asked about the history of last period and dietary management at every visit. At the same time physical examination, measurement of body weight and that of blood pressure in both supine and standing positions, were performed. ECG, fundus, and neurological examinations, and chest x-ray were performed at least annually.

Statistical evaluation of data was carried out on an IBM-AT personal computer. Data input was performed by the modified Lotus Database (Lotus Development Corporation, 1985) programme. The results have been evaluated statistically by analysis of variance (ANOVA) and chi-test by a modified version of Systat (Version 3.0, 1985) programme. The mean values $\pm$S.E. are given.

Results

During the observation period of 23 years 576 patients (265 men, 311 women) died of cardiac (n = 268), peripheral arterial (n = 17), cerebrovascular (n = 105), renal (n = 22), gastrointestinal (n = 16), haematological (n = 9), cancerous (n = 76), and other (n = 38) diseases; 15 died after an accident and 10 committed suicide. Protocols of autopsies being only sporadically available were not evaluated. The distribution of cardiac and other causes of death was dissimilar in the differently treated groups. In diabetic subjects treated with the first generation of sulphonylurea compounds the cause of death was cardiac in 66% (150 cases) and vascular or renal in 9% (20 cases). In diabetic patients treated with regime alone the death was cardiac in 13% (37 cases) and vascular or renal in 9% (25 cases), in those treated with insulin it was cardiac in 14% (54 cases) and vascular or renal in 18% (70 cases).

Both the duration of observation (8 $\pm$ 1 years) and the age (at the beginning of observation 55 $\pm$ 1 years) were the same in the differently treated groups. Only the insulin-treated subjects were somewhat younger (52 $\pm$ 1 years). The duration of diabetes was shorter in the group on diet only (4 $\pm$ 1 years) and longer in those treated with insulin (10 $\pm$ 1 years), compared with the others (8 $\pm$ 1 years).

Angina pectoris occurred in 46 diabetic subjects on first-generation sulphonylurea therapy, in 52 cases treated with glibenclamide, in 94 insulinized patients, and in 52 diabetic subjects on regime alone. In the last three groups the survival time of patients suffering from angina pectoris

did not differ significantly, whereas it shortened ($P < 0.001$) under the influence of first-generation sulphonylurea therapy considerably (Table I). The onset of angina pectoris occurred practically at a similar age and after a similar duration of diabetes in the patients treated with first-generation sulphonylureas or glibenclamide (Table I). Significant alterations ($P < 0.05$) in these variables could be found only in the groups treated with insulin and that kept on regime alone (Table I).

Acute myocardial infarction occurred in 45 diabetics treated with first-generation sulphonylureas, in 28 treated with glibenclamide, in 46 insulinized and in 28 dietetized patients. Survival of diabetic patients on first-generation sulphonylurea proved to be markedly ($P < 0.001$) shorter than in the other groups (Table I). Acute myocardial lesion was observed at a similar age and after a similar duration of diabetes in subjects treated with the first-generation of sulphonylurea compounds or glibenclamide (Table I). Similarly to angina pectoris, duration of diabetes and age at first acute myocardial infarction were found dissimilar ($P < 0.05$) only in the groups treated with insulin or regime alone (Table I).

At the beginning of the observation, data for the diabetic subjects suffering from angina pectoris or myocardial infarction did not differ notably among the patients treated with different antidiabetic drugs concerning history, physical status, x-ray-, and ECG-results. However, the incidence of arrhythmias in patients with angina pectoris (20-25%) or myocardial infarction (23-30%) was higher during first-generation sulphonylurea therapy than during other treatments (0-14%). Explanation for this phenomenon may be the fact that 186 of the 227 patients on first-generation sulphonylurea compounds were already treated with such compounds before their entry in the present study. At the beginning of the observation, severity of angina pectoris did not differ markedly among differently treated groups as estimated by frequency of anginal attacks (weekly 11 ± 1 attacks) as well as by the consumption of nitroglycerin pills (weekly 8 ± 1 pills). In subjects suffering from myocardial infarction, frequency of anginal attacks was considerably ($P < 0.5$) higher in the glibenclamide treated group (56%) than in the group treated with first-generation sulphonylurea compounds (35%). Accordingly, differences in cardiac state following acute myocardial infarction could not explain the shorter survival of patients on first-generation sulphonylurea medication. During the survey, diabetic patients did not show different impairment of cardiac state (cardiac enlargement, dyskinesis of ventricular wall, deterioration in ECG

Table I

Common data for diabetic patients suffering and those not suffering from coronary heart disease

Kind of therapy	Survival after first cardiac attack	Age at first cardiac attack	Duration of diabetes	Duration of observation	Cigarettes	Body mass index	Blood pressure systolic in supine position	Blood pressure diastolic in supine position	Number of patients
	yr	yr	yr	yr	per day	kg/m^2	mmHg	mmHg	n
	Data for diabetic patients suffering from angina pèctoris								
D:	10 ± 1	59 ± 1	9 ± 1	7 ± 1	6 ± 1	27.6 ± 1.1	157 ± 3	92 ± 2	52
1-Su:	6 ± 1	62 ± 1	15 ± 1	9 ± 1	5 ± 1	28.5 ± 1.2	171 ± 3	93 ± 2	46
2-Su:	9 ± 1	61 ± 1	15 ± 1	9 ± 1	6 ± 2	26.1 ± 1.3	160 ± 3	88 ± 1	52
I:	10 ± 1	57 ± 1	21 ± 1	11 ± 1	6 ± 1	26.8 ± 1.1	162 ± 2	90 ± 1	94
	Data for diabetic patients suffering from acute myocardial infarction								
D:	10 ± 1	63 ± 1	9 ± 1	6 ± 1	6 ± 2	28.3 ± 1.3	155 ± 3	88 ± 2	28
1-Su:	6 ± 1	61 ± 1	16 ± 1	10 ± 1	4 ± 1	29.0 ± 2.4	166 ± 2	91 ± 1	45
2-Su:	10 ± 1	61 ± 2	15 ± 1	8 ± 1	8 ± 2	25.2 ± 1.4	154 ± 3	86 ± 1	28
I:	10 ± 1	57 ± 1	20 ± 1	8 ± 1	5 ± 1	26.3 ± 1.1	162 ± 3	90 ± 1	46
	Data for diabetic patients without any signs of coronary heart disease								
D:	—	—	10 ± 1	8 ± 1	5 ± 1	27.7 ± 1.2	154 ± 2	89 ± 1	208
1-Su:	—	—	14 ± 1	9 ± 1	3 ± 1	28.0 ± 1.0	164 ± 2	91 ± 1	161
2-Su:	—	—	14 ± 1	8 ± 1	5 ± 1	27.0 ± 1.2	158 ± 2	91 ± 1	128
I:	—	—	17 ± 1	8 ± 1	6 ± 1	26.1 ± 1.4	155 ± 2	88 ± 1	211
2 S.E.	2	2	2	2	2	2.6	5	2	

Legend:

D = diabetic patients treated with regime alone,
1-Su = diabetic patients treated with first-generation sulfonylurea compounds,
2-Su = diabetic patients treated with a second-generation sulfonylurea, glibenclamide,
I = diabetic patients treated with insulin,
2 S.E. = twice value of S.E.

signs) in the various treatment-groups except the appearance of an apico-basal discrepancy in the lungs ($P < 0.05$) between the groups of patients treated with first-generation sulphonylurea compounds (2%) or with glibenclamide (24%) following acute myocardial infarction. Supraventricular (30 and 4%, respectively) and ventricular (23 and 0%, respectively) ectopic beats occurred at an elevated frequency ($P < 0.05$) following acute myocardial infarction in group of patients treated with first generation sulphonylurea compounds. It was only the insulinized group (Table II) in which parameters of carbohydrate metabolism were found to be considerably (blood glycated haemoglobin: $P < 0.05$; blood glucose: $P < 0.05$; glucose excretion: $P < 0.05$) higher both in patients suffering from angina pectoris and those with myocardial infarction. The level of serum triglyceride was found to be higher ($P < 0.05$) in diabetic subjects treated with glibenclamide, while that of HDL-cholesterol ($P < 0.05$) in those on first-generation sulphonylurea therapy (Table II).

As to cardiovascular risk factors, overweight at the first visit on the body mass index value 27.4 ± 0.2 kg/m^2 did not differ notably among the investigated groups and did not change considerably during the observation period (Table III). Similarly, the tobacco smoking habits did not altered during the survey (Table III). Systolic blood pressure in patients treated with first generation-sulphonylureas was markedly ($P < 0.05$) higher compared with those either on glibenclamide, or on regime alone both at the beginning or later on (Table III). In this respect we should remember that 186 from the 227 patients were already treated with first-generation sulphonylurea compounds before joined the present survey.

Diabetic retino- and nephropathies occurred more often in fatal cases than in survivors, however, the incidence of diabetic retinopathy considerably increased in the survivors during the observation period (Table IV).

During follow-up of the diabetic patients blood glucose ($P < 0.001$) and glycated haemoglobin ($P < 0.001$) levels as well as glucose excretion ($P < 0.001$) decreased considerably (Table II). These parameters showed the lowest level in the group treated with regime alone, while the highest one in that on insulin therapy (Table III). The levels of serum creatinine ($P < 0.001$) and uric acid ($P < 0.01$), glycated haemoglobin ($P < 0.01$) and systolic blood pressure in supine position ($P < 0.001$) were found to be higher in patients who died during the survey period than in the survivors (Table IV). The serum sodium level was found to be considerably lower ($P < 0.01$) in all

Table II

Metabolic data for diabetic patients suffering or not suffering from coronary heart disease

Kind of therapy	Serum creatinine μM	Serum uric acid μM	Serum triglyceride mM	Serum HDL-cholesterol mM	Serum cholesterol mM	Blood glycated haemoglobin %	Blood glucose mM	glucose excretion mmol/day	Number of patients n
	Data for diabetic patients suffering from angina pectoris								
D:	102 ± 3	362 ± 9	2.28 ± 0.14	1.35 ± 0.09	6.3 ± 0.1	5.94 ± 0.22	7.05 ± 0.20	6 ± 3	52
1-Su:	95 ± 1	343 ± 14	2.03 ± 0.25	1.44 ± 0.13	6.5 ± 0.2	7.04 ± 0.36	7.76 ± 0.20	17 ± 5	46
2-Su:	105 ± 4	350 ± 11	2.89 ± 0.29	0.96 ± 0.08	6.4 ± 0.2	6.68 ± 0.24	8.11 ± 0.21	13 ± 4	52
I:	104 ± 4	348 ± 10	2.12 ± 0.17	1.31 ± 0.09	6.6 ± 0.1	7.82 ± 0.22	8.91 ± 0.27	48 ± 7	94
	Data for diabetic patients suffering from acute myocardial infarction								
D:	109 ± 5	358 ± 10	2.27 ± 0.23	1.17 ± 0.09	6.2 ± 0.1	5.58 ± 0.19	6.38 ± 0.19	1 ± 1	28
1-Su:	97 ± 1	333 ± 10	1.81 ± 0.16	1.66 ± 0.09	6.5 ± 0.1	6.79 ± 0.23	7.69 ± 0.16	13 ± 3	45
2-Su:	108 ± 6	376 ± 12	2.49 ± 0.22	0.75 ± 0.08	6.4 ± 0.3	6.75 ± 0.20	7.52 ± 0.11	7 ± 2	28
I:	110 ± 5	353 ± 11	1.95 ± 0.11	0.97 ± 0.09	6.7 ± 0.1	8.03 ± 0.28	8.58 ± 0.28	49 ± 8	46
	Data for diabetic patients without any sign of coronary heart disease								
D:	95 ± 1	367 ± 6	1.88 ± 0.08	1.67 ± 0.07	8.6 ± 2.3	5.79 ± 0.11	7.05 ± 0.08	3 ± 1	208
1-Su:	103 ± 3	358 ± 7	1.64 ± 0.10	1.78 ± 0.10	6.5 ± 0.1	7.57 ± 0.23	8.01 ± 0.11	21 ± 3	161
2-Su:	99 ± 3	347 ± 7	2.11 ± 0.12	1.51 ± 0.07	6.1 ± 0.1	6.65 ± 0.16	8.30 ± 0.19	13 ± 3	128
I:	99 ± 3	348 ± 6	1.90 ± 0.10	2.20 ± 0.71	6.5 ± 0.1	7.91 ± 0.19	8.88 ± 0.16	51 ± 4	211
2 S.E.	7	21	0.39	0.31	0.8	0.46	0.43	9	

Legend: see in the footnote of Table I.

Table III

Data for the diabetic patients treated with different types of antidiabetic drugs

Body mass index	Serum sodium	Serum triglyceride	Serum HDL-cholesterol	Serum cholesterol	Blood HbA_{1c}	Blood glucose	Urine glucose excretion	Cigarettes	Arterial blood pressure systolic in supine position	Arterial blood pressure diastolic in supine position	Number of patients
kg/m^2	mM	mM	mM	mM	%	mM	mmol/day	per day	mmHg	mmHg	n
Patients treated with carbutamide											
B: 27.2 ± 0.4	141 ± 1	1.53 ± 0.11	1.69 ± 0.11	6.8 ± 0.1	9.69 ± 0.37	9.60 ± 0.19	82 ± 10	5 ± 1	163 ± 2	91 ± 1	93
E: 27.0 ± 0.5	138 ± 1	1.71 ± 0.09	1.58 ± 0.10	6.6 ± 0.1	7.81 ± 0.28	8.17 ± 0-12	26 ± 4	5 ± 1	165 ± 2	92 ± 1	83
Patients treated with chlorpropamide											
B: 27.5 ± 0.7	140 ± 1	1.30 ± 0.22	1.56 ± 0.17	6.2 ± 0.2	8.14 ± 0.60	9.64 ± 0.33	92 ± 19	2 ± 1	169 ± 4	90 ± 2	39
E: 27.3 ± 0.8	136 ± 1	1.39 ± 0.25	1.53 ± 0.15	6.1 ± 0.2	7.19 ± 0.33	7.88 ± 0.16	18 ± 4	1 ± 1	172 ± 4	91 ± 2	39
Patients treated with glicazide											
B: 26.5 ± 0.9	139 ± 1	1.40 ± 0.12	1.87 ± 0.06	5.8 ± 2.1	8.94 ± 0.25	8.87 ± 1.56	23 ± 9	1 ± 1	160 ± 10	87 ± 2	2
E: 26.7 ± 0.8	134 ± 1	1.47 ± 0.11	1.88 ± 0.05	5.7 ± 1.8	7.61 ± 0.16	7.26 ± 0.95	1 ± 1	1 ± 1	180 ± 10	88 ± 3	2
Patients treated with tolbutamide											
B: 27.5 ± 0.5	141 ± 1	1.57 ± 0.16	1.90 ± 0.10	6.5 ± 0.1	7.32 ± 0.29	8.37 ± 0.28	34 ± 8	5 ± 1	161 ± 3	87 ± 2	95
E: 27.6 ± 0.6	138 ± 1	1.96 ± 0.17	1.78 ± 0.09	6.5 ± 0.1	6.53 ± 0.17	7.40 ± 0.11	5 ± 2	4 ± 1	164 ± 3	90 ± 2	88
Patients treated with glibenclamide											
B: 27.8 ± 0.6	141 ± 1	1.94 ± 0.10	1.32 ± 0.26	6.3 ± 0.1	7.21 ± 0.15	9.40 ± 0.19	48 ± 6	7 ± 1	156 ± 1	87 ± 1	144
E: 27.9 ± 0.5	138 ± 1	2.37 ± 0.11	1.23 ± 0.05	6.3 ± 0.1	6.68 ± 0.12	8.09 ± 0.12	12 ± 2	6 ± 1	158 ± 2	89 ± 1	231
Patients treated with regime alone											
B: 27.8 ± 0.4	143 ± 1	1.74 ± 0.07	1.60 ± 0.05	6.3 ± 0.1	6.24 ± 0.11	7.47 ± 0.12	15 ± 3	6 ± 1	154 ± 1	90 ± 1	282
E: 27.2 ± 0.5	143 ± 1	2.03 ± 0.07	1.53 ± 0.05	6.6 ± 0.4	5.80 ± 0.09	6.97 ± 0.07	3 ± 1	5 ± 1	155 ± 1	89 ± 1	257
Patients treated with insulin											
B: 26.5 ± 0.4	143 ± 1	1.80 ± 0.08	1.40 ± 0.05	6.7 ± 0.1	8.58 ± 0.17	10.29 ± 0.17	115 ± 7	7 ± 1	156 ± 1	89 ± 1	387
E: 26.8 ± 0.5	143 ± 1	1.97 ± 0.07	1.69 ± 0.37	6.6 ± 0.1	7.91 ± 0.13	8.83 ± 0.12	50 ± 3	6 ± 1	158 ± 1	89 ± 1	352
2 S.E.	2	0.34	0.31	0.6	0.47	0.34	7	2	7	3	

Legend: see in Table I. Begin (B) or end (E) of follow-up.

Table IV

Data of diabetic patients controlled in the outpatient clinic

Serum creatinine μM	Serum uric acid μM	Serum triglyceride mM	Blood HbA_{1c} %	Blood glucose mM	Urine glucose excretion mmol/day	Cigarettes per day	Blood pressure systolic in supine position mmHg	Blood pressure diastolic in supine position mmHg	Alterations of fundus %	Alterations of kidney %
				Data of all diabetic patients (n = 1040)						
				At the first presenting in the outpatient clinic						
97 ± 1	355 ± 3	1.77 ± 0.05	7.50 ± 0.10	9.18 ± 0.10	66 ± 4	6 ± 1	158 ± 1	90 ± 1	19	34
				During the observation in the outpatient clinic						
100 ± 1	354 ± 3	2.04 ± 0.05	6.85 ± 0.08	8.04 ± 0.06	23 ± 1	5 ± 1	158 ± 1	90 ± 1	23	36
				Data for the diabetic survivors (n = 464)						
				At the first presenting in the outpatient clinic						
91 ± 1	345 ± 5	1.85 ± 0.07	7.31 ± 0.14	9.14 ± 0.16	65 ± 6	7 ± 1	151 ± 1	89 ± 1	13	26
				During the observation in the outpatient clinic						
93 ± 1	346 ± 4	2.05 ± 0.07	6.72 ± 0.11	8.17 ± 0.11	28 ± 3	5 ± 1	151 ± 1	89 ± 1	21	30
				Data for fatal cases of diabetes (n = 576)						
				At the first presenting in the outpatient clinic						
101 ± 2	364 ± 5	1.66 ± 0.07	7.75 ± 0.14	9.22 ± 0.11	67 ± 5	5 ± 1	164 ± 1	90 ± 1	23	40
				During the observation in the outpatient clinic						
105 ± 2	361 ± 4	2.02 ± 0.07	7.02 ± 0.11	7.94 ± 0.07	19 ± 2	5 ± 1	164 ± 1	90 ± 1	24	41
2 S.E.	11	0.18	0.31	0.29	10	2	3	2	—	—

Legend: see in Table I.

diabetics treated with sulphonylureas than in those kept on insulin or diet (Table III). Serum potassium level showed no group difference.

It is possible that the development in the field of the therapy of coronary heart diseases, hypertension, coronary care and concomitant diseases may be responsible for the differences in the outcome of coronary heart disease between the groups treated with first-generation sulphonylurea compounds or glibenclamide and the other groups. For this reason, the observation period of 24 years was divided into four parts, and cardiac effect of antidiabetic drugs was compared in each period. The survival time of diabetic subjects with angina pectoris or myocardial infarction on first-generation sulphonylurea therapy showed shorter survival time and an elevated systolic blood pressure in each period. The incidence of concomitant diseases showed no notable group difference. Therefore it seems unlikely that therapeutical progression would considerably influence the survival time of patients with acute myocardial infarction or angina pectoris.

Among the patients without any signs for coronary heart disease 119 were treated with first-generation sulphonylureas, 899 with glibenclamide, 83 with insulin, while 161 patients were kept only on regime. Variables of cardiac and metabolic states showed a similar distribution in these groups both at the beginning and during the observation period (Tables I and II).

Discussion

Almost all of the diabetic patients followed-up at the Outpatient Clinic of our Institute showed some manifestations of heart and vascular complications of diabetes mellitus. This fact could serve as an explanation for the more than 55% of death in our diabetic patients during the 24 years of observation.

Furthermore the higher mortality of the diabetic patients with acute myocardial infarction in coronary care units is attributed to the more frequent onset of cardiogenic shock and that of fatal arrhythmias, compared with a metabolically healthy population /18/. The present work makes another explanation possible. Arrhythmogenic effect of first-generation hypoglycemic sulphonylureas /3—6, 26—28/ is namely supposed to be responsible for the higher mortality rate in coronary heart disease since, with the exception of higher systolic blood pressure, no difference was detected relating metabolic or risk factors among the investigated groups.

In a previous report /33/ systolic blood pressure remained unaltered, but diastolic blood pressure was diminished in a group, in which tolbutamide was administered to patients with impaired glucose tolerance and the drug prevented manifest diabetes from processing. On the other hand, tolbutamide was described by Wales /36/ as a drug increasing systolic blood pressure. Furthermore, various sulphonylurea compounds influence blood pressure in experimental animals differently. These data refer to a group-specific property of sulphonylureas (based on chemical structure) responsible for their divergent effect on blood pressure /2, 3, 26/. The present work contributes further support to this hypothesis.

In contrast to Laube and Krausch /19/, we did not find a decreased HDL-cholesterol level in the diabetic subjects treated with sulphonylureas. It should be noted that the cited work does not give any reference to the applied sulphonylurea compounds. Additionally, similar findings could be made by this experiment with no difference between the sulphonylurea generations. Furthermore it is worth mentioning that the investigated patient groups differ neither in serum potassium level nor saluretic therapy.

The results of the present observation suggest that glibenclamide should be preferred to the first-generation sulphonylurea compounds, especially in the case of manifest coronary heart disease (or if its risk is existing), when satisfactory metabolic control cannot be achieved with regime alone and sulphonylurea treatment becomes necessary in type-2 (non-insulin-dependent) diabetes.

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EXPERIENCES WITH FUNCTIONAL INSULIN SUBSTITUTION: A FOLLOW-UP STUDY ON CONTROL AND PATIENT COMPLIANCE

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Functional insulin substitution, an insulin regimen made up of two daily injections of intermediate-acting insulin and prandial boluses of regular insulin, was to be introduced in 49 type 1 diabetic patients, as previous regimens consisting of two or three daily injections proved to be inefficient due to the patients lifestyle or inherent metabolic lability. Forty-five patients were treated with human insulin injected by NovoPen. In 38 cases therapy was changed in the frame of a one-week, small-group, inpatient, structured educational course. After a mean 14 months of follow-up metabolic status improved in 33 cases while there was further derangement in 16. Eighteen patients were practising true functional therapy, i.e. doing blood glucose tests before each injection. Further 22 diabetics were trying to achieve better metabolic control through 2-3 daily blood glucose tests and insulin dose corrections. The metabolic status was not affected by the frequency of blood glucose testing, rather by raising of the daily dose of short acting insulin in conjunction with the switch in therapy acting beneficially. All patients insisted on using NovoPen further on.

Keywords: Diabetes mellitus, functional insulin substitution, small-group diabetic education, blood glucose self-testing

Introduction

In Europe, the concept and methodology of functional insulin substitution were first developed by Waldhäusl and Howorka /5, 6, 10, 21/. The main point of this method is the separation of basal and prandial insulin doses. Actually, it is a version of intensified, fractional insulin therapy. The latter became popular in the mid 80s. Functional insulin therapy has two

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essential characteristics distinguishing it from other arts of intensified insulin treatment. 1. The diabetic patient does alter the treatment not only according to general conditions (menses, illness, etc.) influencing the daily insulin need. Besides injecting basal intermediate-acting insulin twice daily, the doses of short-acting prandial insulin are adjusted on each occasion according to the current blood glucose level (measured by the patient), the carbohydrate content of the planned meal, the glucose need of the forthcoming physical activities, etc. Moreover, the patient has to respond promptly to any deviation of the blood glucose level from the set target range (correction). By these means the lifestyle of these patients is liberalized to some extent: meals can be omitted or taken at any time and the carbohydrate content of the meals need not be preset. 2. All these advantages can only be achieved if the patient checks the blood glucose level at least four times a day. This is inevitable as the patient must inject short-acting insulin before each principal meal and the dose should be calculated according to the current blood glucose level /10/. The usage of pen devices makes frequent insulin injections easier, resulting in a switch to human insulin.

High motivation and diabetes knowledge level of the patient are the preconditions for any kind of intensified insulin therapy. The most effective way of teaching diabetic patients is group teaching /2/. According to the technique of Waldhäusl and Howorka the completion of a diabetes course is an essential introduction to functional therapy /10/ as well. The correcting and prandial insulin doses could not be defined exactly but some more or less personal algorithms could be computed based on studies in physiology /20/ and some patient parameters. These algorithms were published in a tabular form /10, 21/ and provide substantial help when initiating the functional treatment of a given patient.

This open, uncontrolled study looked at the advantages of the functional insulin treatment of brittle diabetics and at the factors determining therapeutic success or failure.

Patients and Methods

Forty-nine type 1 (insulin-dependent) patients (17 males, 32 females) were included in the study (Table I). Their age at the time of switching to functional treatment was 29.6 ± 1.9 years (mean $\pm$ S.E.). Duration of diabetes was 13.2 ± 1.3 years.

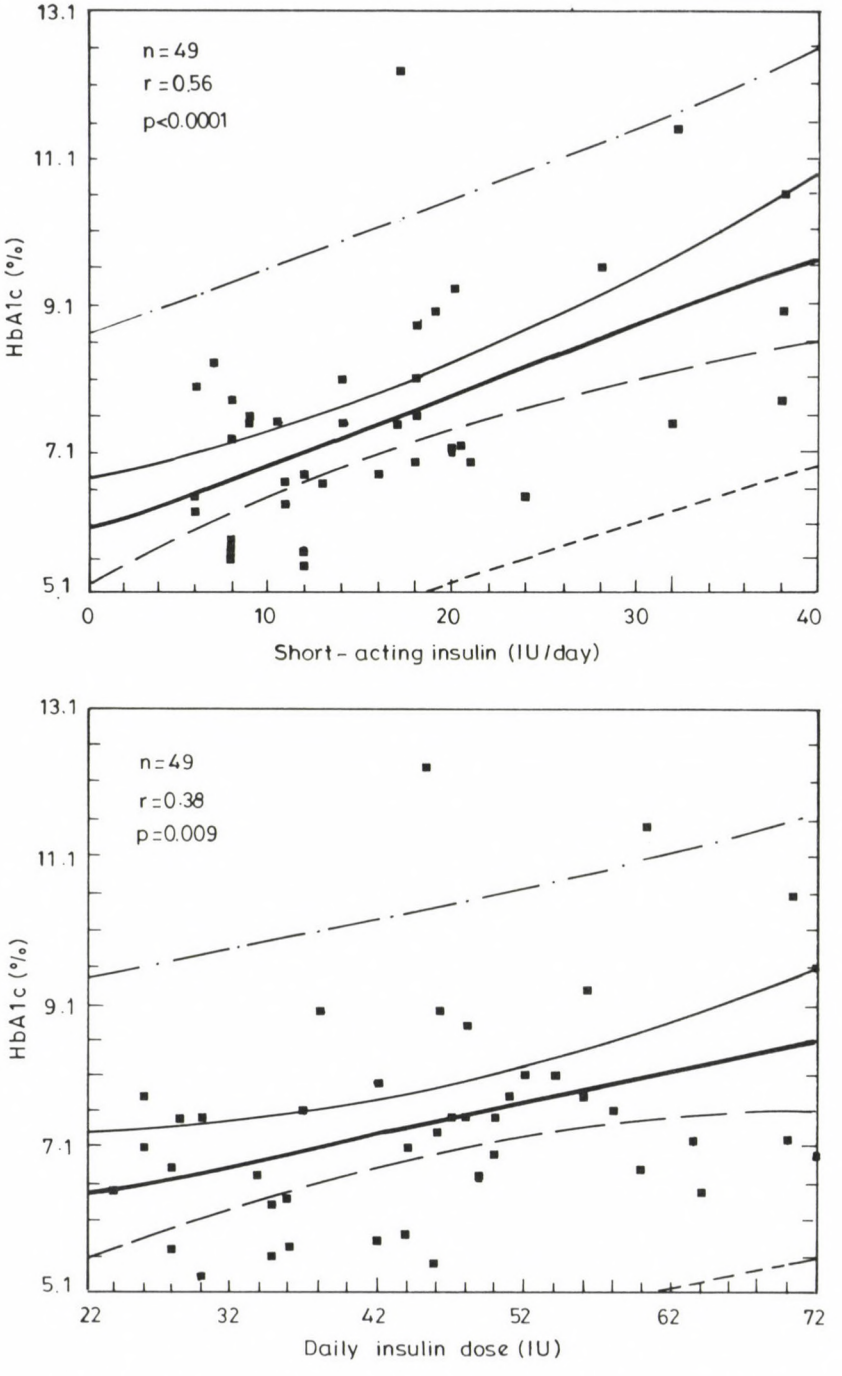

Fig. 1. Regression of HbAlc measured during functional insulin therapy on the short-acting insulin dose (a) administered during conventional therapy and on the previous total daily insulin dose (b)

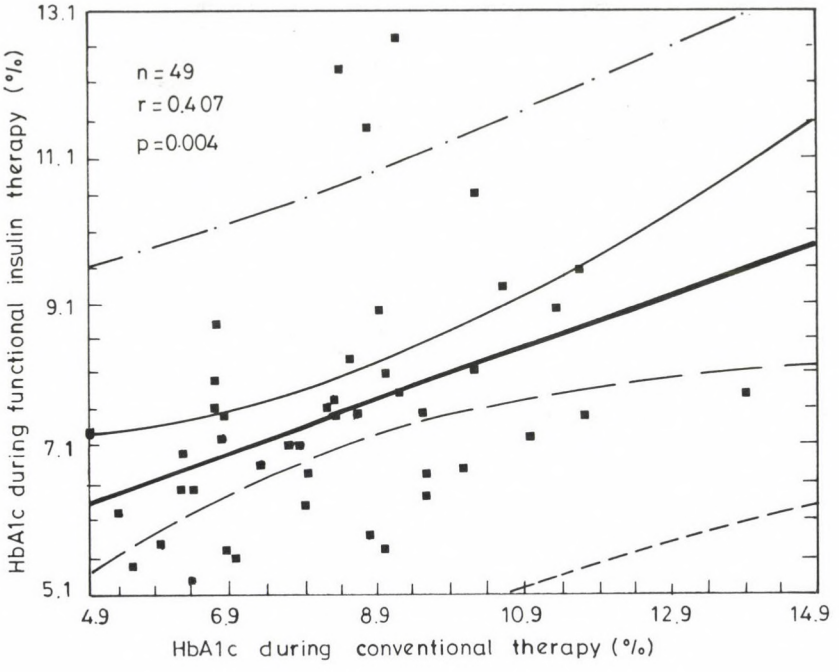

Fig. 2. Regression of HbA1c measured during functional insulin therapy on the HbA1c measured during conventional therapy

Functional insulin therapy was initiated because two or three times daily insulin administration regimens (Actrapid MC/Monotard MC) proved not to be satisfactory amid the patients. Eighteen patients enjoyed excellent metabolic control (HbA1c $< 7\%$) even before changing to functional therapy. These patients had been consulting our team for years and did not choose functional therapy because of bad control but in the hope of a more liberal lifestyle. The remaining 2/3 of the patients switched to functional therapy mainly because of metabolic problems. Forty-five diabetics started functional therapy using NovoPen I and NovoPen II to inject Actrapid HM and Protaphane HM, respectively. Four patients injected Actrapid MC and Monotard MC by no-dead-space disposable syringes. Basal insulin was injected in two nearly equivalent doses (2 x 25% of the daily dose) in the morning and at bedtime /8, 12, 14, 21/. The obligatory morning Actrapid dose, the prandial Actrapid doses to be injected for each 10 grams of carbohydrate and the correcting Actrapid doses were set according to the tables of Waldhäusl and Howorka /10, 21/ and partly based on practical experiences.

Thirty-eight patients learnt functional insulin therapy in the frame of five-day, inpatient, small-group, structured courses, the others were instructed individually.

Patients were seen at months 3, 6 and 12 after being switched to functional therapy and at least yearly thereafter. However, patients were typically seen more frequently, at any time they needed. In this report only the results of the first and last visit are shown, thus the follow-up period was 14 ± 2.0 months.

Overall glucose control was checked by HbA1c determination (BioRad HPLC method, normally 4-6%). Diabetic diary keeping and the monthly number of blood glucose self-determinations were the measure of patient motivation and compliance. This report focuses on the relationships between therapy (insulin doses), metabolic control and patient compliance. Several other

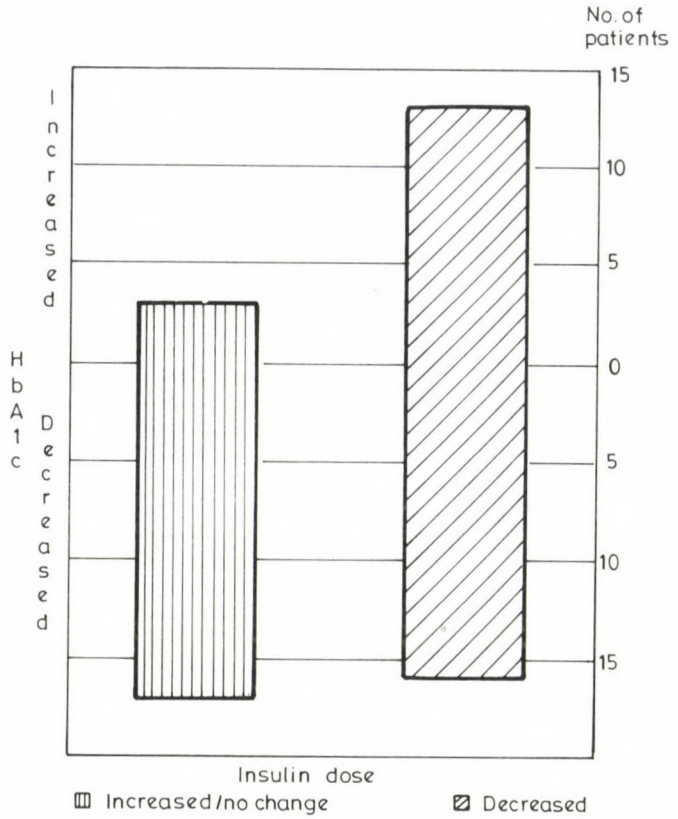

Fig. 3. The changes in control and insulin dosage after the switch in therapy

parameters were also checked (complications, frequency of hypoglycaemias, etc.) but these are not detailed here.

Statistical analysis was done using conventional methods (Chi^2-test, Student's *t*-test, simple and multivariate analysis of variance, simple and multiptle regression analysis). Results were given as mean ± S.E.

Results

The changes of short-acting and intermediate-acting insulin doses are detailed in Table I. The total daily insulin dose did not change but, according to the type of therapy /16/, the daily dose of the short-acting insulin increased for 33 patients while the daily dose of the intermediate-acting insulin decreased for 42.

Table I

Patient material, average insulin doses and changes in insulin dosage during functional treatment

Patients (mean ± S.E.)

n = 49 17 men and 32 women
Age: 29.6 ± 1.9 yrs
Duration of diabetes: 13.4 ± 1.3 yrs
38 patients completed a diabetes course

Average insulin doses (IU/day)

	Before functional mean ± S.E.	During insulin treatment mean ± S.E.	
Short acting	15.2 ± 1.4	20.4 ± 0.7	$P < 0.01$
Intermediate acting	29.7 ± 1.6	22.6 ± 1.1	$P < 0.001$
Total	45.0 ± 2.0	43.0 ± 1.6	$P = 0.17$

Changes in insulin dosage during functional treatment

	Increased	Decreased/did not change
Short-acting insulin dose	33 patients	16 patients
Intermediate acting insulin dose	7 patients	42 patients

Significant decrease of the HbAlc levels was found (Table II).

Nine patients checked their blood glucose 60 times a month or less frequently. Twenty-two diabetics checked it 60 to 120 times and 18 patients did 120 tests or more (Table III). Thus, patient cooperation was far from being satisfactory as only the last group tested properly. Group teaching proved to be an efficient way of motivating patients. The monthly number of

Table II

HbAlc levels during conventional and functional insulin treatment

HbAlc	Control	Therapy Conventional	Therapy Functional	
7% >	(normoglycaemia)	18 patients	20 patients	
7% < < = 8%	(acceptable)	6 patients	17 patients	
8% < < = 10%	(poor)	16 patients	8 patients	
10% <	(bad)	9 patients	4 patients	
HbAlc mean ± S.E. (%)		8.2 ± 0.3	7.5 ± 0.2	$P < 0.05$

	Increase	Decrease	During functional treatment
HbAlc	16 patients	33 patients	

Table III

Monthly number of blood glucose self-tests during functional treatment

Tests/month	60>	61—119	120<
No. of patients	9	22	18
Diabetes course	Completed		Not completed
No. of patients	38		11
Blood glucose tests/month (mean ± S.E.)	104 ± 5.3		83.7 ± 10.1
P		< 0.05	

blood glucose tests was 104.0 ± 5.3 in the case of those who completed the course while the others did only 83.7 ± 10.1 tests a month ($P < 0.05$). Other factors (age, duration of diabetes, duration of functional insulin treatment, sex, previous insulin regimen, age at diagnosis) showed no significant difference.

Looking for determinants of control, insulin dose changes were analysed by insulin types using ANOVA. Analysing the rise and/or lowering of short-acting and/or intermediate-acting insulin doses due to the switch in therapy, we found that the HbAlc of those patients whose intermediate-acting insulin doses were not decreased during functional therapy was much higher on the long run than the HbAlc of the others (9.0 ± 1.1 vs 7.2 ± 0.2%, $P < 0.05$).

Significant linear correlation was found on simple regression analysis between the HbAlc measured while on functional therapy and the previously administered dose of the short-acting insulin, the whole previous daily insulin need ($r = 0.56$, $P < 0.001$, $r = 0.38$, $P < 0.01$, Fig. 1) and the HbAlc values measured during conventional therapy ($r = 0.4$, $P < 0.01$, Fig. 2), respectively. Only the previous short-acting insulin dose proved to be significant in a multiple regression model. Several other variables (age, duration of diabetes, duration of functional treatment, the dosage of intermediate-acting insulins during conventional and functional treatment, the amount of short-acting insulin administered during functional treatment and the monthly number of blood glucose tests) also failed to influence the HBAlc change.

Addressing the same question from a different point of view the parameters of the patients falling into different HbAlc classes were analysed (ANOVA and Chi^2-test, Table IV). It was found that the patients whose con-

Table IV

Insulin dosage changes according to HbAlc classes.
Prediction of expectable metabolic control based on the changes in therapy

Functional treatment		Conventional treatment		Changes in short-acting insulin dosage after switching to functional therapy: increase/decrease or no change
HbAlc (%)	No. of patients	Daily insulin dose (IU)		
		Short-acting mean ± S.E.	Total mean ± S.E.	
7%>	20	11.2 ± 1.5	39.0 ± 2.8	17/3
7%< ≤ 8%	17	16.3 ± 2.1	48.9 ± 3.5	12/5
8%< ≤10%	8	26.0 ± 4.9	55.2 ± 5.4	2/6
10%<	4	15.3 ± 2.6	44.0 ± 2.5	2/2

trol was worse, even during functional treatment, injected higher doses of short-acting insulin on conventional therapy ($F = 5.2$, $P < 0.01$) and had a higher daily insulin need ($F = 3.3$, $P = 0.03$). The trend of the short-acting insulin dosage seemed to influence control. The distribution of patients among the HbAlc classes was roughly equal if their short-acting insulin dose was decreased or was not changed during functional treatment. On the contrary, if the short-acting insulin dose was raised the HbAlc fell in the lower classes ($Chi^2 = 14.5$, $P < 0.01$ with correction). There was no significant difference among the patients of the four HbAlc classes in any other respect (age, sex, duration of diabetes, duration of functional treatment, initial dose of intermediate-acting insulin, insulin dosage during functional treatment, number of monthly blood glucose tests, completion of a diabetes course, daily number of insulin injections on conventional therapy).

A decrease of the daily insulin dose in conjunction with the switch of therapy led to worse control ($Chi^2 = 4,8$, $P < 0.05$, Fig. 3). This means that the better distribution of the daily insulin dose does not necessarily improve the patient's control.

Discussion

The patients studied were partly brittle diabetics and partly had to switch to functional treatment because of an irregular lifestyle. Thus, the study yielded a rather peculiar material. Moreover, no control group could be formed as it seemed to be unethical to treat these fairly young patients,

who in several cases were suffering from lata complications, using methods that previously turned out to be ineffective. This would also mean that with respect to the literature /5, 6, 7, 15, 18/ we did not consider the method to be experimental.

The patient's control changed for the better by the end of the roughly one year follow-up period. This is certainly a significant achievement, especially in light of the above patient characteristics. The background of this change was analysed based on a limited number of parameters. The number of patient referrals, for example, was omitted for technical reasons. According to the personal experiences gained abroad we supposed that the potential inherent benefits of the treatment method will outweigh the drawbacks and thus blood sugar self-determination will not pose any problem. It is noteworthy that while in many publications on other forms of intensive and "intensified" insulin treatment the authors go into details on the necessary frequency of blood glucose self-tests to achieve best results, the true practice of their patients is barely documented in the "Results" section /1, 3, 7, 11, 13, 14, 16, 18, 19/. If accurate data are available /4, 15, 17, 22/, the frequency of blood glucose tests is usually only satisfying in the case of pregnant diabetics. Only 36.7% of our diabetics checked their blood glucose satisfactorily. Another 45% did at least two tests a day. One of the major obstacles was the pain they experienced. The bulky Hungarian made D-Cont glucose meter operating practically only in conjunction with an AC/DC adapter was also perceived as an obstacle. The patients are to be praised for doing so many tests in spite of this condition. A large selection of automated finger pricking devices has become available recently. A new, tiny piece of the new generation of the D-Cont glucose meter family can also be purchased at a bargain price. In the light of the multivariate analysis group education is an effective form of patient motivation. It is interesting that several factors, previously thought to be most important, did not influence the HbAlc at all, e.g. blood glucose self-determination (as experienced by /15/) and completion of a diabetes course. Patients undoubtedly experienced an improvement in their quality of life. This was attributed mainly to the more flexible method of insulin therapy and to the NovoPen injection system (and to the decrease in the number of hypoglycaemias, not analysed in this report). In summary, the blood glucose control of 2/3 of our patients already uncontrollable with conventional methods, improved significantly. No significant differences in age, sex and type distribution, insulin doses, etc., was found between the group practising

true functional therapy and the patients who checked their blood glucose less frequently. Five of the 16 patients whose metabolic status changed for the worse checked their blood glucose satisfactorily, while 11 did not. In these cases a switch to the previous insulin regimen would not be the solution as the patients would not tolerate the fixed regimen. Strict follow up is planned to amend control.

Main determinants of control were the ratios of previous and current insulin doses. It was found that the control achievable by functional insulin therapy was in significant inverse relationship with the daily insulin need, the short-acting insulin dose and the HbAlc during conventional insulin treatment. Thus, the higher these parameters the less successful the therapeutic switch is likely to be. More precise analysis stressed the significance of the short-acting insulin dosage. This means that if a given patient has received most of the insulin in short-acting form during conventional therapy, the increase in the number of insulin injections on functional therapy is not enough to achieve better control, the amount of the short-acting insulin should be increased as well. On the other hand, no further deterioration of control can be foreseen on the basis of the initial insulin dosage. However, an increasing HbAlc value is more likely to be found on functional insulin therapy if the original insulin dose has not been changed or had been lowered. Increasing the dose of the intermediate-acting insulin is, of course, not beneficent.

Although both control and quality of life improved during the follow-up period it is not possible to assess the true value of functional insulin therapy due to the mentioned problems. It must be noted, that while during the first period patients had to be persuaded to accept the theraputic switch, later other patients applied for the change themselves after having been informed on the benefits by the diabetics on functional therapy. Neither did anybody want to switch back to conventional therapy nor did the NovoPen users want the syringes they used formerly. In spite of our previous expectations the use of the method should be studied in a controlled, randomized pattern with special regard to the selection of the proper patients and possible forms of their motivation on the long run.

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LDL MOLECULAR SIZE AS RISK FACTOR IN CORONARY ARTERY DISEASE

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Sera of 65 fasting human subjects — 32 patients with coronary artery disease (CAD) aged 42–80 years and 33 healthy individuals — were tested for determination of nine lipid-related laboratory parameters, including protein-enriched LDL (low density lipoprotein cholesterol (LDL apo B) which is proportional to the amount of cholesterol per LDL particle. Three of the investigated parameters: protein-enriched LDL, HDL cholesterol and apo B level differed significantly in the two groups (corrected $P < 0.001$, $P < 0.009$ and $P < 0.009$, respectively). Discriminant analysis revealed that protein-enriched LDL, LDL cholesterol, apo B and fasting triglyceride levels, but not HDL cholesterol, were the major discriminating factors for CAD in this study.

Pearson correlation coefficients were calculated to describe the association between this size-related parameter and those which in both groups seem to be most strongly associated with it: apo B/A-I ratio (i), triglyceride (ii) and LDL/HDL ratio (iii). The analysis was done separately in the two groups. In the patients with CAD the influence of these three parameters were less decisive in the determination of the protein-enriched LDL than in the controls (corr. coeff.: (i) -0.155 vs -0.358; (ii) -0.624 vs -0.791; (iii) -0163 vs -0.471). In healthy volunteers the size-reducing effect of the same parameters was more profound, and at high values of LDL/HDL ratio, apo B/apo A-I ratio and triglyceride no distinction in LDL particle size can be made anly longer between CAD patients and controls. Thus the improvement of the atherogenic profile does not seem to result in the reduction of risk for CAD in terms of LDL size and composition.

Keywords: LDL, molecular size, risk factor, coronary artery disease, apolipoprotein B, A-I

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Introduction

Research on cardiovascular risk factors is amply justified by the importance of coronary artery disease (CAD) as the most common and highly lethal manifestation of atherosclerosis. There is evidence to suggest that apart from total or even LDL cholesterol content, the apo B concentration in the LDL fraction is another very important factor in the estimation of susceptibility to CAD /4, 22, 26/. The apo B level in LDL is involved in the term "protein-enriched LDL", a designation applied to a quantity defined as LDL cholesterol/LDL apo B /29/.

If it is assumed that cholesterol constitutes about 50% of LDL particles and an additional 20% represents protein — which, unlike lipids, is not subjects to any kind of exchange /10, 19/ — then the amount of cholesterol in a particle is proportional to the particle size. Since each LDL particle bears only one apo B molecule on its surface /19/, the LDL-C/LDL apo B ratio is proportional to the cholesterol content of an individual LDL particle. Thus, LDL molecular size is mainly governed by its cholesterol content. The smaller a LDL molecule usually the higher is its density as a result of the increased proportion of the higher density of apo B molecule /10, 20, 31/.

It seems likely that the progress of atherosclerosis is influenced by the size of low density lipoprotein (LDL) particles in the blood. A plausible explanation of such an association /9/ may be the fact that small particles penetrate the arterial wall more readily than larger ones /14/. There is also a difference in the binding capacity of LDLs to arterial proteoglycans in terms of molecular weight and particle size /32/.

In individuals with premature coronary heart disease LDL particles are smaller and of higher density than those in normal subjects /9, 14/. These observations are consistent with those of Sniderman et al. /26/ and Teng et al. /30/, who reported a decreased LDL cholesterol/LDL apo B ratio in this disease. There is a suggestion that this decreased ratio may be the result of the increase of an LDL subfraction with small relative cholesterol content and particle size /16, 17/.

One of recent controversies started with the introduction and study of protein-enriched LDL as one of the risk factors in CAD. While its importance in this respect is generally agreed, there is argument that it cannot be regarded as an independent risk factor, and implication that HDL cholesterol appears to be the best indicator for CAD /29/. In the present study we

attempted to determine which ones of the studied lipid-related parameters display significantly different values in the CAD patients' group, and also to estimate that as a consequence of their interaction, which one of the routinely — thus frequently — measured parameters is in close correlation with the one of the best predictive value in this disease. These associations are described in detail and a comparison between the investigated groups is made to assess their difference in extent and character.

Subjects and Methods

Patients and controls

Blood samples were taken after overnight fasting (at least 14 h) from 65 subjects of whom 32 were patients with myocardial infarction (27 males and 5 females) from Newfoundland. They were treated and taken regular medical care at the Department of Cardiology of the General Hospital in St. John's. Their average age, 56.5 years, ranged from 42 to 80. Three of the patients were steady smoker, 21 of them quitted smoking during recovery and eight had never smoked. Four patients were on treatment for diabetes mellitus and 6 received antihypertensive medication consisting of beta-blocker and Ca-channel blocker (propranolol and verapamil). The diagnosis of myocardial infarction was based on symptoms, ECG findings as well as enzyme changes (CPK, SGOT or LDH). Among the drugs prescribed upon discharge from hospital there was no cholesterol lowering agent. The patients, however, were instructed to keep a high-fibre cholesterol-poor diet.

Thirty-three healthy individuals (22 males and 11 females) voluntarily gave blood sample for the study as controls. Their age ranged from 17 to 78 years with a mean of 53.2 years.

The blood was drawn into a 10 ml vacutainer container with a 15% solution of EDTA anticoagulant. The plasma was freshly separated after centrifugation at 1500 g for 30 min at room temperature.

At the first step the samples were tested for plasma apo B and A-I concentration by using an immunoturbidimetric assay kit (Isolab Inc.) /25/. An enzymatic triglyceride assay was also performed (Boehringer Mannheim) /18/.

Apo B and A-I determination

0.1 ml of plasma was diluted with 0.5 ml of an aqueous solution containing 0.3% Tween, 0.3% NaCl and 0.1% sodium azide. For apo B determination 0.1 ml, for apo A-I 0.02 ml of the diluted sample was added to a labelled disposable plastic cuvette equipped with cap (available from Isolab: code ID-8200) and containing 1 ml of reaction buffer (PBS of pH = 7.4, 3% PEG-6000 and 0.1% sodium azide). After 0.1 ml of goat antiserum was added the cuvettes were capped and gently shaken. The incubation time at room temperature was 30 min. Light absorbance was tested by using a spectrophotometer at 340 nm. Turbidity is linear with apolipoprotein concentration up to 200 mg/dl. Calibrators are supplied with the kit in the form of lyophilized human plasma with sodium azide as preservative. They are standardized against the CDC-IUIS reference pool for serum apolipoproteins /7/.

Triglyceride assay

The enzymatic triglyceride assay was applied for the BM/Hitachi Systems 704/705. The assay utilizes EDTA plasma samples. The first step was an enzymatic hydrolysis by lipase. The hydrolysis produced glycerol, which was measured as described by Pinter et al. /21/. The substrate mixture consisted of 10 ml of phosphoenolpyruvate (3.5 mol/l), 0.25 ml of ATP (75 mmol/l), 1 ml of NADH (2.5 mg/ml), 0.1 ml pyruvate kinase (2 mg/ml) in 2.2 mol/l ammonium sulfate aqueous solution and 0.1 ml of lactic dehydrogenase (0.5 mg/ml) in 2.2 mol/l ammonium sulfate solution. Light absorbance was measured spectrophotometrically at 340 nm.

Separation of lipoprotein fractions by affinity chromatography

In order to separate the plasma beta-lipoprotein and alpha-lipoprotein (HDL) fractions, the plasma was applied to a heparin-agarose chromatographic column supplied with LDL-Direct Plus kit (Isolab Inc.) /6/.

This procedure is based on the principle of affinity chromatography. The beta-lipoproteins, the term includes those exhibiting beta or pre-beta electrophoretic mobility, bind to the column specifically. The binding is mediated by apolipoprotein E and B at near-physiological pH and ionic strength. However, at higher salt concentration the bound lipoproteins (mainly LDL and less IDL and VLDL) are to be desorbed /13/. Alpha-lipoproteins pass through the column together with several other serum proteins, this fraction contains HDL(2) and HDL(3).

The advantage of this separation method here was its simplicity in comparison with analytical ultracentrifugation, on the one hand, and that the fractions could readily be used for further assays without interference from earlier applied reagents, on the other. Quantitative comparison between the two medtods is given by Bentzen et al. /6/.

In the eluted beta-lipoprotein fractions, which mostly contain LDL, less amount of IDL and VLDL particles, the apo B level and cholesterol concentration were measured. Since the blood was taken after fasting, the amount of chylomicrons can be ignored. The method for apo B determination in the beta elution fraction was the same as the one referred to: immunoturbidimetric assay.

Cholesterol assay

The total plasma cholesterol as well as the cholesterol in elution fractions was measured by a modification of the method described by Allain et al. /1/. The assay procedure requires enzyme reagent (1.6 mmol/l 4-aminoantipyrine, 5560 U/l peroxidase and 400 U/l cholesterol oxidase in phosphate buffer of pH = 6.7) and activator (40 mmol/l phenol in aqueous solution). Incubation period 20 min at room temperature. Light absorbance was measured with spectrophotometer at 505 nm.

The obtained values of apo B and cholesterol levels in the beta elution fraction were applied to estimation of the LDL cholesterol/apo B ratio. The determined values of apo B and A-I levels in the unfractionated plasma, on the other hand, were used for calculating the apo B/apo A-I ratio.

Statistical methods

Difference between patients and control was tested for significance by using Student's *t*-test. In order to describe the studied associations we calculated Pearson correlation coefficients for statistical evaluation. A stepwise discriminant analysis was carried out to find a set of variables (laboratory tests) which, as discriminant functions, can be used to achieve a maximal segregation of CAD patients and controls. The stepwise analysis also estimates the order of importance of these variables in terms of their discriminative power. The computer programme automatically stops when the addition of new data does not result in a higher segregation rate than the indicated one.

Results

Serum lipids in patients and controls

The results of the apolipoprotein (B and A-I), triglyceride and cholesterol determinations in the investigated blood samples are shown in Table I.

Table I

Serum lipids and lipoproteins in coronary artery disease patients and normal subjects

Parameters	CAD patients n = 32		Controls n = 33		Significance level (corr.P)
Apo B (mg/dl)	88.5	(19.7)	71.7	(15.6)	$P < 0.009$
Apo A-I (mg/dl)	99.6	(17.2)	96.3	(20.8)	ns
Apo B/A-I	0.917	(0.254)	0.768	(0.197)	ns
Tot.chol. (mmol/l)	5.03	(0.87)	5.13	(0.97)	ns
LDL chol. (mmol/l)	3.44	(0.76)	3.76	(0.91)	ns
HDL chol. (mmol/l)	0.92	(0.31)	1.22	(0.39)	$P < 0.009$
LDL-C/LDL apo B	0.0508	(0.007)	0.0573	(0.006)	$P < 0.001$
Triglyc. (mmol/l)	1.77	(0.97)	1.53	(0.66)	ns
LDL/HDL chol.	4.17	(1.67)	3.28	(0.95)	ns

All values are mean $\pm$ S.D., ns: non significant

The size-related protein-enriched LDL, which is proportional to the cholesterol content of an individual LDL practicle, and HDL cholesterol were found to be significantly decreased in CAD patients (corrected $P < 0.001$ and $P < 0.009$, respectively) (Table I). We also experienced an elevated apolipoprotein B concentration in the patients' group (corrected $P < 0.009$) despite their moderately reduced LDL cholesterol level, which might be due in part to a cholesterol-poor diet, for the patients have already been on treatment for several months.

The apo A-I level was about the same in CAD patients as in the controls — thus it was not considered to be an important risk factor, though Yano et al. /33/ found it otherwise. The rest of the parameters — apo B/A-I, triglyceride and LDL/HDL cholesterol, on the other hand, showed a somewhat elevated value, never reaching the limit of significance.

The elevated serum triglyceride level in patients was thought to be responsible for the observed higher apo B concentration, but after closer

Table II

Discriminant analysis in 65 subjects with the diseased state as grouping variable

Step	Function	Parameter	Equivalent F	Significance: P<
1.	add	apo B	11.411	0.0017
2.	add	LDL-C	12.061	0.0001
3.	add	triglyceride	14.879	0.00001
4.	add	LDL-C/LDL apo B	13.300	0.00001

Percent of cases correctly classified: 90.57%

scrutiny it was revealed that apo B level was higher in CAD than in controls in cases with practically the same triglyceride levels.

In order to estimate the discriminative power of these parameters for CAD we applied a discriminant analysis to the experimental data. When all the variables were included in the analysis, the best segregation of patients and controls based on laboratory data was achieved after step 4, by introducing the data of protein-enriched LDL (LDL-C/LDL apo B) (Table II).

The parameters which are not shown in Table II, although were included at the start, -- including HDL-C -- were not necessary to achieve the maximal segregation of patients and controls. Since the addition of these data could not have made a better distinction possible between the two groups, the predictive value of these missing parameters is less than of those in Table II. This also suggests that the significant association between HDL-C and CAD may be only secondary to the connection between HDL-C and apo B or LDL-C/LDL apo B, data also showing strong association with CAD.

Table III

Correlation coefficients between lipid-related parameters in coronary artery disease and normal control

Investigated parameter	Associated parameters	Correlation coefficients Patients	Controls
Protein-enriched LDL	Triglyceride	-0.6242	-0.7906
	HDL cholest.	0.4409	0.3757
	apo B/A-I	-0.1551^{+}	-0.3577
	LDL/HDL cholest.	-0.1626^{+}	-0.4713
			P < 0.05

We then focused our attention on the parameter of the most significantly decreased value: protein-enriched LDL. Calculating the Pearson correlation coefficients in each of the two groups, the results are summarized in Table III.

Protein-enriched LDL: different patterns of variation in CAD

In patients with CAD the size-related protein-enriched LDL exhibits the best correlations with triglyceride, HDL, LDL/HDL ratio and apo B/A-I ratio, in a decreasing order (Table III) while in the control group, these associations are all stronger, except for that of HDL with protein-enriched LDL. An association between these lipid-associated parameters is not really unexpected, but it is questionable whether there are differences in the extent and relative prominence of these associations between patients and controls.

LDL-C/LDL apo B in both groups is decreasing with triglyceride concentration but there is an obvious difference in the correlation coefficients. The relative cholesterol content of LDL and consequently its size, though smaller in CAD, does not depend so much on triglyceride level as in controls, the decrease is less pronounced.

The second parameter associated with protein-enriched LDL is HDL cholesterol -- which by itself showed a significant association with the disease. The correlation between these two risk factors makes it even more difficult to tell whether this association of HDL with CAD is a primary one or only secondary to that of LDL-C/LDL apo B.

In our study, with limited number of patients though, it is suggested that the association of CAD with HDL is only secondary as shown by the result of the discriminant analysis (Table II). HDL level, significantly reduced as it is in CAD patients, did not belong to the variables of most discriminative power, between diseased and controls.

The correlation coefficients featuring the two remaining parameters, LDL-C/HDL-C and apo B/A-I ratios, with protein-enriched LDL are very similar to, and consistent with, the strong correlation between the two ratios (not shown here). In the control population LDL-C/LDL apo B values are higher for most cases and for both parameters. It is worth mentioning that protein-enriched LDL is much more dependent on both ratios in the control group than in patients. This is clearly shown by the Pearson correlation coefficients, being twofold in controls (Table III).

Discussion

Significantly low levels of protein-enriched LDL and HDL cholesterol were found in CAD patients, confirming literary data /16, 26, 30/. Although, based on our unpublished data, there is a slight association between protein-enriched LDL and age, in this study the different mean age in our groups is to account for only one-seventh of the standard deviation for LDL-C/LDL apo B in the patients. Normal levels of LDL-C were detected, associated with a disproportionate increase in apo B as has already been reported in CAD and is thought to contribute to the acceleration of atherosclerosis /4, 22, 26/.

It is worth mentioning that the LDL-Direct Plus kit we used for the isolation of the beta-lipoprotein fraction cannot distinguish between VLDL, IDL and LDL, though LDL certainly represents the majority of the particles. In spite of this pitfall, the determined LDL-C/apo B ratios were still acceptable for a statistical evaluation, because IDL-C/apo B values are approximately the same /12, 24/ and VLDL, on which no similar data have yet been available, constitutes only 7-8% of the particles in the beta fraction. Therefore, the presence of IDL and VLDL is thought not to have a profound effect on the associations outlined here.

The conventional method for LDL-C calculation (LDL-C = Tot.C — HDL-C — triglyc./5) was not applicable here either, because VLDL-C was not to be subtracted when VLDL-apo B was measured.

Although the separation of LDL by ultracentrifugation could have been a more exact approach, the procedure we used is rapid, more convenient to perform and as such, it has a better chance to become daily routine for screening to detect disease susceptibility for CAD.

In a discriminant analysis we found apo B, LDL-C, triglyceride and protein-enriched LDL to be the best discriminative variable of all the studied serum lipids, as reported also by Barbir et al. /5/. The 90.57% segregation of cases required the involvement of these parameters, but no HDL cholesterol came into play in the analysis in spite of its significant difference in the Student's t-test between the two investigated groups. In our opinion — unlike the conclusion of Swinkels et al. /29/ — the size-related protein-enriched LDL has more discriminative power for CAD than does HDL.

There is no doubt about the existence of a correlation between protein-enriched LDL and HDL cholesterol but the first seems to be a risk

factor to CAD more important than the latter. Steiner et al. /28/ also rejected an association between CAD and HDL cholesterol by using stepwise logistic regression analysis. They claimed, however, that CAD showed a strong association with smoking and intermediate-density lipoprotein apo B. Since we only included serum lipids in the analysis therefore smoking was omitted and because the antibody we used in the immunoturbidimetric assay cannot distinguish between IDL and LDL apo B we can not be more specific here. However, there are indications that apo B/C ratios in IDL closely approximate those in LDL /12, 24/. Among serum lipid parameters the triglyceride level showed the strongest association with protein-enriched LDL /11, 15, 23/. A plausible explanation for the fact lies in the action of the concentration-dependent triglyceride:cholesteryl ester exchange protein, whereby LDL and IDL particles become enriched in triglyceride and lose cholesteryl ester /10/. The size reduction is probably the consequence of continuing lipase action on triglyceride. Although this correlation was significant in both groups alike, triglyceride level did not seem to effect the cholesterol depletion of the LDL particles in the patients as much as it did in controls.

Amos et al. /2/ reported that results of segregation analysis were consistent with major gene determination of apo B and HDL-C levels, the HDL-C to apo A-I ratio, the LDL-C to apo B ratio and a measure of relative content of cholesterol in HDL-C and LDL-C. If such gene(s) predisposing to CAD does exist then it would certainly set limits to the influerce on LDL size of environmental factors like triglyceride level in blood, which may be an explantation for the reduced triglyceride dependency. One of the possible assumptions may be that as a result of this gene effect the activity of the mentioned lipid exchange protein is altered in CAD /27/. Nevertheless, hypertriglyceridaemia is an important risk factor for CAD as recently confirmed by Barbir et al. /5/, using a discriminant function analysis. In this work LDL apo B was the second most important discriminant variable, suggesting an, at least partly, independent way of action of these two parameters in conferring susceptibility to CAD.

As with triglyceride, similar conclusions were drawn with regard to LDL/HDL cholesterol and apo B/A-I ratios. The LDL-C/LDL apo B ratio in the patients did not vary with these parameters considerably. In contrast to triglyceride, these associations proved to be significant in the control group alone.

Earlier observations indicated that individuals with normal levels of total and LDL cholesterol were still at an increased risk of CAD if their apo B level is high (hyperapo B syndrome) /26/. Our findings confirm this and are in accordance also with the observation that apo B correlates with the disease better than LDL cholesterol does /4, 8/. Part of the higher risk in these cases can apparently be attributed to the smaller size of the LDL particles.

The small relative cholesterol content and particle size is probably due to the predominance of LDL subclass pattern B in CAD, reportedly associated with increased apo B and triglyceride levels and decreased HDL cholesterol, recently claimed to be inherited as an autosomal trait /3/. Such a genetic determination might explain for the observation that factors of strong influence in controls like triglyceride level or apolipoprotein B/A-I and LDL-C/HDL-C ratios do not seem to effect LDL molecular size or composition in CAD so much.

In the light of our data it is suggested that in patients with strong genetic predisposition any improvement in atherogenic profile characterized by LDL/HDL cholesterol and apo B/A-I ratios must be regarded and interpreted more cautiously in terms of its real value in reducing the risk represented by low LDL molecular size for CAD. To a less extent this also applies to serum triglyceride level. Genetic predisposition is certain in case of patients with multiple family cases. The presence of cardinal non-genetic and non-lipid risk factors like smoking, stress, etc., may have similar consequences. However, it is important to emphasize that overall risk for CAD as a multifactorial disorder consists of several components, LDL molecular size being only one of them. Thus the effect of the same parameters, of course, can be quite different on the overall risk, the investigation of which is beyond the scope of this study.

Conclusion

According to our findings it seems likely that the cholesterol content and size of apo B bearing lipoprotein particles in the beta fraction are factors of major importance in coronary atherosclerosis. Unfavourable atherogenic profile expressed as high LDL/HDL cholesterol as well as apo B/A-I ratios and high serum triglyceride level are associated with small LDL molecular size, but in the possible presence of genetic predisposition

and/or other major environmental risk factors like smoking, stress, etc. the risk for CAD represented by low protein-enriched LDL may remain considerably high in spite of an improvement of the mentioned lipid parameters.

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OCCUPATIONAL HEALTH

GENOTOXIC EFFECTS OF OCCUPATIONAL EXPOSURE IN THE PERIPHERAL BLOOD LYMPHOCYTES OF PESTICIDE PREPARING WORKERS IN HUNGARY

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Venous blood samples of 240 donors including 33 industrial and 60 historical controls were investigated in order to assess the genotoxic risk of pesticide preparing workers manufacturing monochlorinated benzene in Hungary. Mutation frequencies were determined in the hypoxanthine-(guanine)-phosphoribosyl transferase genes located on the X chromosome. DNA repair capacity was estimated following hydroxyurea treatment with subsequent UV irradiation of separated lymphocytes. Smoking as confounding factor of genotoxicity was also taken into consideration. Mutation frequencies were increased among the workers exposed to monochlorinated benzene in correlation with the duration of working time, compared to the controls. Mutation frequencies were lower than expected among non-smoker, long-exposed workers. Smoking itself proved to be an effective confounding factor in the enhancement of point mutations in the case of long-exposed workers. Smoking, however, caused no significant increase in the mutation frequency among the controls, and did not influence the DNA repair capacity of any of the groups.

Keywords: Genotoxicity, lymphocyte, pesticide, HPRT-locus, DNA-repair

Abbreviations: HPRT = Hypoxanthine-(guanine)-phosphoribosyl transferase; LI = Labeling index; MAC = Maximum allowable concentration; MF = Mutation frequency (LI(PHA)); PBL = Peripheral blood lymphocyte(s); PCA = Perchloric acid; PHA = Phytohaemagglutinin; PHA% = Proliferative response of cells to PHA treatment; RC = De novo DNA synthesis, 'DNA repair capacity'; SCE = Sister chromatid exchange; SSB = DNA single-strand break; 6-TG = 6-thioguanine; UV = Ultraviolet light (approximately 250 nm); VF = Variation frequency of HPRT mutations

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Introduction

The estimation of the genotoxic effects including the frequency of mutations (MF) and the repair capacity (RC) of the existing mutations arising from occupational and/or environmental exposure. is especially important at those populations which are known (or suspected) to be exposed to genotoxic hazards in working places. These are e.g. the workers of the chemical industry.

The problems are the detection, estimation and evaluation of the effects of long-lasting and low-level exposure to carcinogens and co-carcinogens. The human population monitored by different biological markers including cytogenetical methods may help to solve these branching problems /10, 11/ and repeated examinations would show the tendencies of the followed cytogenetic alterations. Although there are molecular and cytogenetical techniques in the human risk assessment /4, 19/, the dosimetry and the dose-response relationships are still unsolved /3/.

The detection of mutations in the hypoxanthine-(guanine)-phosphoribosyl transferase (HPRT) locus of the X chromosome is a frequently used test system in the human risk assessment among other biological markers /10, 12, 17/. As the HPRT locus is located on the X chromosome in humans, a single copy is only active in lymphocytes irrespectively of the donor's sex. However, any form of the mutations is sufficient to the loss of HPRT phenotype, i.e., the manifestation of the resistance to the cytotoxic purine analogue 6-thioguanine (6-TG) treatment /3/. An autoradiographic assay has been developed by Strauss and Albertini /12/ in order to detect the mutant (i.e., 6-TG resistant) lymphocytes.

The estimation of the capacity of DNA excision repair induced by short wave ultraviolet light (UV) irradiation by measuring the unscheduled DNA synthesis can also serve the assessment of the genotoxic effects of an exposure as DNA repair can eliminate the consequences partly or totally /9/. The measurement of unscheduled DNA synthesis requiring a relatively small amount of cells is simple and it is a practical method to estimate the DNA repair capacity (RC).

In a previous publication Tompa compared the changes in MFs among cancer patients and industrial workers /15/. Although these studies gave significant results the disadvantages of the lack of the comparability of the results to other end points of the same sample were clear. Parallel estimations of different end points as e.g. MFs and RCs of the same sample

may give a better understanding of the late genotoxic effects of industrial hazards at working places.

The aim of our present study was to estimate the genotoxic effects of the working environment in samples of peripheral blood lymphocytes (PBLs) of workers manufacturing the pesticide Lutidin of monochlorinated benzene. The comparabilitiy of two parallelly determined end points may improve the confidence of the risk assessment. Therefore, we have measured 6-TG resistance of PBL (VF) /14/ and the unscheduled DNA synthesis after 250 nm UV light irradiation (RC) /1/. Data taken from the exposed groups of pesticide workers were compared to those of historical and industrial control groups. Smoking as a confounding factor of genotoxicity /6, 16/, was also taken into consideration.

The genotoxic effects of chlorinated benzenes /18/ and some derivatives were extensively studied in vitro by Fishbein /5/, and Gad-el-Karim et al. /7/ who demonstrated that these benzene derivatives were tumour promoters. Boutwell et al. reported similar conclusions in their early observation /2/.

Materials and Methods

1. Separation of lymphocytes

Samples from 240 donors were taken by venipuncture and prepared immediately. The donors included 147 exposed workers, 33 industrial controls working at the same firm but in a harmless environment and 60 historical controls. All the samples were taken and prepared in the same way. Grouping 1 to 5 of the donors by sex, age and smoking habit is presented in Table I.

Table I

Distribution of control and exposed donors by sex, age, and smoking habits

Groups	n	Average age ± S.E.	Men		Women		Smokers		Non-smokers	
			n	%	n	%	n	%	n	%
1 Historical control	60	39.1 ± 1.6	10	16.7	50	83.3	28	46.7	32	53.3
2 Industrial control	33	36.1 ± 1.5	24	72.7	9	27.3	20	60.6	13	39.4
3 Workers exposed for 0 to 1 year	45	28.0 ± 1.9	40	88.9	5	11.1	24	53.3	21	46.7
4 Workers exposed for 1 to 10 years	41	30.0 ± 1.9	25	61.0	16	39.0	19	46.3	22	53.7
5 Workers exposed for > 10 years	61	45.8 ± 2.6	46	75.4	15	24.6	38	62.3	23	37.7
Total	240	36.2 ± 1.7	145	60.4	95	39.6	129	53.8	111	46.2

Twenty ml of heparinized and again 20 ml of citrated blood samples were taken from each donor in order to determine 6-TG resistance (VF) and the unscheduled DNA synthesis (RC), respectively. Lymphocytes were separated on a Ficoll-Hypaque density gradient as it was described elsewhere /15/.

2. Determination of 6-TG resistance

Lymphocytes separated from heparinized blood samples were washed twice in Hanks' balanced saline buffer (Gibco). Cells were counted in Buerker's chamber then were adjusted to 10^6 cells/ml in RPMI-1640 culture medium (Gibco) supplemented with 30% fetal calf serum (Gibco) without antibiotics. Three groups of cultures were made in triplicates of each donor. Untreated control cells of group a) were only cultured in RPMI-1640 medium, cells of group b) were stimulated with 0.1% (v/v) Phytohaemagglutinin-P (PHA, Difco) in culture medium, while in group c) the PHA stimulation was supplemented with 10^{-4} M 6-thioguanine (6-TG, Sigma) treatment. Cells were cultured in glass centrifuge tubes and incubated for 24 h at 37 °C in a humidified CO_2 thermostat (Labor MIM, Hungary). Six hours before termination, cells of each culture were labeled with 1 µCi/ml (^{3}H)-thymidine (Amersham).

3. Estimation of DNA repair capacity

Lymphocytes separated from citrated peripheral blood samples were washed twice in Hanks' balanced saline (Gibco), supplemented with 10% of 0.12 M citric acid (Reanal, Budapest). After counting in a Buerker's chamber lymphocytes were adjusted to 3×10^6 cell/ml in citrated Hanks' balanced saline. Control (group a) remained untreated. The cells of group b) were treated with 40 µl/ml, 0.125 M hydroxyurea (HU, Reanal, Budapest). Those of group c) were treated with HU plus irradiated with 250 nm UV light (10 s, 60 cm). All groups were made in duplicates in Petri dishes. Cells were subsequently labeled with 5.0 µCi/ml (^{3}H)-thymidine (Amersham) and incubated for 60 min at 37 °C in thermostat. DNA was pelleted by a three times repeated washing of the labeled nuclei with 1 M perchloric acid (PCA, Reanal) then hydrolysed by 0.5 M PCA (30 min, 88 °C). After centrifuging at 2000 G the supernatant was cooled down and 0.3 ml aliquot was transferred into 10 ml scintillation cocktail containing toluene (Reanal), Triton-X-100 (Reanal), 0.2% 2,5-diphenyl-oxasol (Reanal) and 0.05% 1,4-bis-2-(5-phenol-oxasolyl)benzene (Reanal). DNA contents were measured by photometry at 580 nm (Spekol, K. Zeiss, Jena). Scintillometry was performed in LKB 1211 RACK BETA scintillometer /1/.

4. Biometrical analysis

a) Calculation of VF

The ability of cells to incorporate (^{3}H)-thymidine was determined by autoradiography. 2500 PHA stimulated cells were counted in order to calculate the labeling index for PHA (LI(PHA)). In the TG-treated group (PHA + TG) the total number of cells placed on coverslips was counted in a Buerker's chamber before smears were prepared. MF is identical with the labeling index of the 6-TG treated cells, therefore MF = LI(PHA + TG). VF (i.e., variation frequency of mutations) was calculated by the equation of VF = LI(PHA + TG)/LI(PHA) /17/. VF is different from MF for including the frequency of the lectin-stimulated cells. The significance of elevations in MFs and VFs were statistically analysed using the non-parametric Mann-Whitney test applied to the ranked measurement /20/. The selection of workers both for exposed and non-exposed groups was performed randomly. Sex, age, and smoking habit were considered as confounding factors.

b) Estimation of RC

In order to estimate the RC values we computed the DNA content of the samples using the equation DNA(µg) = ε(st)x100 µg/ε (sample), where ε(st) is the extinction of the 100 µg/ml DNA

standard (DNA, Sigma) and ε(sample) is the extinction of the given sample. RC was estimated following the formula RC = (R-HU)/HU, where R is the average dpm/DNA(μg) value of the UV irradiated and HU is the average dpm/DNA(μg) value of the UV-plus-HU treated samples of the donor /1/. Statistical analysis was performed with the Student's t test.

Results

The concentration of aromatic solvents in air samples of the studied working places measured regularly by the Safety Department of the firm are summarized in Table II and are compared to the Hungarian MAC values. It is clear that the actual aromatic solvent concentrations exceed the corresponding MAC values.

Table II

A comparison of concentrations of aromatic solvents in the air samples of the working rooms to the Hungarian MAC values

Solvent	Concentration in air samples (mg/m^3) Average ± S.E.	Range	MAC values (mg/m^3) (Hungary)	Phenol concentration in urine (mg/L) mean ± S.E.
Benzene	21.2 ± 7.06	2.8 - 134.0	5.0	Control donors:
Monochlorbenzene	33.5 ± 17.77	1.9 - 173.4	50.0	n = 23 50.0 ± 40.7
Chloroform	72.2 ± 6.35	30.0 - 99.0	20.0	Exposed donors:
Methanol	39.1 ± 5.78	16.0 - 80.0	50.0	n = 18 602.3 ± 89.3
Toluene	91.2 ± 12.54	41.0 - 153.0	100.0	

The 147 exposed donors were examined for genotoxicology end points as well as for routine clinical check up. Increased phenol excretion was found in the urines of 23 donors with a mean of 602 mg/L, which is 12 times as high as the expected (normal) level (cf. Table II). Eight of these 23 donors had also qualitative alterations in blood smears although they had no significant differences in their genotoxicology tests when compared to non-exposed (phenol-free) control donors. Detailed results of the routine clinical check up will be published later.

Proliferative response of lymphocytes to PHA treatment

Table III summarizes the results of the genotoxicity test. Within each groups (groups 1 to 5) the proliferative response of T-lymphocytes (PHA%) of smokers and of non-smokers to PHA stimulation did not differ significantly except group 2 (industrial controls) where PHA% of smokers and of non-smokers were on the average of 21.2% and 13.8%, respectively ($P < 0.01$). In group 1 (historical control) the corresponding PHA% values were 19.5% and 18.3%, respectively. The average PHA%s of the (exposed) groups 3, 4, and 5 were 20.8%, 21.3%, and 18.3%, respectively.

Mutation frequencies

In group 1 the frequency of the mutant cells (MF) according to the labeling index (LI(PHA + TG) was on the average 0.57. The MF values were calcu-

Table III

Results obtained on the genotoxical end points of the exposed and control groups of donors

Groups	n	PHA stimulation index (PHA%) mean ± S.E.	Mutation frequencies (MF) mean ± S.E.	Variation frequencies (VF) mean ± S.E.	DNA-repair capacity (RC) mean ± S.E.
1 Historical control	60	18.90 ± 1.43	0.57 ± 0.07	3.27 ± 0.42	5.07 ± 0.29
Smokers	28	18.32 ± 1.46	0.54 ± 0.10	3.31 ± 0.63	5.89 ± 0.11
Non-smokers	32	19.52 ± 1.45	0.59 ± 0.10	3.19 ± 0.56	4.40 ± 0.77
2 Industrial controls	33	18.32 ± 2.19	1.83 ± 0.32	10.00 ± 1.74*	8.03 ± 0.39
Smokers	20	21.20 ± 1.74	2.19 ± 0.49	10.80 ± 2.42*	8.84 ± 1.98
Non-smokers	13	13.81 ± 1.83	1.31 ± 0.36	8.83 ± 2.45*	6.83 ± 1.88
3 Workers exposed for 0 to 1 year	45	20.77 ± 1.30	3.75 ± 1.17	16.41 ± 3.21*	8.25 ± 0.36
Smokers	24	21.45 ± 1.68	1.81 ± 2.06	12.80 ± 2.44*	9.33 ± 0.42
Non-smokers	21	19.99 ± 2.00	4.61 ± 1.51	20.43 ± 6.30*	7.11 ± 0.53
4 Workers exposed for 1 to 10 years	41	21.31 ± 1.66	3.13 ± 0.58	25.01 ± 9.11*	6.78 ± 0.48
Smokers	19	19.04 ± 2.57	3.12 ± 0.75	32.33 ± 16.38*	7.75 ± 0.67
Non-smokers	22	19.83 ± 2.06	4.67 ± 1.58	20.86 ± 6.56*	7.32 ± 0.53
5 Workers exposed for > 10 years	61	18.29 ± 1.30	3.53 ± 0.99	23.03 ± 5.16*	7.89 ± 0.35
Smokers	38	19.04 ± 1.78	4.49 ± 1.49	28.32 ± 7.45*	8.35 ± 0.43
Non-smokers	23	16.83 ± 1.86	1.73 ± 0.36	13.94 ± 4.59*	6.90 ± 0.53

*Significantly different from historical controls, $P < 0.01$ (Mann–Whitney test).

lated for 10^5 cells. In the exposed groups 3, 4, and 5, MFs slightly increased, the average MFs were 3.8, 3.1, and 3.5, respectively. MF values for the smokers and non-smokers differed significantly in the exposed group 3 (cf. Table III) where MF of smokers proved to be identical with that of group 2, while in the case of the non-smokers, MFs were 3.5 times as high as that for the industrial control (4.6, and 1.3, respectively). The difference between MFs for smokers and non-smokers did not increase in group 4 (3.1, and 4.7, respectively). In group 5 the tendency, however, changed as MFs of the smokers proved to be still increasing to 4.5, while in the case of the non-smokers of Group 5 the average MF fell to the control level (MF = 1.7).

Figure 1 indicates the distribution of the variation frequencies (VFs) of the mutations in a point diagram. As there were no significant differences between PHA%s for smokers and those for non-smokers of the exposed groups there were also no essential differences in the correspondig VFs. In

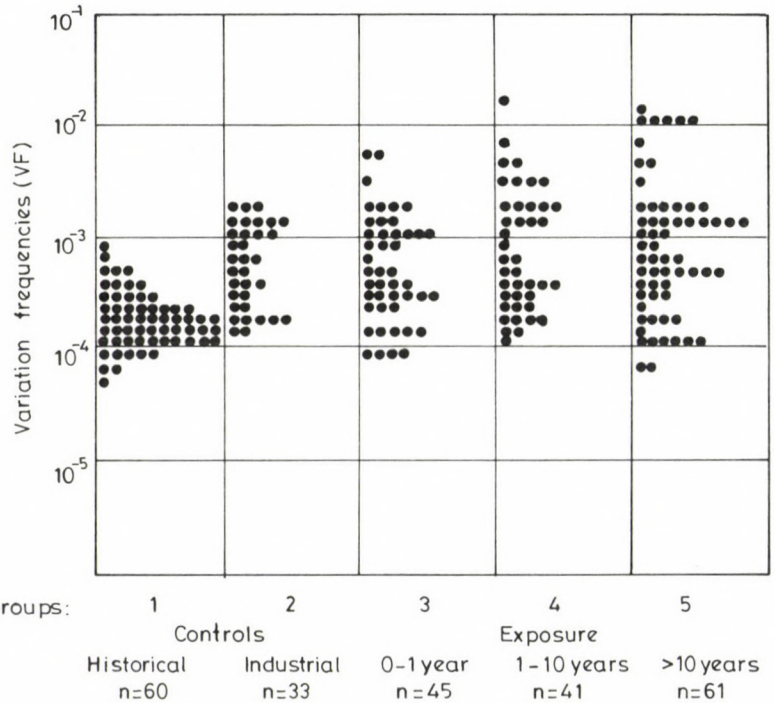

Fig. 1. Point diagram of the VF values in the examined groups of workers (VF values increase in each exposed groups parallel to the length of exposure, cf. details in the text)

contrast with the effect of smoking as a confounding factor, VFs of groups 1 to 5 were on the average of 3.27, 10.0, 16.4, 25.0, and 23.0, respectively, showing slight increase according to the length of exposure.

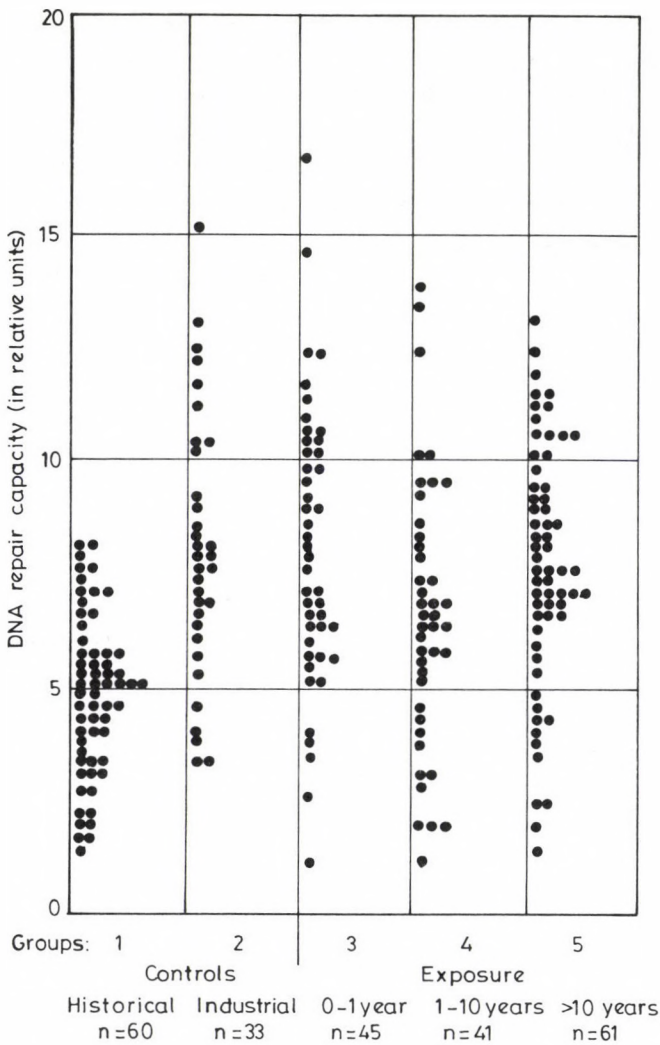

Fig. 2. Point diagram of the distribution of DNA repair capacity values in the examined groups of donors

(An increase of RCs in the industrial controls' group is observable when compared to the historical controls. Chemical exposure, however, did not produce a further increase of RCs, cf. details in the text)

DNA repair capacity

The last column of Table III demonstrates the results obtained from the DNA repair capacity tests. There were no significant differences in RCs between smokers (5.9) and non-smokers (4.4) of group 1. In group 2 only RCs of non-smokers (6.8) correspond to the controls (on the average 5.07) while smokers' RCs, however, were higher (8.8). A virtually similar tendency of the smokers' and non-smokers' RCs was also found in the case of the exposed groups, but the differences were not significant. The detailed RC data are demonstrated in the point diagram shown in Fig. 2.

Discussion

In the present study we examined 240 samples taken from donors of 60 healthy (not exposed, historical control), 33 industrial control donors and of 147 employees of a firm producing pesticides in Hungary. This latter exposed donors' group was divided into subgroups according to the duration of the exposure. The smoking and the non-smoking habits of all the donors were also taken into consideration as confounding factors of the genotoxicity.

The expected level of PHA% according to the controls was on the average 18.9%. In group 2 (industrial control) the non-smokers' PHA% values (13.8%) reduced significantly ($P < 0.01$) when compared to that of the smokers (21.2%). This finding is exceptional and difficult to explain since no similar difference is virtually existing in the case of the exposed donors regardless of the duration of exposure. PHA% is indeed increasing according to the duration of the exposure as it is in groups 3, and 4, but smoking does not seem to be an effective differentiating factor. In the case of donors of group 5, who suffered from the longest exposure, the capacity of lectin stimulation of T lymphocytes slightly reduced and returned to the control level.

Examining the frequency of the mutant cells we observed that the short-term exposure increased MFs, the medium exposure resulted in no further increase, while long-term exposure over 10 years elevated again MF to a certain extent. In the case of non-smokers the short-term and medium exposure resulted in a significant increase of MFs when compared to that of smokers of the same group. In long exposed donors, however, MFs for non-

smokers fell to the control level, while MFs for smokers of group 5 still increased. These suggest that smoking as a confounding factor enhances the genotoxic effects of the halogenated benzene derivatives in the case of a chronic exposure. This enhancing effect of smoking is, however, hidden when in the case of an acute or sub-chronic exposure, an illusory reduction of MFs occurs. The true background of this illusion is not clear but adaptive mechanisms as e.g. an increase of DNA repair capacity is suggested. The exhaust of these adaptive mechanisms leads probably to the dramatic increase of MF in the case of the long-term exposed workers.

Stimulation (PHA%) is an important factor of VF according to the mathematical formula of Strauss and Albertini /14/. As no significant differences of PHA% values were found among the exposed smoker and non-smoker donors no significant alterations were observed in their VFs. VF values increase parallel to the PHA% according to the length of exposure and remain stable at a high level in group 5. Chemical exposure depending upon the time increases the average variation frequencies of mutations. Figure 1 demonstrates that there are subpopulations of the chemically exposed groups (groups 3, 4, and 5) with considerably high VF.

The average DNA repair capacity of the industrial controls (group 2) increased 1.58 times as compared to that of the historical control (group 1, in Figure 2). No further increase was observable in the exposed groups indicating that the influence of the chemical exposure is not significant. The working environment itself regardless of the dominating exposing factor has a stimulatory effect upon the RC. A tendency of RCs is clear in Figure 2, i.e., chemical exposure increases RCs independently of its lenght. Smoking, as it is indicated in Table III, trends to be a confounding factor increasing RCs slightly but not significantly in all cases.

These results seem to confirm our hypothesis /15/ suggesting that human T lymphocytes are able to accommodate to chemical affections by adaptive mechanisms. Smoking itself may give a permanent stimulatory effect helping the immune system in the development of adaptive responses. In non-smokers, such an adaptation to chemical exposures takes approximately 10 years. However, chemical exposure induced lower MFs in smokers during the same period, but over 10 years of exposure MF showed a further increase. In contrast, VF do not increase permanently according to the duration of exposure it remained stable in the long-exposed group. As VF includes PHA% which had been decreased to the control level in group 5, this stabilization seems to be explainable by a reduced PHA stimulation of PBLs. Tompa observed the

same tendency among uranium miners and aluminium plant workers (unpublished data).

The expression of the DNA damage as a mutation needs certain time /13/ depends upon the individual sensitivity, the actual proliferation rate, and DNA repair capacity of the target T lymphocytes. The repair processes can minimize or even eliminate mutations as consequences of DNA damage /9/. Therefore donors with intact repair system have also less risk of persisting mutations at a given chemical exposure than those with decreased repair capacity.

Since the estimation of RC is an essential informative factor in risk assessment, it is important to measure the amount of the primary DNA lesions e.g. single-strand breaks (SSB) /8/, or DNA adducts. For this purpose there is an urgent need for adequate and accurate methods in population monitoring /10, 11/. In the case of occupational risk assessment we also need further information about other end points as chromosome aberrations reflecting to true mutational events, and SCE. In this prescreening we would have data about the very first steps of DNA damage occurring as SSBs and SCEs forecasting the conversion of DNA damage into mutation.

This proposed more complete panel can help in amore accurate understanding of the genotoxic consequences of the occupatuonal and/or environmental chemical exposure and can also help in a correct occupational risk assessment.

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IMMUNOLOGY

RELATIONSHIP OF SERUM ANTIHISTONE ANTIBODY LEVEL TO THE PATIENT'S AGE

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The serum antihistone antibody (AHA) positivity of patients with various autoimmune diseases was compared with their positive reaction for antinuclear factor, rheumatoid factor, lupus erythematosus factor, kryoglobulin, immunocomplex, C-reactive protein, total protein, gamma globulin, IgG and IgM. In non-drug-induced SLE cases the predictive value of the AHA test was not higher than that of the other tests. It was striking that in 42% of patients with non-autoimmune disease aged over 70 the AHA test was positive. Elevated IgM values were recorded in about 70% of positive AHA samples.

Keywords: Antihistone antibody, autoimmune diseases, age, immunoglobulins, myeloma

Introduction

A special feature of autoimmune (AI) diseases is an immune response characterized by the appearance of antibodies generated to antigens hardly demonstrable, if at all, in healthy subject. The commonly known antigens are localized in intracellular compartments and are highly conserved molecules such as DNA, histones and essential enzymes. Currently the view is held that autoimmune diseases can be diagnosed, first of all, by a variety of what are called "sets" of antinuclear autoantibodies (ANA) raised against the nucleus, nucleolus or cytoplasmic antigens. These "sets" are either disease-specific (anti-native DNA; anti-SM: in SLE) or may be present in several diseases, varying in prevalence (antihistone in SLE, in drug-induced lupus)

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/1, 5, 15/. Of the disease-specific "sets", the histone antigens were of special interest for us.

In our earlier studies on the bio- and histochemistry of glycated histones /8, 11, 12/ we also reported that in the liver cell nuclei of patients who had died of diabetes mellitus the level of glycated histones was found slightly elevated — in contrast with healthy subjects, who showed no such elevation /9/.

Therefore, in collaboration with the Biotechnical Laboratory of the University of Pécs, we launched a programme to prepare an antiglycohistone antibody and to develop an ELISA test. These will be reported elsewhere. On the other hand, we have studied the incidence and prevalence of antihistone antibody (AHA) in autoimmune diseases /4, 15, 18, 25/. It was in the course of these studies that an increased AHA positivity of elderly subject, mainly in those over 70 years of age, made itself conspicuous. Our investigation into this problem is presented here as a first attempt.

Material and Method

We examined the serum AHA activity in patients treated for autoimmune diseases in the 1st Department of Medicine, Department of Dermatology and Department of Neurology of the University Medical School of Pécs for years. The clinical diagnoses were lent support to by the results of routine measurement of the antinuclear factor (ANf), rheumatoid factor (Rhf), lupus erythematosus factor (LEf), immunocomplex (IC), cryoglobulin (cry), C-reactive protein (CRP), total protein, gamma globulin calculated from electrophoretic separation, determination of IgG and IgM by nephelometry in 61 patients with autoimmune disease and in 28 with nonautoimmune diseases, all aged between 30 and 60 years: The currently accepted routine methods recommended by the IFCC were used throughout.

Furthermore, AHA examinations were conducted in 42 dermatological and neurological patients aged less than 70, the diagnosis of autoimmune disease was established by various laboratory methods. The AHA activity of 34 multiple myeloma (MM) patients was determined in the course of their haematological treatment and regularly checked by us.

A separate group comprising a total of 55 surgical, ophthalmological, orthopaedic, traumatological and nonsurgical patients aged over 70, admitted with non-immune diseases on the basis of their case histories and clinical pictures, also had their AHA determined.

Serum antihistone was determined with the semiquantitative ELISA kit of the firm BIOLAB (Wavre, Belgium). The principle of the test is that the total histone (H1, H2A, H2B, H3 and H4) adsorbed to the wall of the well forms a complex with the serum antihistone antibody, then the complex is reacted with anti-IgM, IgG globulin, marked with peroxidase; then it is solubilized and the colour developed from the ABTS (azino-benzthiazoline sulphonate) peroxidase substrate is measured at 405 nm. At evaluation, the OD values of the negative and positive controls and $\pm$ 2 S.D. of the healthy population were taken into consideration.

Table I

The results of the various examinations of autoimmune (n = 61) and control (n = 28) patients

	Diagnosis	n	ANF	LEf	RHf	IC	KRYO	CRP	IgG	IgM	AHA	TPROT	γ glob
Autoimmune patients	SLE	7	6/7	6/7	4/7	3/7	4/7	1/7	2/2	1/2	2/7	2/7	4/7
	Chronic active hepatitis	12	6/12	3/12	5/12	6/12	4/12	4/12	3/3	2/3	3/12	5/12	9/12
	Raynaud's disease	16	3/16	2/16	0/16	2/16	1/16	0/16	0/6	1/6	3/16	0/16	5/16
	Collagenosis	13	8/13	5/13	3/13	2/13	3/13	5/13	1/4	0/4	1/13	2/13	2/13
	Rheumatic arthritis	10	7/10	1/10	7/10	10/10	6/10	7/10	0/4	2/4	2/10	0/10	2/5
	Scleroderma	3	3/3	1/3	3/3	3/3	1/3	0/3			0/3	0/3	0/3
	Total:	61											
Control patients	Asthma bronchiale	3	ALL TESTS ARE NEGATIVE										
	Migraine	1											
	Polymyositis	4											
	Hepatic cirrhosis	4										2/4	2/4
	Others	16											
	Total:	28											

Results

Table I shows the results of the ten different tests widely used in the diagnosis of autoimmune diseases in addition to the AHA test. In spite of the low patient number within the individual autoimmune disease groups, none of them did all the traditonal tests give positive results. Just the opposite was true. This also holds true of the AHA tests whose reliability was occasionally inferior to the other tests used here or whose positivity was, at the most, identical with them. In 10 healthy controls of similar age, the results of all examinations were negative.

Table II presents the results of 42 patients aged between 40 and 65 followed only by the AHA test and clinically diagnosed as affected with autoimmune disease. In Table III the surprising result obtained for the 34 MM patients is indicated /20/. The rate of positive AHA values was essentially the same as in Table I: 10% of the cases were positive in contrast with the 44% and 33% incidence of SM and SLE cases, respectively.

Table II

Serum values of autoimmune patients aged under 70 examined with the AHA test

Diagnosis	n	AHA-positive
SLE (children)	3	1
Dermatological autoimmune patients	21	2
Multiple sclerosis	18	8
Total:	42	11

Table III

Results of the AHA reaction in 34 multiple myeloma patients

	n	AHA-positive
Multiple myeloma	34	3
under 70 years	29	0
above 70 years	5	3
IgG type	24	2
IgA type	7	1
IgM type	3	0

It was remarkable that the 3 positive samples (2 IgG, 1 IgA of the 34 MM patients) were those of 5 MM patients aged over 70, while in the 29 MM patients under 70 the results of the test were negative. This finding raised the idea of a possible relationship between the patients' age and serum AHA activity.

According to the results in Table IV, of the 55 patients over 70 years of age treated for nonautoimmune diseases, 23 (42%) gave positive AHA reaction. Here, in addition to the 28 patients under 70 presented in Table I, the 10 patients aged between 18 and 30 years also admitted with non-autoimmune diseases whose sera showed no AHA activity served as negative controls.

Table IV

Results of the AHA examination of 55 patients aged over 70 treated for nonautoimmune diseases

	n	AHA-positive
Internal medicine	38	17
Ophthalmology	2	1
Orthopaedics	6	2
Traumatology	4	1
Surgery	5	2
Total:	55	23

The serum total protein, gamma globulin, IgG and IgM values of the AHA positive and AHA negative subjects aged under 70 years were compared. In total protein (Fig. 1/a) there was no difference between the two groups, in contrast with the more elevated value of gamma globulin in the AHA positive group (Fig. 1/b). The IgG value (Fig. 2/a) showed no appreciable deviation (33% and 38%). At the same time, the IgM concentration (Fig. 2/b) was elevated in about 70% of the AHA-positive cases, while in negative cases in only 37%.

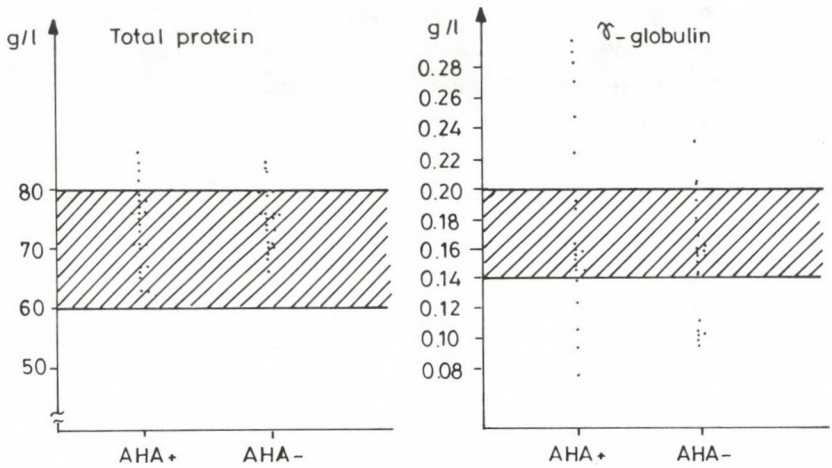

Fig. 1. Serum (a) total protein and (b) gamma globulin concentration of AHA positive and negative patients aged under 70

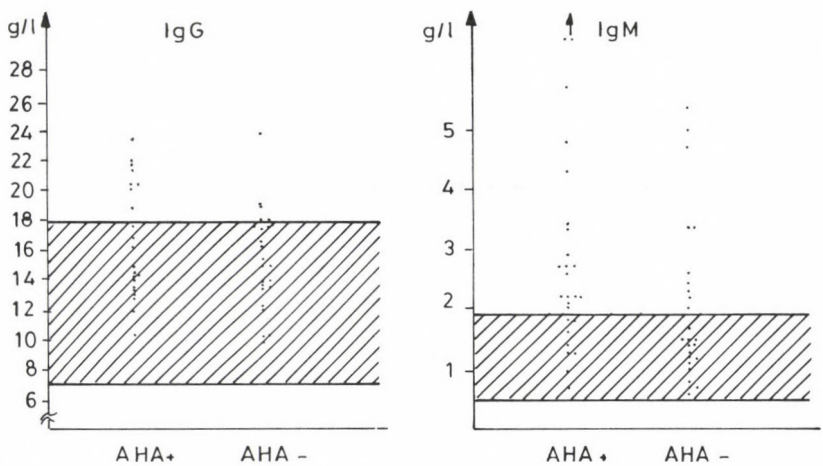

Fig. 2. (a) IgG and (b) IgM levels of AHA positive and negative patients aged under 70 years

Discussion

According to current views /23/, with which the present authors agree, an immune response is characteristic of each of the autoimmune diseases: on the one hand, a particular antibody is recognizable in one disease and not in another; on the other hand, each of the autoimmune diseases can be characterized by a particular antibody profile. It is largely these two traits that enable the various autoimmune diseases to be easily differentiated and diagnosed. Historically the antibodies to DNA and histones were the first to be written of, then antibodies to nucleocytoplasmic nonhistone proteins and RNA complexes were identified and characterized /16/.

Research on the different antibodies of great diversity is hampered, to a considerable degree, by thousands of intracellular proteins, hardly identified, there may certainly be more effective antigens than the nucleic acids of conservative structure. Low immunogenicity applies also to core histones, in contrast with extranucleosomal histones, which give antiserum of high titre with Freund's adjuvant /16/. According to the literature /3, 22/, antibodies to ss-DNA (single-stranded DNA) and histones are of diagnostic importance, first of all in drug-induced autoimmune diseases /7, 17, 19, 24/. In this respect our investigations have furnished no direct data. On the one hand, no such patients were available for us, and on the other, we made neither anti-ss-DNA nor anti-ds-DNA (double stranded DNA) examinations.

Our results (Table I) show that positivity of the AHA test is no more characteristic of our middle-aged autoimmune patients than are the other tests mentioned above /6, 21/. On the basis of this, we believe that none of the disease groups studied by us is characterized by an elevated AHA titre. Occasionally, the explanation of their positivity is just as open as in the case of the other tests. There is no doubt that higher IgM values were found in a higher number of patients with elevated AHA titre, while IgG showed no such difference. This observation is in agreement with the data on immunoglobulin published in connection with total histone and histone fractions /10/.

Besides the communications on the diagnostics of AI diseases published to date, elevated AHA values in about 42% of AI negative patients over 70 years may be regarded as a new observation. In accordance with this, the IgM concentration of AHA positive patients was 70%.

It was not the aim of the present study to re-examine the wide range of autoimmune diseases with the currently available methods, neither to assess their diagnostic value or to draw conclusions as to aetiology. Others had done this before us. Our earlier investigations into histones enabled the present pilot study of the diagnostic value of AHA, on which only few literary date are available. In the course of this study the AHA reaction turned out to be positive in about 42% of old subjects who showed no sign indicative of an autoimmune process. This observation also confirmed our view that the results of tests used in the diagnosis of autoimmune diseases should be evaluated cautiously. The presence of an autoimmune disease should be only be established if, in addition to the result of a combination of several antibodies, the patient's age has also been considered. A further piece of information may be expected from the AHA test, which, however, gives just as little information as the diagnostic procedures used so far. This opinion may be suprising if one considers that in SLE, the most thoroughly studied disease /22/, the pathogenetic importance of antibodies is accepted. At the same time, it has not yet been clarified whether the rest of autoantibodies exert their pathogenetic effect through immune complexes or via other mechanisms. The question is further complicated e.g. by the immunogenicity of H1 histone being more or less evenly distributed along the polypeptide chain. This enables specific antibodies, corresponding to the different structural domains of the molecule, to be formed /2, 13/. These antibodies might some day play a role in the study of proteins as well as chromatin and thus also in the diagnosis of the different autoimmune diseases.

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EFFECT OF VITAMIN E ON THE IMMUNOREACTIVITY OF SPLEEN CELLS IN HYPERLIPIDAEMIC RATS

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Atherogenic (lipid-rich) diet suppressed mitogen-induced lymphocyte blastogenic responses in rats. Supplementation with vitamin E completely abolished the suppressive effect of the diet. The atherogenic diet also decreased the tumour necrosis factor alpha (TNF-α) activity produced by spleen macrophages, however, vitamin E supplementation failed to abolish this effect. Diet or supplementation had no measurable action on interleukin-1 (IL-1) production of macrophages.

Keywords: Vitamin E, hyperlipidaemia, immunoreactivity

Introduction

The regulatory effect of hyperlipidaemia concerning cellular immune functions is well known: plasma lipoproteins inhibit mitogen-induced lymphocyte activation /6, 11, 16, 17, 20, 21, 24/. In a previous study /31/, we have shown that lipid rich diet markedly suppressed mitogen induced blastogenesis; PHA and Con-A induced blast transformation of spleen cells isolated from hyperlipidaemic rats was significantly decreased.

Vitamin E supplementation is known to enhance humoral and cell-mediated immunity and augment the efficiency of phagocytosis in laboratory animals, farm animals, and humans /34/. Therefore, there is a considerable

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interest of vitamin E therapy of diseases in which free-radical reactions are involved /1, 2, 7, 12, 14, 18, 22, 36/. Vitamin E protects membranes from oxidative damages by interfering with free radical-initiated chain reactions of polyunsaturated fatty acids /18/. The pathogenic role of free radical reactions and lipid peroxidation have already been described in hyperlipidemia /3, 4/, as well as, in the deranged function of lymphocytes and monocytes /28, 32/. The effect of vitamin E on serum lipids and on lipoproteins varies, but in most studies no significant changes were observed /9, 13, 15, 19, 27, 29, 30, 33, 35/.

In the present study the effects of vitamin E treatment were determined on cell-mediated immunoreactivity in hyperlipidaemic rats.

Materials and Methods

Experimental hyperlipidaemia

Young male Wistar albino rats weighing 150-200 g were used. Animals were divided into 3 groups, each consisting of 20 animals. The animals of group I were fed with normal LATI chow (Gödöllő, Hungary). The animals of group II were fed with atherogenic diet consisting of 2.0% cholesterol, 20% sunflower oil and 0.5% cholic acid added to the control LATI chow. Animals of group III were fed with the same lipid rich diet and treated with 8.56 mg/kg body weight vitamin E (HEK-Pharma, GFR) for 9 days, mixed into the diet. The rats were killed by decapitation at the 9th day /3/.

Lymphocyte proliferation assay

Cells obtained from perfused rat spleens were suspended in RPMI 1640 tissue culture medium (Gibco, USA) supplemented with 10% heat inactivated fetal calf serum (Phylaxia, Hungary), 25 mM HEPES buffer (SERVA, GFR), 2 mM L-glutamine (Gibco, USA), 10 IU/ml penicillin, 100 µg/ml gentamicin and 7.5 µg/ml amphotericin B. 4×10^5 spleen cells in 200 µl medium were placed in each flat-bottomed microplate wells (Greiner, GFR) using four parallel samples. Concanavalin A (Con-A, Pharmacia, Sweden), in doses of 1, 5 and 10 µl/ml, was added to the cell suspensions. Control cultures without lectin were included in each experiment. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 72 h. The cultures were pulsed with 0.4 µCi ^{3}H-thymidine (UVVR, Czechoslovakia) 24 h before termination. The cells were harvested on filter paper disks by an automated sample harvester (Skatron, Norway). Isotope determinations were made in a liquid scintillation counter (Nuclear Chicago Isocap 300, USA). The results were expressed in cpm using the arithmetic mean of four replicate values /10/.

Preparation of monocytes

Rat splenocytes were isolated as described above. Monocytes were separated by adherence to plastic surface. 10×10^6 cells/ml were seeded in 24-well tissue culture plates and allowed to adhere for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Nonadherent cells were removed by vigorous washing with warm culture medium. The adherent cells were harvested from the monolayer by scraping with a rubber policeman, then washed twice and finally resuspended in complete medium RPMI 1640 to a concentration of 1.5×10^6 cells/ml /25/.

Triggering IL-1 and TNF-α release

Monocytes (1.5×10^6 cells/ml) were incubated in the presence or absence of 20 µg/ml lipopolysaccharide (LPS from E. coli O111:B4, Sigma, USA). After 24 h, cell-free supernatants were harvested by centrifugation to remove cellular debris and filter sterilized using 0.22 µm Millipore membrane filter.

Interleukin-1 assay

Interleukin-1 activity was assayed in the culture supernatants as described previously /25/. Briefly, thymocytes from 5- to 7-week-old C3H/HeJ mice were prepared as single cell suspensions and adjusted to the density 1.5×10^6 cells/well. They were cultured for 48 h at 37 °C in 96 well flat-bottomed microtiter plates in the presence of 1 µg/ml Con A and test supernatant fluids. After 48 h, the cultures were pulsed with ^{3}H-thymidine. Thymidine incorporation was determined in a liquid scintillation counter. The results were expressed in c.p.m. using the arithmetic mean of quadruplicate cultures. In the majority of experiments the supernatants were diluted 1:8 prior to assay.

TNF-α assay

TNF-α activity was assayed as described elsewhere /26/: TNF cytotoxic activity was measured by the ability of LPS-stimulated monocyte supernatants to cause detachment from the monolayer of ^{3}H-thymidine labelled HEp-2 target cells. In brief, HEp-2 target cells were cultured in Eagle's MEM enriched with 10% heat-inactivated fetal calf serum, 25 mmol/l HEPES, 2 mmol/l L-glutamine and antibiotics. After discarding detached, dead cells with the supernatant medium, HEp-2 cells were resuspended with 0.5 ml of 0.1% trypsin (Sigma, USA) in medium TC 199, then washed twice. Resuspended HEp-2 target cells (2.5×10^3 cells/well) were placed in 96-well flat bottom microtiter plates, then labelled with 0.4 µCi/well of tritiated thymidine. To each well test supernatant fluid was added and the culture plates were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere. Target cells incubated in medium alone were included in each experiment. After 24 h, the detached target cells were removed. The remaining adherent cells were frozen at −20 °C. After thawing, the content of wells was sucked off on to filter mats with a cell harvester. Incorporated radioactivity was determined by scintillation counting. Results from six replicate values were expressed, as mean c.p.m. The percentage of cytotoxicity was calculated taking the medium control as baseline /26/.

Assays of serum lipids

Sigma kits were used for measuring serum lipids, HDL-cholesterol, triglycerides and cholesterol.

Statistical analysis was performed with Student's *t*-test.

Results

Triglyceride levels of sera failed to change in groups II and III as compared with those of controls (1.294 ± 0.359 mmol/l). The total cholesterol level of sera was significantly higher (4.3 fold) in hyperlipidaemic rats (7.970 ± 2.57 mmol/l), but vitamin E treatment did not diminish the elevated level in this "short-term" experiment. The HDL-cholesterol level was

Table I

The effect of atherogenic diet and vitamin E treatment on the lymphoblast transformation of rat spleen cells (thymidine incorporation, mean cpm ± S.E., n = 20)

Treatment Group		Con A (μg/ml) 0	1	5	10
I	Control	627 ± 79	58324 ± 8242	81181 ± 7447	72061 ± 6340
II	Atherogenic	441 ± 67	25738 ± 5566	47928 ± 7166	34996 ± 6947
III	Atherogenic + vitamin E	616 ± 76	56072 ± 9007	83084 ± 6070	73100 ± 4198

significance:		P
Con A 1 μg/ml	I vs II	< 0.01
	I vs III	n.s.
	II vs III	< 0.001
Con A 5 μg/ml	I vs II	< 0.01
	I vs III	n.s.
	II vs III	< 0.001
Con A 10 μg/ml	I vs II	< 0.001
	I vs III	n.s.
	II vs III	< 0.001

Table II

Effect of atherogenic diet on the TNF-alpha activity of rat spleen macrophages (mean cytotoxicity % ± S.E., n = 20)

Treatment group		Untreated	LPS-treated	P*
I	Control	-5.9 ± 6	20.8 ± 3.4	< 0.01
II	Atherogenic	-6.2 ± 4.6	1.8 ± 4.6	n.s.
III	Atherogenic + vitamin E	-8.4 ± 3.4	-5.1 ± 4.7	n.s.

significance:		P
untreated	I vs II	n.s.**
	I vs III	n.s.
	II vs III	n.s.
LPS-treated	I vs II	< 0.01
	I vs III	< 0.001
	II vs III	n.s.

*significance (untreated versus LPS-treated)

**n.s.: not significant

Table III

Effect of atherogenic diet on the IL-1 activity of rat spleen macrophages (thymidine incorporation, mean c.p.m. ± S.E.)

Treatment group (n)		Untreated	LPS-treated	P*
I Control	(13)	762 ± 163	1938 ± 209	< 0.01
II Atherogenic	(13)	1383 ± 227	1864 ± 155	n.s.
III Atherogenic + vitamin E	(8)	742 ± 85	1779 ± 336	< 0.01

significance:		P
untreated	I vs II	< 0.02
	I vs III	n.s.**
	II vs III	< 0.05
LPS-treated	I vs II	n.s.
	I vs III	n.s.
	II vs III	n.s.

*significance (untreated versus LPS-treated)

**n.s.: not significant

1.543 ± 0.215 mmol/l in control animal sera, and it was lower in hyperlipidaemia (1.33 ± 0.40). Vitamin E treatment was similarly ineffective.

The decrease of lymphocyte blast transformation of lymphocytes caused by lipid rich diet was abolished by vitamin E treatment (Table I). Lipid rich diet significantly decreased the LPS-induced TNF-α activity of monocytes. Vitamin E treatment failed to abolish the suppressed TNF-α activity (Table II). As shown in Table III, the LPS-induced IL-1 activity of monocytes was not changed by either the lipid rich diet or the vitamin E treatment.

Discussion

As shown earlier /31/, atherogenic diet resulted in a suppressed blastogenic response to plant mitogens. Vitamin E, added to the diet, completely abolished the suppression. TNF-α activity of spleen cells was also decreased by the diet, however, vitamin E supplementation failed to abolish the suppressive effect. The diet exerted no measurable effect upon LPS-induced IL-1 activity of spleen cells.

Since vitamin E supplementation failed to alter serum lipids, we suggest that its effect should be exerted indirectly. Vitamin E, as an anti-

oxidant, protects the cells of the immune system from peroxidative damage; possibly through a modulation of lipoxygenation of arachidonic acid, vitamin E alters cell membrane functions and cell-cell interactions /8, 34/. Short-term vitamin E supplementation improves immune responsiveness in healthy elderly individuals; this effect appears to be mediated by a decrease in prostaglandins and/or other lipid-peroxidations products /23/.

According to the protective effect upon immune reactivity of vitamin E, a continuous dietary supplementation is recommended, in particular in cholesterol rich diet, and possibly, in hypercholesterolaemic states.

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HEPATOLOGY

THE INCIDENCE OF HEPATITIS DELTA VIRUS INFECTION IN CHRONIC LIVER DISEASES IN HUNGARY

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A study of hepatitis B and D virus markers in 118 hepatitis B virus seropositive patients suffering from histologically confirmed chronic liver disease is reported. The prevalence of hepatitis delta infection amounted to 13.6%, whereas active hepatitis delta virus replication was proven in 6 of the cases. On the basis of these findings, conclusions — similar to those published earlier — are drawn about the role of hepatitis delta virus in the progression of chronic liver diseases. It is suggested that HBsAg-IgM complex seropositivity in patients suffering from anti-delta positive chronic liver disease supports active hepatitis delta virus replication.

Keywords: Hepatitis viruses, hepatitis delta agent, chronic liver diseases

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Abbreviations: HBsAg: hepatitis B virus surface antigen; anti-HBs: hepatitis B virus surface antibody; anti-HBc: hepatitis B virus core antibody; IgM anti-HBc: IgM class antibody to hepatitis B virus core; HBe: hepatitis B virus 'e' antigen; anti-HBe: hepatitis B virus 'e' antibody; HBsAg-IgM complex: a complex of hepatitis B virus surface antigen and an IgM class antibody to hepatitis B surface antigen; anti-HD: hepatitis delta virus antibody; IgM anti-HD: IgM class antibody to hepatitis delta virus antigen; HBV: hepatitis B virus; HDV: hepatitis delta virus (agent); HDAg: hepatitis delta virus antigen; HDV-RNA: hepatitis delta virus RNA; CAH: chronic active hepatitis; CPH: chronic persistent hepatitis

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Introduction

The delta agent (hepatitis D virus) was first described by Rizzetto et al. in 1977 /20/.

HDV infection may occur as coinfection and superinfection. The overwhelming majority of chronic HDV hepatitises develop subsequent to a HDV superinfection. Chronic HDV infection causes serious liver injury: infected patients often develop CAH or liver cirrhosis /2, 4, 10, 11, 18, 22, 24—26/.

HDV infection occurs worldwide; its prevalence in a population is determined by the number of HBsAg carriers. Anti-HD seropositivity in symptomless HBsAg carriers greatly varies by country and continent. For example, it was claimed to amount to 20-30% in Italy and France, 10-20% in Sweden, Switzerland and North Africa, 0-5% in the USA, England, Austria and Japan, and to exceed 60% in Roumania /12, 15, 19, 26, 27/.

Ternák et al. /28/ confirmed the occurrence of HDV infection in Hungary in 1985. However, the small number of the cases reported by these authors prevented them from determining its incidence rates in individual liver diseases.

In this paper, we intend to analyse the following issues:

1. The ratio of HDV infection demonstrable in chronic liver diseases,
2. The ratio of active HDV replication for patients having acquired HDV infection,
3. The influence of HDV infection on the severity of chronic liver diseases,
4. Possible connections between HDV infection and the serologic markers of HBV.

Patients and Methods

Our study includes the examination of 118 (86 males and 32 females) patients. The diagnosis in all cases was confirmed histologically according to internationally accepted morphological criteria /1, 7/. The distribution of the patients by histological diagnosis is shown in Table I.

The HDV and HBV markers were determined in the same serum samples. The blood samples drawn by venipuncture were immediately centrifuged, the serum samples were stored at -25 °C until tested. The virus markers were determined in our Radio-Immuno-Assay Laboratory by use of commercial Sorin Biomedica kits, viz. AB-DELTAK (anti-HD) AUK-3 (HBsAg), AB-AUK3 (anti-HBs), AB-COREK (anti-HBc), CORE-IGMK (IgM anti-HBc), EBK (HBe, anti-HBe) and AU-IGMK (HBsAg-IgM complexes). The IgM anti-HD measurement was performed by Alan G. Shattock in Dublin (Ireland) using the Wellcome kit in the ELISA method.

Table I

The distribution of patients by histological diagnosis

Histological diagnosis	Number of patients
Chronic persistent hepatitis	2
Chronic active hepatitis	50
Chronic active hepatitis and liver cirrhosis	13
Liver cirrhosis	37
Other liver diseases (Steatosis hepatis, hepatopathia toxica, chronic reactive hepatitis, granulomatous hepatitis, primary hepatocellular carcinoma, metastases hepatis)	16
Total:	118

All liver patients admitted to our department are tested for HBsAg, anti-HBs and anti-HBc. If all these prove negative, both actual and earlier HBV infection may be excluded. If any of the three markers proves positive, we determine the other B and D virus markers as well. Earlier HBV infection was proven in all of 118 patients.

Results

The rate of HDV infection for each disease group is shown in Table II.

Of the 118 patients, 16 (12 males, 4 females) have proved anti-HD positive (13.56%), 14 of them suffered from CAH or CAH with liver cirrhosis.

Of the 16 anti-HD positive patients, IgM anti-HD was determined in 14 cases. The detailed findings of the B and D virus marker tests of these patients are shown in Table III.

Six (4 males, 2 females) of the 14 patients tested proved IgM anti-HD positive (a sign of active HDV infection). Of these 6 patients, 5 suffered from CAH, and the CAH of 1 patient had progressed to liver cirrhosis.

The findings of hepatitis B and D virus marker tests are summarized as follows:

All the 118 patients examined proved seropositive for anti-HBc, 54 proved HBsAg positive and 40 anti-HBs positive. Furthermore, 9 patients proved positive for both HBsAg and anti-HBs simultaneously.

Table II

The ratio of HDV infection in the individual disease groups

Histological diagnosis	Number of patients	Number of anti-HD positive patients	
		No.	per cent
Chronic persistent hepatitis	2	0	0
Chronic active hepatitis	50	11	22
Chronic active hepatitis and cirrhosis	13	3	23
Liver cirrhosis	37	1	3
Other liver diseases (steatosis hepatis, hepatopathia toxica, chronic reactive hepatitis, granulomatous hepatitis, primary hepatocellular carcinoma, metastases hepatis)	16	1	6
Total:	118	16	13.6

Table III

The results of the detailed hepatitis B and D virus marker tests

Name	Histological diagnosis	HBsAG	anti-HBs	anti-HBc	HBe	anti-HBe	IgM anti-HBc	HBsAg IgM complex	anti-HD	IgM anti-HD
1. B. M.	CAH + CIR	-	-	+	-	-	-	-	+	-
2. T. J.	CAH + CIR	-	+	+	-	-	-	-	+	-
3. O. I.	CAH	+	-	+	-	+	-	-	+	0
4. B. P.	CAH	+	-	+	-	+	-	+	+	0
5. N. S.	CAH	-	+	+	-	+	-	-	+	-
6. K. G. ♀	CAH	-	+	+	-	+	-	-	+	-
7. N. R. ♀	HEP. TOX.	-	+	+	-	+	-	-	+	-
8. G. G.	CIR	+	-	+	-	+	-	-	+	-
9. F. F.	CAH	+	-	+	-	+	-	+	+	-
10. M. B.	CAH	-	-	+	-	-	-	-	+	-
11. V. L.	CAH + CIR	+	-	+	-	+	-	+	+	+
12. Z. I.	CAH	+	-	+	-	+	-	+	+	+
13. S. I. ♀	CAH	+	-	+	+	-	-	+	+	+
14. N. I.	CAH	+	-	+	-	+	-	+	+	+
15. L. A.	CAH	+	+	+	-	+	-	-	+	+
16. H. J. ♀	CAH	+	-	+	-	+	-	+	+	+

Abbreviations: CAH: chronic active hepatitis; CIR: cirrhosis hepatis; HEP. TOX: hepatopathia toxica
+: positive; -: negative; 0: no test performed

Of the 16 anti-HD positive patients, 2 proved positive only for anti-HBc, 9 for HBsAg, and 4 for anti-HBs, in 1 case we observed the simultaneous presence of both of the latter markers. We emphasize that all IgM anti-HD seropositive patients were positive for HBsAg, too.

The presence of HBsAg-IgM complex, a marker indicating the immune response to HBsAg was observed in the sera of 34 of the 118 patients. Of the 34 cases, 7 belonged to the group of the 16 anti-HD positive patients. Of the 6 patients suffering from active HDV infection, 5 proved to be seropositive for the HBsAg-IgM complex.

The sixth — HBsAg-IgM complex seronegative — patient (A.L., patient no. 15) — is identical with the patient in whom we observed the simultaneous presence of HBsAg and anti-HBs. Of the two other patients positive for HBsAg-IgM complex, one proved negative for IgM anti-HD (F.F., patient no. 9), and we were not in the position to examine the other patient (B.P., patient no. 4) for IgM anti-HD. IgM anti-HBc, one of the most sensitive markers of active HBV infection, proved positive in 22 of the 118 cases. None of the 22 cases belonged to the HDV infected group. The other tested marker indicating HBV replication, HBe, proved positive in 17 of the 118 cases. One of these 17 cases belonged to the anti-HD seropositive group. This patient (S.I., patient no. 13) proved positive for IgM anti-HD, too.

Discussion

Among our 118 cases, the incidence of HDV infection in HBV positive chronic liver patients was 13.6%.

Of our 16 anti-HD positive patients, 15 suffered from serious active liver disease: CAH and/or cirrhosis. It should be noted that all the 6 patients suffering from active HDV infection belonged to the CAH or the 'CAH with liver cirrhosis' group.

Several publications have appeared about a connection between HDV infection and chronic liver disease. Arico et al. examined 2487 symptomless HBsAg positive patients, 112 (5%) of whom proved to be seropositive for anti-HD. They reported laboratory findings indicating liver malfunction for 43 of the 112 anti-HD-positive persons (38.4%), while for only 215 cases (9.14%) of the 2375 anti-HD-negative persons. Of the former group, histological tests were performed in 31 cases. In 19 (61%) of these serious liver disease (CAH or cirrhosis) was observed, as contrasted with the anti-

HD-negative cases, in 97 cases of which liver biopsy was performed, and histological examination showed serious liver disease in only 18 (19%) cases /2/.

Govindarajan et al., who examined 80 histologically confirmed chronic HBsAg positive patients, reported similar findings. They found anti-HD positivity in patients suffering from CPH in 4.3%, whereas in patients suffering from CAH and/or cirrhosis, they found it in 31.5% /12/. According to the data reported by Brunetto et al. on the basis of a study of cirrhosis cases distributed by age group, the major aetiological factor of cirrhosis in young adults in Italy was HDV infection /3/.

Our findings, which correspond to those of several other authors and the above-mentioned data quoted in the literature, confirm the observation that HDV infection leads to serious liver injury, and enhances the progression of existing liver disease /2, 3, 5, 10—11, 12, 17, 21, 26/.

Conclusions based on the analysis of hepatitis B and D virus markers of our HDV-infected patients:

IgM anti-HBc positivity, one of the markers of active HBV infection was observed in none of our patients. HBe positivity occurred in only one of the 16 cases. Our findings correspond to those reported by other authors in that the markers of active HBV replication are usually absent in anti-HD-seropositive patients, or even in patients showing active virus replication at an early stage soon HBe, anti-HBe seroconversion develops /2, 8, 12, 14, 21, 23, 26/.

Our one and only HBe seropositive patient (S.I., patient no. 13) may belong to the latter group, though during her follow-up — up till the time when our paper was written — we had not observed seroconversion in her case, yet the IgM anti-HBc negativity observed may be interpreted as a sign of the suppression of HBV replication.

All our patients suffering from active HDV infection were positive for HBsAg, which is inevitable in all cases with active HDV replication, as HBsAg is a constituent of the envelope of the delta virus.

We also observed HBsAg-IgM complex seropositivity in 5 of the 6 patients suffering from active HDV infection. This marker may be considered to be a sign of active immune response to the HBsAg being present in the envelope of HDV. The sixth patient was negative for the HBsAg—IgM complex despite his IgM anti-HD seropositivity. However, in his case the simultaneous presence of HBsAg and anti-HBs suggests that, as a result of the organism's immune response, chronic virus infection reached the stage of

HBsAg—anti-HBs seroconversion, HBe—anti-HBe seroconversion had taken place, the elimination of HBsAg was going on, and the HBsAg-IgM complex had disappeared from the circulation. Negative HBsAg and IgM anti-HD tests to be obtained during further examinations can prove the assumption that in this case the efficient immune response resulted in the elimination of both viruses /6, 13, 16/ and the observed virus marker constellation is a sign of one stage of this process. On the basis of what has been mentioned above, we believe that HBsAg-IgM complex seropositivity observed among other markers (IgM anti-HD, HDAg, HDV-RNA) in anti-HD seropositive patients may be the evidence of HDV infection with active virus replication.

The main significance of serological diagnostics lies in the fact that it is critical in choosing the appropriate therapy. By the tests described, it is possible to exclude or prove active virus replication. In the former case, — according to appropriate criteria — immunosuppressive treatment is to be considered, while in the latter case anti-viral treatment is necessary. For several years antiviral treatment has been successfully applied in liver diseases with active HBV replication, and recently good results of the antiviral treatment of active HDV infection have been reported /9/.

As a summary, the following is stated:

1. HDV infection was proved in 16 (13.5%) of our 118 HBV positive patients suffering from histologically proved chronic liver disease.

The rate of anti-HD seropositivity was the highest in the patients belonging to the CAH or the 'CAH with cirrhosis' group (in 14 of the 63 patients).

2. Of the 16 anti-HD seropositive patients, active HDV replication was proved — on the basis of IgM anti-HD seropositivity — in 6 cases, all belonging to the CAH or the 'CAH with cirrhosis' group.

3. Fifteen of the 16 anti-HD seropositive patients suffered from severe progressive liver injury. This is in accordance with literary data and is suggestive of a connection between HDV infection and the severity of liver disease.

4. On the basis of detailed virus serological tests we found that

a) the markers of the active HBV replication are usually absent in HDV infected patients,

b) all cases of active HDV replication are accompanied by HBsAg seropositivity,

c) HBsAg-IgM complex seropositivity in anti-HD seropositive liver patients may support the existence of active HDV infection with virus replication.

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THE SIGNIFICANCE OF DETAILED HEPATITIS B VIRUS SEROLOGY IN CHRONIC LIVER DISEASES

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Hepatitis B virus (HBV) markers were studied with Sorin RIA kits in serum samples from 390 patients suffering from histologically confirmed chronic liver disease. On the basis of negative HBsAg, anti-HBs, anti-HBc tests, HBV infection was excluded in 235 of the cases. The diagnosis was fatty liver and/or alcoholic hepatitis in 52%, while chronic active hepatitis and/or liver cirrhosis only in 21.7%. Part or present HBV infection was proven in 155. In 53% of these cases the diagnosis was chronic active hepatitis and/or liver cirrhosis, whereas fatty liver and alcoholic hepatitis occurred in 27.7%. Detailed HBV marker analysis was performed in 76 patients. Previous infection without replication (positive anti-HBs and/or anti-HBc and/or anti-HBe) was proven in 48 cases, 12 patients had active HBV infection (positive HBsAg, HBe, IgM anti-HBc), while in 16 cases HBV integration (positive HBsAg, anti-HBc, anti-HBe) was proven. HBsAg-IgM complex seropositivity was shown in every case with active HBV replication. Because of therapeutic, prognostic and epidemiologic reasons, the significance of detailed HBV serology in chronic liver diseases is stressed.

Keywords: Hepatitis viruses, chronic liver diseases

Abbreviations: HBsAg: hepatitis B virus surface antigen; anti-HBs: hepatitis B virus surface antibody; HBcAg: hepatitis B virus core antigen; anti-HBc: hepatitis B virus core antibody; IgM anti-HBc: IgM class hepatitis B virus core antibody; HBe: hepatitis B virus 'e' antigen; anti-HBe: hepatitis B virus 'e' antibody; HBsAg-IgM complex: a complex of hepatitis B virus surface antigen and an IgM class antibody to hepatitis B surface antigen; HBV: hepatitis B virus; HDV: hepatitis D (delta) virus; CAH: chronic active hepatitis; CPH: chronic persistent hepatitis; HBV-DNA: hepatitis B virus DNA; RIA: Radio-Immuno-Assay

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Introduction

Trail-blazing work in the identification of HBV is associated with the names of Blumberg et al. and Dane et al., in the first place /3, 4, 10/. After the surface(s) (Australia) antigen-antibody system was described, Almeida et al. reported on the core antigen-antibody system in 1971 /1/. In 1972 Magnius and Epsmark described the 'e' antigen-antibody system /21/. These antigens and antibodies — as a consequence of HBV infection — can be detected in the patients' serum and their liver tissues. According to data published in the literature, HBV infection may be proved in various chronic liver diseases. The incidence rate of HBV positivity depends on the population examined and the sensitivity of the laboratory methods applied /9, 15, 23/. The conclusions drawn on the basis of the seropositivity and dynamics of HBV antigens and antibodies are of utmost significance in the therapy, prognostics and epidemiology of liver diseases.

In this paper, we make attempts to answer the following questions:

1. In what rate is earlier or actual HBV infection detectable in patients with chronic liver disease?

2. Does HBV infection affect the severity of liver diseases?

3. What conclusions can be drawn from the results of detailed HBV marker tests?

4. Is there any connection demonstrable between the various stages of HBV infection and the severity of liver disease?

Patients and Methods

Our examinations concerned 390 (333 males, 57 females) patients aged 15—74 (average: 43.05) yrs. The diagnosis in each case was confirmed histologically according to internationally accepted morphological criteria /2, 13/. The distribution of the patients according to histological diagnosis is shown in Table I.

The various HBV markers were determined in the same serum samples. The blood drawn by venipuncture was immediately centrifuged, and the serum samples were stored at -25 ^{o}C until tested. The virus markers were examined with commercial Sorin Biomedica kits in our Radio-Immuno-Assay Laboratory, viz. HBsAg with AUK-3, anti-HBs with AB-AUK-3, anti-HBc with AB-COREK, IgM anti-HBc with CORE-IGMK, HBe and anti-HBe with EBK and HBsAg-IgM complex with AU-IGMK kits.

All liver patients admitted to our department, as a routine, undergo HBsAg, anti-HBs and anti-HBc tests. If all three prove negative, both actual and earlier HBV infection may be excluded. If any of the three markers proves positive, the other HBV and HDV markers are also determined.

Table I

The distribution of the patients by histological diagnosis

Histological diagnosis	Number of patients
Intact liver tissue	2
Steatosis hepatis and alcoholic hepatitis	165
Chronic persistent hepatitis	46
Chronic active hepatitis	39
Chronic active hepatitis with liver cirrhosis	31
Cirrhosis hepatis	63
Other liver diseases (nonspecific hepatitis, granulomatous hepatitis, glycogenosis, hepatocellular carcinoma)	44
Total:	390

Results

Of the 390 patients examined, the HBV marker, a sign of HBV infection, was positive in 155 cases (125 males, 30 females, average age: 48.21 yrs), i.e. in 39.7%.

On the basis of negative HBsAg, anti-HBs and anti-HBc tests both actual and earlier HBV infection was excluded in 235 cases (208 males, 27 females, aged 15-70, average age 39.65 yrs) (HBV-negative group). In this group acute hepatitis was listed in the anamnesis of 7.2% of the patients, while in the HBV positive group the rate was as high as 21.3%.

Table II shows the distribution of the patients in the HBV positive and negative groups according to histologically confirmed diagnosis.

The disease of 122 patients (52%) of the 235 belonging to the HBV-negative group was steatosis or alcoholic liver disease, while 51 of them (21.7%) suffered from serious, progressing liver disease (CAH and/or cirrhosis). In the HBV positive group, on the other hand, more than half of the patients suffered from serious, active liver disease (CAH and/or cirrhosis), and only 27.7% belonged to the milder liver disease group (steatosis or alcoholic liver injury).

Table II

The distribution of HBV-positive and HBV-negative patients by histological diagnosis

Histological diagnosis	HBV negative (n = 235)	HBV positive (n = 155)
Intact liver tissue	2	0
Steatosis hepatis and alcoholic hepatitis	122 (52%)	43 (27.7%)
Chronic persistent hepatitis	29	17
Chronic active hepatitis	15	24
Chronic active hepatitis with liver cirrhosis	12 (21.7%)	19 (53%)
Cirrhosis hepatis	24	39
Other liver diseases: (nonspecific hepatitis, granulomatous hepatitis glycogenosis, hepatocellular carcinoma)	31	13

Table III

The results of the detailed HBV serology of 76 HBV-positive patients

Histological diagnosis	n	HBV infection: past	HBV infection: active	HBV infection: integrated
		positive anti-HBs and/or anti-HBc and/or anti-HBe	HBe and/or IgM anti-HBc positive, HBsAg and HBsAg-IgM complex positive, anti-HBc positive	HBsAg, anti-HBc, anti-HBe positive, HBe, IgM anti-HBc negative
Steatosis hepatis and alcoholic hepatitis	15	10	2	3
Chronic persistent hepatitis	1	1	0	0
Chronic active hepatitis or chronic active hepatitis with liver cirrhosis	28	11	9	8
Cirrhosis hepatis	29	24	0	5
Other liver disease (granulomatous hepatitis, aspecific hepatitis, hepatocellular carcinoma)	3	2	1	0
	76	48	12	16

We were in the position to perform all HBV marker tests in only 76 of the 155 HBV-positive cases. The results obtained are shown in Table III.

With the help of detailed HBV marker tests we were able to confirm 48 past infections ending with elimination of the virus (positive anti-HBs and/or anti-HBc and/or anti-HBe). It should be noted that in 2 of the 48 cases only anti-HBs seropositivity was detected, all the rest — 46 patients — proved anti-HBc seropositive, in 12 of these the presence of only this single marker proved past HBV infection, while in 25 cases anti-HBc and anti-HBs seropositivity, and in 3 cases simultaneous positivity of all the three markers proved previous HBV infection.

HBV infection with active virus replication was proved in 12 patients (positive HBsAg, anti-HBc, HBe and/or IgM anti-HBc).

In 5 cases virus replication was proved by positive IgM anti-HBc tests, in 4 patients by positive HBe, while in the serum of 3 patients both markers were positive. All the patients suffering from HBV infection with active virus replication proved seropositive for HBsAg-IgM complex.

The HBV infection of 16 patients had reached the stage of integration (positive HBsAg, anti-HBc, anti-HBe and negative HBe and IgM anti-HBc).

Table III also shows the distribution of the patients in the various stages of the infection according to histologically confirmed diagnosis. The incidence rate of the liver diseases of different severity in the various stages of infection does not differ significantly.

We studied the marker indicating active immune response to HBsAg: HBsAg-IgM complex seropositivity. The results obtained are shown in Table IV.

We did not observe the presence of this marker in serum samples from any of the patients belonging to the 'fought-off HBV infection' group.

Table IV

The results of the detailed HBV serology of 76 HBV-positive patients

HbsAg-IgM comples

	HBV infection		
	Past	Active	Integrated
HBsAg-IgM complex seropositivity	0/48	12/12	5/16[+]

[+] 4 of the 5 patients suffered from HDV infection with active virus replication, which accounts for their HBs-Ag-IgM complex positivity.

Whereas, all the patients suffering from infection with active virus replication were HBsAg-IgM complex seropositive. Five of the 16 patients in the 'integration phase' proved HBsAg-IgM complex seropositive, and of these 4 suffered from HDV infection with active virus replication. This is what explains the presence of the marker mentioned in their case /19/.

Discussion

The incidence rate of HBV infection in patients suffering from chronic disease amounts to 39.7% in the present study. The literary data about the incidence of HBV infection in chronic liver disease very widely. Apart from geographical, genetic and social factors, the applied laboratory methods of different sensitivity may account for these differences /6, 9, 15, 16, 20, 23, 26/. According to our observations, the most sensitive marker is anti-HBc; it proved positive in all cases with active virus replication and integration. In the overwhelming majority (46 of 48) of the cases of past HBV infection anti-HBc was detectable. It should be emphasized that in one-fourth of the patients belonging to the latter group the seropositivity of only this one marker proved the earlier HBV infection. This observation of ours — anti-HBc seropositivity being the most sensitive marker of HBV infection —, which we have already stressed /11, 12/, corresponds to the findings of Kater and Vogten /20/, Gerber et al. /16/, Bories et al. /6/, and Pár et al. /23/.

The incidence rate of HBV infection in cases of steatosis hepatis and alcoholic liver affection was 26%, a rate roughly corresponding to literary data /22, 23, 26/.

Though in the pathogenesis of these diseases primarily alcohol consumption and metabolic diseases are listed, the literary data and our observations show that — among the known injuring factors — the potentiating role of HBV infection should also be considered, especially in cases with active virus replication and infections reaching the integration phase /12/.

The incidence rate of HBV infection in patients suffering from chronic hepatitis and/or liver cirrhosis in our study amounted to 55.3%, a rate which is lower than the 82% found by Renner et al. in Austria /24/, and the rate 71% found by Pár et al. in Hungary /23/, but higher than the rate of 31.6% reported by Coates et al. /8/.

In our study, serious progressive liver diseases occurred two and a half times as often in the HBV-positive group than in the HBV-negative group /53% vs 21.7%).

In the anamnesis of the patients in the HBV-infected group acute virus hepatitis occurred three times as often as in that of the patients belonging to the HBV-negative group. However, even in the former group, only every fifth patient had had acute virus hepatitis. This fact, on the one hand, suggests that acute HBV infection in the most part of the cases proceeds latently, in a mild anicteric form /18/. On the other hand, it sheds light on the necessity to consider the possibility of HBV infection and its pathogenic role even in the lack of relevant data in the anamnesis. We believe this is a further reason why it is essential to perform the virus-serological tests in every liver patient. The main significance of detailed virus serology lies in the determination of the actual stage of HBV infection.

Past infection ending with elimination of the virus is marked by anti-HBc or anti-HBs seropositivity alone, or jointly, or also combined with anti-HBe seropositivity. The most sensitive marker of these is anti-HBc.

In our study, we diagnosed active virus replication on the basis of positive HBe and/or IgM anti-HBc tests. However, it is important to note that today the tracing of HBV-DNA or HBV-DNA polymerase is also needed to prove or exclude active virus replication, as we now know that active HBV replication may go on even in cases with HBe and IgM anti-HBc seronegative cases, moreover, even with anti-HBe seropositivity. Active virus replication may be proved by HBV-DNA, HBV-DNA polymerase, or by the detection of HBcAg in the liver tissue /5, 25/.

Positive HBsAg, anti-HBc and anti-HBe with simultaneous seronegativity of the markers indicating active virus replication show that HBV infection has reached the stage of integration, i.e. the virus genome having integrated in the nucleus of the liver cell is responsible for HBsAg production, without maintaining active replication of the virus. The inflammatory infiltration of the liver tissue in the integration phase resembles that in autoimmune chronic active hepatitis /25/.

The literature is poor in data about the significance of HBsAg-IgM complex determination /17/. We observed HBsAg-IgM complex seropositivity in 17 cases, in 16 of which simultaneous virus replication was also observed (4 cases concerned HDV replication). Thus, our findings show that HBsAg-IgM complex seropositivity may support the continuance of active virus replication, i.e. — if HDV infection can be excluded — it suggests active HBV

replication /19/. Careoda et al. /17/ and Toti et al. /27/ have reported similar findings.

The knowledge of the various stages of HBV infection and proving or excluding active virus replication is essential to the correct choice of therapy, since in the latter case, immunosuppressive therapy is to be considered, while in the former case antiviral therapy is necessary /14/. Furthermore, it should be borne in mind that in the integration phase, the HBV genome integrated in the nuclei of the liver cells pose a potential threat of hepatocellular carcinoma, so the close follow-up of these patients is needed (abdominal ultrasonography and serial alpha-1-fetoprotein level test).

Also, the determination of the actual stage of HBV infection has very great epidemiological significance, because it is the only method to decide if a patient may become source of infection (the presence of the markers indicating active virus replication) and if vaccination of the patient or endangered person is necessary or not.

As a summary, the following is stated:

1. In our material HBV infection was proven in 39.7% of chronic liver patients.

Acute virus hepatitis in the anamnesis of the patients belonging to the HBV-positive group is three times as frequent as in the HBV-negative group. However, even in the HBV-positive group, such data are listed only in the history of every fifth patient. This fact underlines the necessity of considering the possibility of HBV infection in the lack of relevant anamnestic information as well.

2. The incidence rate of serious progressing liver diseases (CAH and/or cirrhosis) in the HBV-positive group is two and a half times higher than in the HBV-negative group.

3. a) With the help of detailed virus serological tests the actual stage of HBV infection can be determined. This is of primary therapeutical, prognostical and epidemiological significance.

b) Anti-HBc may be considered the most sensitive of the markers of HBV infection.

c) HBsAg-IgM complex seropositivity shows active virus-replication, so — if HDV infection is excluded — it suggests active HBV infection.

4. In the different stages of HBV infection, the incidence rate of liver diseases of different severity does not differ significantly.

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THE DISTRIBUTION OF ABO(H) ISOANTIGENS IN URINARY BLADDER TUMOURS

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The distribution of blood group isoantigens (ABH) was studied with the specific red cell adherence test (SRCA); the red blood cells were visualized by the benzidine-peroxidase reaction. The H antigen was detected with Ulex europaeus agglutinin I lectin by direct immuno-peroxidase technique.

One hundred and seven bladder tumours were tested. It was found that blood group isoantigens diminished with immaturity (grade) and tumour invasiveness (T stadium). Patients with ABH blood group isoantigen deletion should be considered to be belong to a particularly high-risk group. The preservation of blood group antigens in grade II—III carcinomas may be useful in the choice of treatment (conservative or radical). In six cases in the area of squamous metaplasia of invasive carcinomas a strong false SRCA reaction was noticed detecting presumably the blood group determinants of the epidermal growth factor receptors.

Keywords: Bladder tumour, grade, stage, A, B, O(H) isoantigens

Introduction

The development of a urinary bladder cancer from uroepithelial dysplasia to infiltrating cancer is a long process.

The normal urinary bladder epithelium similarly to other epithelial cells, carries on the cell surface ABO(H) blood group antigens isologous with the patient's blood group antigens /4/.

During the development of cancer the ABH antigens decrease in number or reactivity, or even disappear from the cell surface /7/.

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According to clinical experience, decreased or lacking ABH reactivity is indicative of an aggressive progression of bladder carcinoma /3, 5, 6, 8, 9, 11—18, 21—26, 28/.

In this study we examined urinary bladder tumour tissues from 113 patients. For detecting the A and B isoantigens we applied the specific red blood cell adherence (SRCA) test. The antigen of 0 blood group (H antigen) was examined with Ulex europaeus agglutinin I (UEA-I) by an immunoperoxidase method.

Materials and Methods

The urinary bladder tumour tissue samples were obtained by transurethral recection in years 1983—1992. 2/3 of the biopsy material was sent for routine histoligical examination. The grade and T stadium of the tumours were determined according to the WHO directives /20/.

5-6 μm thin frozen tissue sections (Tissue Tek II) were made and air-dried, then they were incubated with anti-A and anti-B sera (Human, Budapest) in a humidified chamber and washed with phosphate buffered saline (PBS, pH 7.4). Subsequently the cryostat sections were covered with erythrocytes (blood group A and B, 1% suspended in saline) and incubated. The non-adherent red blood cells were removed by washing with saline. The sections were fixed in (2%) glutaraldehyde solution and the benzidine-peroxidase reaction was used to enhance the contrast of erythrocytes.

The tissue distribution of 0(H) blood group antigen was examined with peroxidase-conjugated UEA-I lectin (Sigma), diluted 1 : 100. Sections were incubated for 45 min and washed with saline. Amino-ethyl carbazole was used as chromogen, and Mayer's haematoxylin as counterstain.

The appearance of tissue ABO(H) blood group antigens was evaluated in a plus—minus system /2/, where (+++) means strong (all the epithelial cells are covered); (++) medium sized (2/3 of the cells are covered) and (+) weak reaction (1/3 of the cells are covered by erythrocytes).

Results

ABO(H) blood group isoantigens were examined in 107 papillary uroepithelial cancer and in 6 normal bladder uroepithelial tissues. The blood group distribution of the patients: A: 66, B: 16, AB: 7, 0: 24, which was similar to the blood group distribution of the Hungarian population.

Our observations show that the lack of the blood group antigens from the uroepithelial surface is a sensitive marker of malignant transformation and tumour progression (Figs 1—3). The distribution of ABH antigens according to the grade and stage of the tumours is shown in the Table. However, the grade II carcinomas showed heterogenous tissue blood group antigen distribution from the stage Ta to T3a infiltrations deepness, and a strong (+++) to a negative reaction was observed.

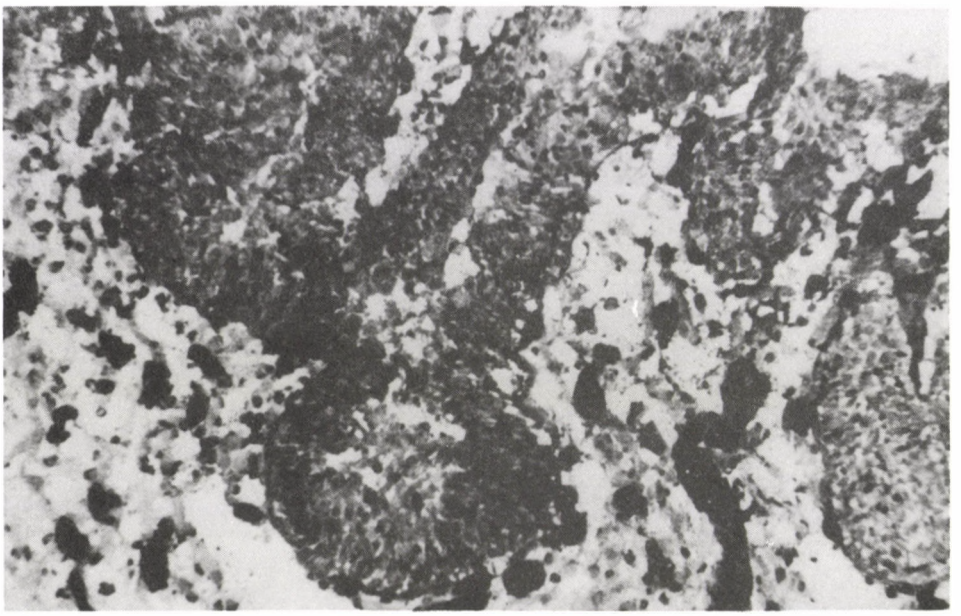

Fig. 1. Normal uroepithelium of a patient with 0 blood group reacts strongly (+++) with the UEA-I lectin. Stroma is negative, capillary endothelial cells also positive. Immunoperoxidase reaction, haematoxylin, x 200

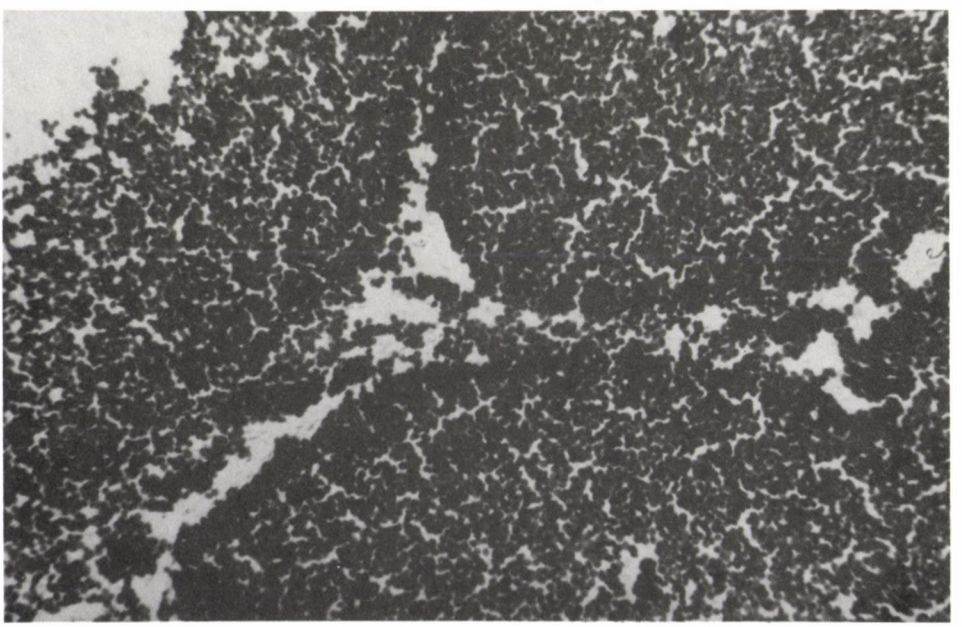

Fig. 2. Papillary uroepithelial carcinoma (grade I) shows strong SRCA reaction (+++). After the benzidine-peroxidase reaction the attached red blood cells show high contrast, x 200

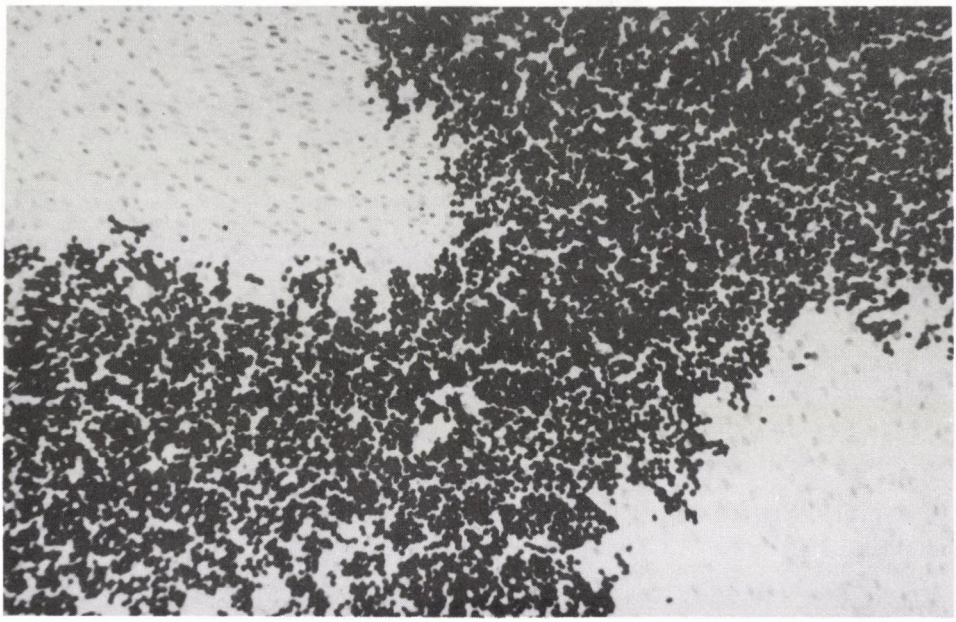

Fig. 3. Grade II papillary uroepithelial carcinoma, (++) positive reaction. Benzidine-peroxidase, haematoxylin, x 200

Table I

Grade, stage and ABO(H) blood group antigen distribution of 107 superficial bladder tumours

Grades / Stages	I					II					III					No. of patients
	-	+	++	+++	all	-	+	++	+++	all	-	+	++	+++	all	
pTA			6	15	21	4	4	8	5	21	3	1		1	5	47
pT_1				1	1	1	2	3		6						7
pT_2				1	1		1	1	2	4	2	1	1	1	5	10
pT_{3a}						7		1	1	9	22	9	3		34	43
Grade No. of patients					23					40					44	107

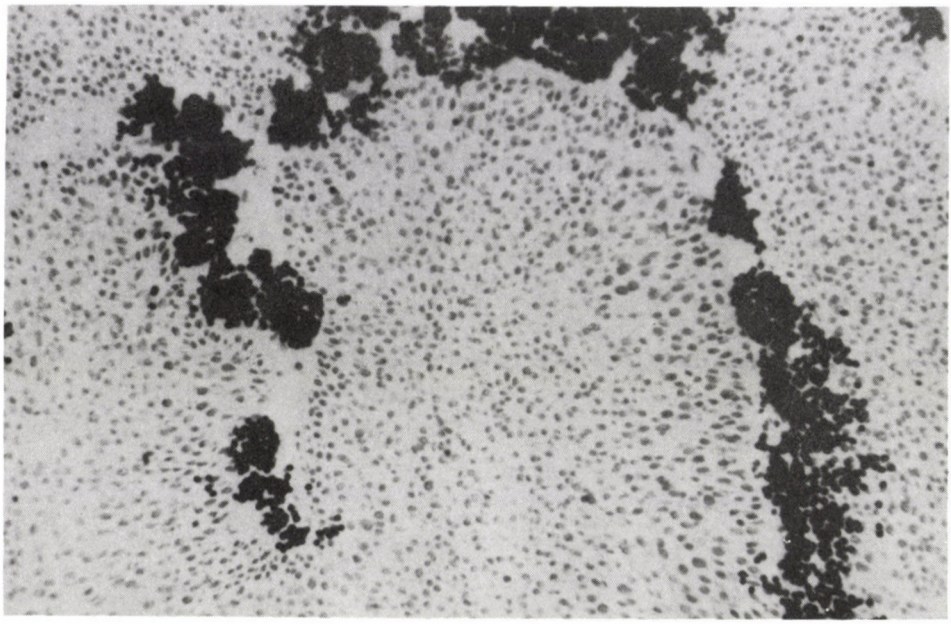

Fig. 4. Grade III papillary uroepithelial carcinoma, negative SRCA test. The positivity of capillary endothelial cells serves as endogenous control. Benzidine-peroxidase, haematoxylin, x 200

Most of the grade III carcinomas showed very weak or negative reaction. However in 6 cases in squamous metaplasia in the area of invasive carcinomas we met strong (+++) SRCA reaction (Fig. 4). In cases with AB blood group antigens A and B tissue antigens decreased parallel.

Discussion

The prognosis of the tumours is based on the degree of differentiation shown by cellular and tissue morphology. Synthesis of specific products, such as keratin and mucins may also be related to differentiation. Increasing evidence exists that cancer is associated with abnormalities in gene regulation expressed in multiple molecules (especially carbohydrates) at the cell surface membranes /10/. The deletion of blood group ABO antigen expression in bladder carcinoma has attracted attention because its possible demonstration by the SRCA test may serve as a prognostic parameter /7/.

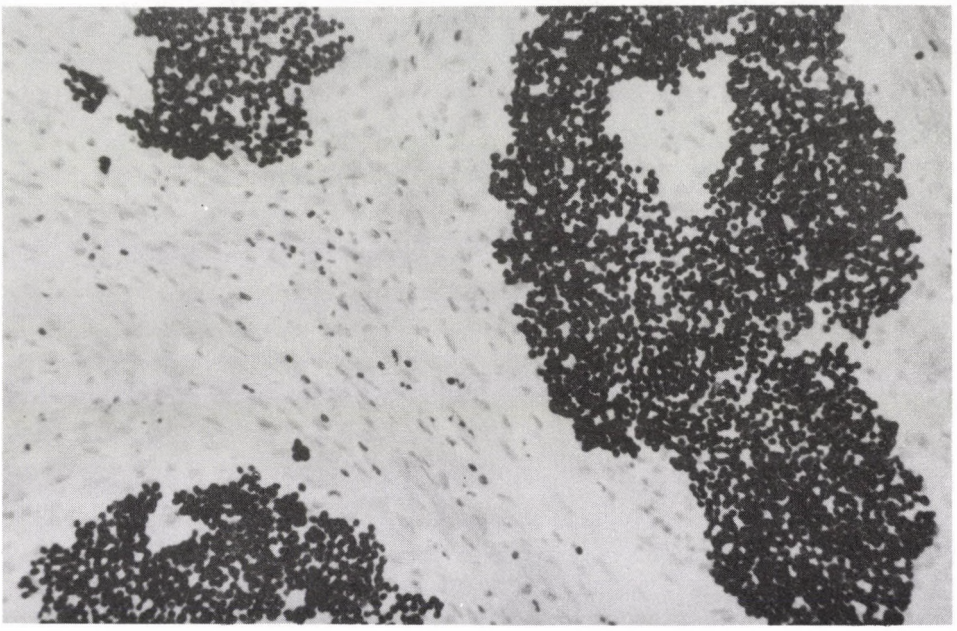

Fig. 5. Strong SRCA reaction in the area of squamous metaplasia of an invasive transitional cell carcinoma. Benzidine-peroxidase, haematoxylin, x 200

We found that the benzidine-peroxidase reaction of the specifically adhered red blood cells (with their dark brown staining) essentially increased the reliability of the SRCA test. Testing of 107 bladder tumours showed that blood group antigens diminish or disappear parallel to immaturity (grade) or invasiveness (T stadium) (Table).

According to long-term clinical experience, decreased or lacking ABH reactivity is indicative an aggressive progression of carcinomas /2, 17, 18, 23, 24/.

The diagnostic value of blood group antigen determinations is based upon the fact that isoantigen deletion may precede aneuploidy as measured with flow-cytometric technique /1/. Deletion of ABH antigen expression has been reported to occur before malignant progression after repeated recurrence of bladder tumours /26, 27/. Our findings also show that ABH blood group isoantigen deletions should be considered to be a particularly high risk for tumour recurrency, invasiveness and that these patients must be followed with special accuracy. The expression of blood group antigens in superficial high grade tumours is a strong indication for a conservative therapy and it is useful in the choice of therapy /14/.

We observed strong SRCA binding in six cases in the area of squamous metaplasia in invasive transitional cell carcinoma. From the 10 reported squamous cell bladder cancer cases four were SRCA positive /cit. 3/. Martin et al. /19/ found that transitional cell carcinomas of the bladder showing squamous metaplasia are mainly resistant to radiotherapy. The preservation of the ABH antigens in the area of squamous metaplasia seems to represent a false positive reactions with the blood group determinant of the epidermal growth factor receptors /18/.

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LONG-TERM PROSTACYCLIN — TREATMENT ACTS ON THE DNA AND RNA CONTENT OF RAT GASTRIC (ANTRAL AND FUNDIC) MUCOSA DOSE-DEPENDENTLY

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The changes in garstric (antral and fundic) mucosal DNA and RNA contents were investigated in long-term (80 days) treatment of rats with orally administered prostacyclin. Prostacyclin caused a dose-dependent, significant increase of the DNA levels in both parts of the gastric mucosa together with a significant thickening of the fundic mucosa. The observed changes (decrease) of the RNA/DNA ratio in the fundic as well as antral mucosa, are interpreted as a sign of accelerated cell renewal, i.e. hyperplasia.

Keywords: Prostacyclin, long-term treatment, DNA, RNA, rat, gastric mucosa

Introduction

Karacsony et al. /8/ published that a long-term (80 days) prostacyclin ($PG\text{-}I_2$) treatment caused a marked modification of the cell composition of rat gastric mucosa, together with a significant increase in the mucosal DNA content.

Balint et al. /4, 12/ gave an account of the effect of $PG\text{-}I_2$ treatment on protein, DNA and RNA content of rat fundic mucosa.

However, these investigations left (at least) two questions open, namely:

(i) What is the effect of a long-term $PG\text{-}I_2$ treatment on the antral part of rat gastric mucosa, and

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(ii) is the encountered effect of PG-I_2 a genuine pharmacological effect of the molecule, i.e. is it dose-dependent or not?

To elucidate these problems, the following investigations were performed.

Materials and Methods

Adult female Wistar rats of 220-230 g of initial body weight were used.

The animals were assigned in groups each consisting of 10 animals. They received standard pellets as food and water was allowed ad libitum.

The experimental animals were given PG-I_2 orally for 80 days.

The daily dose of PH-I_2 was nil for group I (control animals),

20 µg/kg for group II;

100 µg/kg for group III and

200 µg/kg for group IV.

A stock-solution of PG-I_2-methylester (1 mg/ml) was made-up freshly in 0.05 M Tris-buffer of pH 9.6. Dilutions were made immediately prior to use in ice-cold isotonic sodium-bicarbonate solution and given orally through a gastric tube. The control animals received isotonic sodium-bicarbonate solution, the same volume (also through a gastric tube for 80 days).

During PG-I_2 treatment the body weight of the animals was checked and recorded weekly.

After the treatment period the animals fasted for 24 h but allowed water ad libitum. Subsequently, the following investigations were done:

The animals were killed, their stomachs were removed and opened. Specimens for histological investigation were fixed in formalin, dehydrated in an ascending alcohol series, embedded in paraffin, then serially sectioned and stained by a modified Zimmerman's method /10/. Specimens of total mucosal thickness were measured using an ocular micrometer. A minimum of ten areas were checked per slide. Mucosal thickness was expressed in mm.

Following sampling the gastric fundic (oxyntic cell area) and antral mucosa was separately scraped off, and both were divided into two parts, which were homogenized either in ice-cold saline for protein determination /9/ or in 0.8 M ice-cold perchloric acid for DNA and RNA determination. Emphasis was put throughout on rapid processing, and the whole procedure was carried out in an ice-bath.

The RNA content of the homogenate was determined by the orcinol reaction /6/, while the DNA content was measured by the diphelylamine method /1, 7/. Both were expressed in µg/mg protein.

Within each group mean $\pm$ S.E. was calculated and analysed statistically using Student's t-test. Limit of significance was $P = 0.05$.

Results

The long-term application of PG-I_2 enhanced the gastric mucosal DNA contents and gastric mucosal thickness significantly, while no changes were seen in the body weight and gastric mucosal RNA levels (Tables I--III).

Table I

Body weight of the animals
(n = 10 in each group)

$PG-I_2$ dose	Body weight Before $PG-I_2$ treatment	After
Nil (Control)	225.3 ± 9.88	265.1 ± 8.02
20 µg/kg	228.8 ± 8.92	259.9 ± 7.90
100 µg/kg	221.2 ± 7.94	260.6 ± 8.35
200 µg/kg	224.5 ± 8.06	266.4 ± 8.41

Mean ± S.E. in grams

Table II

Gastric mucosal DNA and RNA changes during long-term $PG-I_2$ treatment
(n = 10 in each group)

$PG-I_2$ dose	Antrum DNA µg/mg protein	Antrum RNA µg/mg protein	Antrum RNA/DNA	Fundus DNA µg/mg protein	Fundus RNA µg/mg protein	Fundus RNA/DNA
Nil (Control)	20.49 ± 1.54	37.70 ± 1.61	1.84	14.91 ± 1.19	26.95 ± 1.70	1.81
20 µg/kg	18.75 ± 1.72	31.84 ± 2.27	1.70	16.95 ± 1.18	22.74 ± 1.29	1.34
100 µg/kg	23.68 ± 2.22	35.00 ± 2.82	1.48	19.87 ± 1.64$^+$	24.08 ± 1.38	1.21
200 µg/kg	28.38 ± 2.52$^+$	38.39 ± 2.62	1.35	22.64 ± 2.09$^+$	28.31 ± 2.38	1.25

Mean ± S.E.
$^+P < 0.05$ vs Control

Table III

Gastric mucosal thickness after long-term $PG-I_2$ treatment
(n = 10 in each group)

$PG-I_2$ dose	Antrum	Fundus
Nil (Control)	0.201 ± 0.003	0.465 ± 0.011
20 µg/kg	0.193 ± 0.005	0.449 ± 0.027
100 µg/kg	0.199 ± 0.003	0.509 ± 0.012$^+$
200 µg/kg	0.209 ± 0.004	0.521 ± 0.014$^+$

Mean ± S.E. in mm.
$^+P < 0.05$ vs Control

Conclusions

On the basis of our results the following conclusions might be drawn:

1. The long-term PG-I_2 treatment showed no effect on the body weight of the animals compared to the controls, i.e. there was no sign of a toxic side-effect.

2. After 80 days of PG-I_2 treatment there was a significant thickening of the gastric fundic mucosa, while in the antral part thickening was observed only with the 200 µg/kg dose of PG-I_2. In our opinion this phenomenon corresponds to the hyperplasiogenic effect of PG-I_2 on the gastric (fundic) mucosa /4, 8/. This assumption is strengthened by the results obtained by checking the DNA and RNA levels in this series of investigations.

3. The data presented with regard to the changes in the DNA and RNA content of rat gastric mucosa, are in accordance with our earlier results /3, 5, 9/ namely that:

(i) The DNA level significantly increases in both parts of the gastric mucosa, while

(ii) the RNA content remains at the control level, — also in both parts of the mucosa — resulting in a

(iii) decreased RNA/DNA ratio.

This ratio varies from tissue to tissue (or from species to species) but under physiological circumstances it is stable within a given tissue or organ. Elevation of this ratio refers to de novo protein synthesis, while its decrease is a convincing sign of new cell formation /3, 11/.

We conclude that during long-term PG-I_2 treatment new cell formation takes place in both parts of the gastric mucosa. This phenomenon, together with the thickening of the mucosa, is in accordance with our previously published results /4, 8/ regarding to the fundic mucosa. For the antral mucosa the present series of investigations give the first data in which the hyperplasiogenic effect of PG-I_2 seems to be enlightened. In acute experiments /3, 4/ the antral mucosa reacted differently as compared to the fundic part, namely it showed an elevation in the RNA/DNA ratio, indicating de novo protein synthesis, most probably mucus secretion. It is worth mentioning that in the fundic mucosa in aucte circumstances there is also a new cell formation.

4. The experimental results suggest that during a long-term treatment of rats the effect of PG-I_2 on the gastric mucosal DNA level and RNA/DNA ra-

tio is a dose-dependent genuine pharmacological action in both parts (antral and fundic) of the gastric mucosa.

Finally, it is notable that in acute experiments there is a significant RNA level-elevating effect of PG-I_2 /4/. This effect on the RNA level was not present in the course of our present, 80 days investigations.

Acknowledgement: PG-I_2-methylester was generously donated by the firm Chinoin (Budapest, Hungary).

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LYSOSOMAL ENZYME ACTIVITIES IN FROZEN, NON-CULTURED CHORIONIC VILLI FOR PRENATAL DIAGNOSIS OF ENZYMOPATHIES

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Normal reference values of lysosomal enzyme activities (alpha-glucosidase, mannosidase, fucosidase and arylsulfatase-A) were determined in chorionic villi obtained from arteficial abertion in the first trimester of normal prognancies (gestational weeks 6 to 11). Villi were homogenized comparatively either in saline or in Triton X-100 detergent. The alpha-glucosidase, mannosidase and arylsulfatase-A enzyme activities significantly diminished if homogenization was done in saline instead of Triton-X while the difference in fucosidase activity was not significant. Significant correlation was detected between alpha-glucosidase activity and week of gestation. It is suggested that Triton X-100-homogenization should be used for the lysosomal enzyme determinations in chorionic villi because the solubilization of enzymes from the lysosomes is complete in this case than with homogenization in saline.

Keywords: reference values, lysosomal enzymes, chorionic villi (non-cultured), genetical enzymopathies.

Introduction

Prenatal diagnosis of genetically-determined enzymopathies had become available by analysing chorionic villi obtained in an early period of gestation (9-12 weeks) /5/ due to interruption of gravidity. Most lysosomal enzyme activities can be detected in chorionic villi and in cultured trophoblasts /4/. Differences in the concentration of lysosomal enzyme activities have been detected between chorionic villi and cultured fibroblasts or amniocytes /2/.

For prenatal diagnosis of lysosomal enzymopathies we have studied the normal reference values of enzymes found in frozen, freshly-obtained, non-

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cultured chorionic villi and the pH-dependence of enzyme activities in the early gestational weeks.

Materials and Methods

After 1 to 4 weeks storage at -80 °C the frozen chorionic villi obtained at arteficial abortion were prepared free from maternal decidua under a dissection microscope. The isolated villi were used for enzyme activity determinations. Chronionic tissues samples were homogenized either in saline, or in destilled water containing 0.1% Triton X-100. Lysosomal enzyme assays and protein concentration measurements were performed in the supernatant of such homogenates. The alpha-glucosidase, fucosidase, mannosidase and arylsulfatase-A enzyme activities were determined by colorimetric techniques as described by Poenaru et al. /6/. The activities were calculated as transformed substrate nmol: protein mg:h. Pearson-type correlation coefficients were applied for the detection of the relation of gestational age and enzyme activities.

Results

The mean values of lysosomal enzyme activities are shown in Table I. The alpha-glucosidase, mannosidase, and arylsulfatase-A activities of chorionic villi homogenized in saline were significantly diminished compared to those of the Triton X-100 homogenate, while the fucosidase activity seemed to be independent of the homogenizing medium.

Among the lysosomal enzymes only the alpha-glucosidase activity proved to be in significant correlation with the gestational week ($r = 0.48$, $P < 0.01$) (Table II).

Table I

Activity of lysosomal enzymes from chorionic villi

In Triton X-100-homogenized chorionic tissue

	Gest. weeks	α-glucosidase	Fucosidase	Mannosidase	Aryl-sulfatase A
			nmol/mg protein/h		
$\bar{X}$	9.15	122.9	755.5	147.2	130.7
S.D.	1.69	59.6	164.5	45.0	108.5
n = 40					
		In saline-homogenized chorionic tissue			
$\bar{X}$		19.29	615.6	14.85	79.4
S.D.		3.54	152.5	8.15	22.4
n = 10					
P		< 0.001	> 0.05	< 0.001	< 0.001

Table II

Activity of lysosomal enzymes from chrorionic villi and the correlation with gestational weeks

In Triton X-100-homogenized chorionic tissue

	Gest. weeks	α-glucosidase	Fucosidase	Mannosidase	Aryl-sulfatase A
			nmol/mg protein/h		
$\bar{X}$	9.15	122.9	755.5	147.2	130.7
S.D.	1.69	59.6	164.5	45.0	108.5
r^x		0.43	0.14	0.32	0.12
P		< 0.01	> 0.05	0.0514	> 0.05
n = 40					

r^x = Pearson-type correlation coefficient between the enzyme activity and the gestational week

A pH-dependence of enzyme activities have been observed (Figs 1--4). As shown in the Figures, linearity in the concentration -- and pH -- ranges is satisfactory.

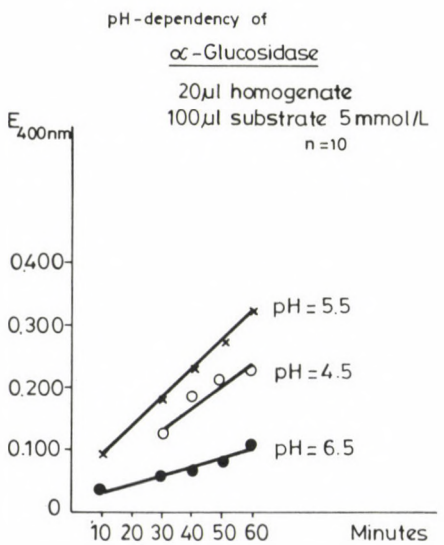

Fig. 1. pH-dependency of alpha-glucosidase activity from chorionic villi

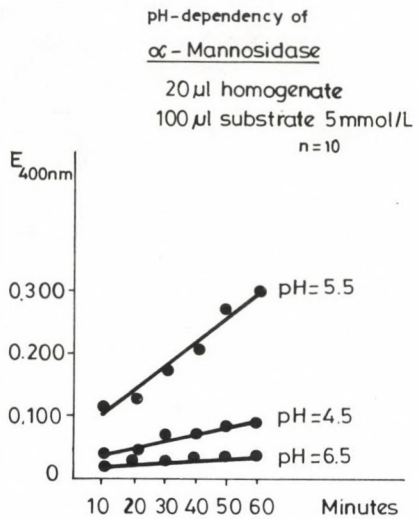

Fig. 2. pH-dependency of alpha-mannosidase from chorionic villi homogenate

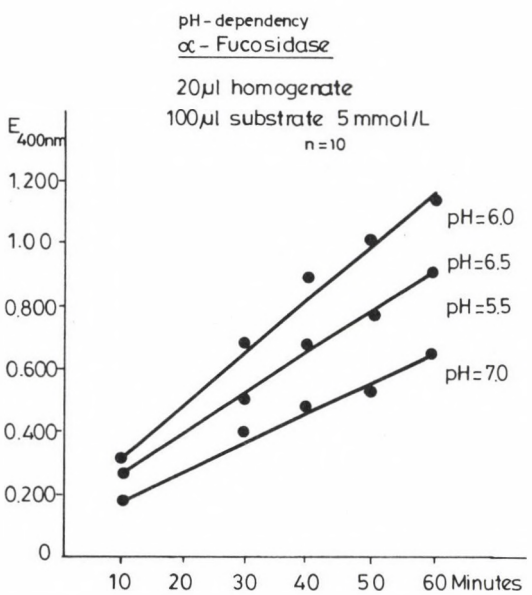

Fig. 3. pH-dependency of alpha-fucosidase from chorionic villi

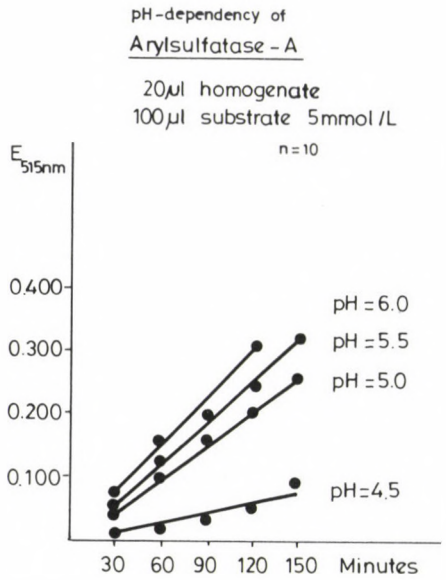

Fig. 4. pH-dependency of chorionic arylsulfatase-A

Discussion

Ben-Yoseph et al. /1/ investigating the lysosomal enzyme acitivities in fresh and frozen chorionic villi and in cultured trophoblasts established that freezing of the tissue specimens had no effect on hexosaminidase-B and alpha-iduronidase activities while arylsulfatase-A and beta-galactosidase activities were slightly increased and other activities were slightly decreased on freezing.

In contrast to the few significant differences between fresh and frozen tissues, highly significant differences were detected by the same authors between the values obtained from experiments on tissues and those performed with cultured cells. Freezing of samples seems to affect only the so-called labile enzymes, alpha-galactosidase and neurominidase.

Pulkkinen /7/ reported in own reference values for arylsulfatase-A and for some steroid-sulfatase in the developing organisms and for the placenta. Arylsulfatase-A activity was 191 $\pm$ 11.2 U/g weight in placental tissue taken from the fetal side.

According to Fukuda et al. /3/ the mean level of alpha-glucosidase activity was 6.3-times higher and that of beta-glactosidase activity was 2.6-times lower in chorionic villi than in cultured amniotic fluid cells.

In the present study, enzyme activities from normal pregnancies are given which were from normal pregnancies are given which were found, in saline-homogenized and Triton X-100-homogenized chorionic tissues. Diminished activities of alpha-glucosidase, mannosidase and arylsulfatase-A were detected in the saline-homogenized chorionic villi compared to Triton X-100-homogenzied ones, while the fucosidase activity did not differ from the Triton X-100-homogenized patterns. Significant correlation was detected between alpha-glucosidase activity and gestational age.

Because there are no universally-accepted, optimized methods for determination of the above-mentioned lysosomal enzymes, it is important to determine the normal reference values in each laboratory. We have get intermediate values for arylsulfatase-A compared to data found by Evans et al. /2/ and those reported by Poenaru et al. /6/. Our alpha-glucosidase values are lower than those published by Poenaru et al. /6/. In case of alpha-fucosidase and mannosidase we obtained enzyme activities similar to those found by other authors /1, 2, 6/.

Our results seem to be important because normal values are essential for promoting and making more specific the prenatal diagnostic of lysosomal enzymopathies.

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Acta Medica Hungarica, Vol. 49/1–2, pp. 149–151 (1992/93)

BOOK REVIEWS

AUTOPSY IN EPIDEMIOLOGY AND MEDICAL RESEARCH

Eds: E. Riboli and M. Delendi

IARC Scientific Publications No. 112. Lyon, 1991.

Since the earliest human dissections practised in Alexandria between 400 and 300 BC autopsy has been one of the oldest methods of medical investigations. From the fundamental work of Morgagni, autopsy contributed first to the evolution of anatomical knowledge and then to an understanding of the organic nature of diseases.

Autopsy means, literally, "seeing with one's own eyes", therefore it has been the starting point for the development of histology, macro- and microscopic pathomorphology and the most recent histopathological techniques. In modern medicine, autopsy is still carried out in order to answer two questions: "What was the cause of death of a particular patient?" and "What can be learnt from a particular case to improve medical knowledge?". Nowadays, autopsy is the only way for quality control in the public health service.

In Trieste (Italy) between 1901 and 1985, there were highly significant increases in the numbers of deaths due to arteriosclerosis and to malignant neoplasms in people of each sex. Overall, infectious diseases accounted for 55% deaths in 1901. In 1985, on the other hand, death was caused by infection in only 3.7%. In Austria, the legal regulations for performing autopsies are based on laws dating back to middle of the 18th century (Holzner), which were introduced by the Empress Maria Theresa and the Protomedicus Gerard van Swieten. So, the autopsy rate is theoretically 100% in all public hospitals, i.e. hospitals under provincial and municipal administration. These include almost all the large hospitals in the country. Maintenance of an acceptable rate is important for continuous quality control of mortality statistics of a population, as well as for continuous quality control of clinical diagnosis in general. It is of great significance regarding the malignant neoplasms, too. In 1987 in Austria, autopsies were carried out in 49.6% of the deaths that occurred in hospitals and in 34.5% of those that occurred at home (external deaths). In 1988, in the Vienna University Hospital the autopsy rate was 75%. Between 1978 and 1987 in Austria on the basis of autopsy data, there was no change in the deaths, newly diagnosed cases and staging both of stomach and lung carcinoma.

The prevalence of cirrhosis at autopsy is high in Trieste and shows no tendency to decrease, as inferred by some clinical studies.

Di Bonito et al. compared the diagnoses on death certifications with the autopsy reports in gynaecological cancers. Complete agreement was found only for 30% of cervical and corpus tumours and for 50% of ovarian tumours. The cause of failure was first erroneous interpretation of codes, second, confusion of the anatomical site of primary cancers. Grundmann and Menke found that the concordance between clinical and autopsy diagnoses was very poor with respect to infectious diseases: lues was rarely recognized clinically, even though it may be fatal; 50% of tuberculous patients died of miliary tuberculosis without a correct diagnosis. Endocarditis in all its forms was underdiagnosed clinically in 75% of the cases. These data, including the histological findings provide substantial arguments in favour of autopsy control of clinical diagnosis. Jonasson and Björnsson concluded

Akadémiai Kiadó, Budapest

that autopsy is indispensable as an instrument of quality control and for generating mortality and morbidity statistics. The introduction of new diagnostic modalities seems not to have improved the accuracy of clinical diagnosis of the immediate cause of death but may significantly have improved the detection of other major diseases. According to Rossi et al., agreement between the diagnoses was 81% for primary disease and 58% for cause of death. They concluded that autopsy was a valid tool for investigation, despite the availability of sophisticated diagnostic techniques. Autopsies can indicate the exposure to asbestos. Mollo et al. found that only post-mortem examination can provide probative data about the presence of a lung cancer and its relationship to exposure to asbestos. Autopsy may suggest a through investigation of occupational exposure to asbestos. The perinatal autopsy is clearly an undervaluated source of information and discovery. Little information is available from the developing countries (Husain and O'Conor).

The dilemma of Peacock et al. "The autopsy: a useful tool or an old relic?" may not be a dilemma. Autopsy is useful today for monitoring the diagnostic processes within wider programmes of assuring the quality of clinical activities. But without active participation of clinicians and in the absence of a change in some institutional inertia, autopsy never reaches the goal it has set itself for centuries: "MORTUI VIVOS DOCENT" (Ferencic and Belicza).

A. BAJTAI

CLINICAL APPLICATION OF RADIOIMMUNOASSAY

Ferenc A. László and Tamás Janáky

Akadémiai Kiadó, Budapest 1992.

The purpose of this book is to provide for clinicians with a working knowledge of current radioimmunoassays in different fields of medicine. It contains 18 chapters on 184 pages, figures, tables and subject index. Each chapter end with a reference lists up to the year 1990.

The first part of the book deals with hormones of the hypothalamic-hypophyseal system and of their target organs; the next part with the parathyroid hormones and insulin, followed by gastrointestinal hormones; references are only up to 1988. In the chapter of the haemopoietic system we can read about vitamin B_{12} ferritin and folic acid. In the chapter of tumour derivatives only CEA and AFP are mentioned. IgE and hepatitis B virus are dealt with in the next two chapters. The last chapter is devoted to pharmacology: digoxin, barbiturates and morphine.

The glossary of radioimmunoanalytical procedures seems to be very useful, but a little bit too much for practitioner clinicians and little for specialists. In most part of this book are very few practical advices for the doctors of ward. It is pity that there is very little information from the last years.

Overall, the book provides new insight into RIA procedures for the clinicians who attend patients with endocrine disorders and use the results of radioimmunoassays. The Reader will be motivated to invest more time and interest in this field. It is recommended as an introduction to radioimmunoassays.

MARIA FARKAS

ATLAS OF FETAL DIAGNOSIS

Edited by Zoltán Papp

Elsevier, Akadémiai Kiadó, Budapest 1992. 255 pages

The development in obstetrics of the past few years can be characterized by the "marriage" of obstetrics and genetics. The truth of this statement is proven by the excellent atlas edited by Professor Zoltán Papp.

Broadening knowledge of genetics has led to an increase in the number of affected families requiring genetic care. Among others, the work of the Editor and the contributors of this book resulted in the creation of genetic counselling centers throughout Hungary.

The number of prenatal diagnostic and therapeutic interventions has rapidly increased and as a result, we have witnessed the sudden development of prenatal ultrasound diagnosis, invasive intrauterine interventions and fetal pathology. Thus postgraduate training of obstetricians working with fetuses and pregnancies affected by genetic disorders or developmental malformations has become extremely important. The Atlas of Fetal Diagnosis is in compliance with this demand and can be considered as a supplement to or a continuation of his previous book entitled Obstetric Genetics (Akadémiai Kiadó, Budapest 1990).

The text as well as the illustrations presented in this new postgraduate training manual are of excellent quality. The book containing 400 colour photographs and 100 ultrasound pictures is a choice collection of fetal developmental malformations and chromosome aberrations. Prenatal diagnosis of the malformations was mainly performed between 1977 and 1989 at the Department of Obstetrics and Gynecology, University Medical School, Debrecen (Hungary) and at the Department of Medical Genetics of the Churchill Hospital, in Oxford, U.K.

The Editor gives an overview on the wide area of fetal malformations and genetic diseases discussed in the twelve chapters.

The co-authors of the Atlas are known experts in their fields, K. Csécsei and G. T. Szeifert in fetal pathology, R. H. Lindenbaum in cytogenetics, Z. Tóth in prenatal ultrasound diagnosis and V. Váradi in pediatric ultrasound.

The high quality professional evaluation of fetal malformations is richly illustrated by the excellent photographs. This atlas is to be of great interest for obstetricians, geneticists, pediatricians and pathologists both in Hungary and abroad. Besides the valuable contents and unique illustrations, the expertise in typography (Alföldi Nyomda, Debrecen) should also be praised.

LÁSZLÓ LAMPÉ

Acta Medica Hungarica, Vol. 49/1–2, pp. 153–155 (1992/93)

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This programme is partially supported by the italian Association for Research on Cancer.

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Two copies of the manuscript typewritten double-spaced with margins at least 4 cm wide should be submitted. Pages should be numbered consecutively. The first page should contain (1) the title of the paper (2) the initials and first name(s) of the author(s), (3) name of the institution where the work was done, (4) name and address of the author to whom correspondence and offprint requests should be addressed — this will appear as a footnote; (5) an abstract not exceeding 250 words which states the purposes of the study, the main findings and principal conclusions. Below the abstract provide 3 to 10 keywords that will assist indexers in cross-indexing the article.

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Unusual abbreviations should be identified in an alphabetical list typed after the abstract and keywords.

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Examples:

1. Stagg, B. H., Temperly, J. M., Wyllie, J. H.: The fate of pentagastrin. Gut 12, 825—829 (1971)
2. Falkner, F.: Prevention in Childhood of Health Problems and Adult Life. WHO, Geneva 1980
3. Fishman, A. P.: Dynamics of pulmonary circulation. In: Hamilton, W. F., Dow, P. (eds): Handbook of Physiology. American Physiological Society, Washington 1963, pp. 65—79

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Figures should be submitted in duplicate. They must be numbered consecutively with arabic numerals. All figures should bear the name of the first author, the figure number and an arrow indicating the top. Cite each figure in the text and indicate its approximate place on the margin. If a figure has been published, acknowledge the original source and submit written permission from the copyright holder to reproduce the material. Figure captions should be submitted typed double-spaced on a separate sheet.

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THE EFFECT OF ATRIAL DILATATION ON REPERFUSION ARRHYTHMIAS: DEVELOPMENT OF SUPRAVENTRICULAR TACHYCARDIAS ON REPERFUSION WITH ATRIAL STRETCHING

F. SOLTI, VIOLA KÉKESI, A. JUHÁSZ-NAGY

Cardiovascular Surgical Clinic, Semmelweis University Medical School, Budapest, Hungary

(Received: December 2, 1992)

The study was aimed at investigating the effect of atrial dilatation on the genesis of supraventricular tachyarrhythmias following myocardial reperfusion. Experiments were carried out in 26 mongrel dogs under pentobarbital narcosis with artificial ventilation. Electrophysiological study was performed for studying the arrhythmic condition of the heart. Investigations were carried out: (i) in normal condition, (ii) during atrial stretching (balloon dilatation of the left atrium), (iii) in reperfusion following myocardial ischemia, (iv) in reperfusion combined with atrial stretching. On reperfusion the irritability of the atrium increased moderately (on atrial extrastimuli in 3 dogs non-sustained atrial tachycardia, in 7 dogs repeated atrial responses could be induced). Reperfusion with atrial stretching, however, very markedly enhanced the atrial vulnerability, and in 19 dogs atrial tachycardia appeared spontaneously. Comparison of the effect of atrial stretching to that of atrial stretching + reperfusion showed that the reperfusion significantly augmented the arrhythmia-inducing effect of atrial stretching. Clinical investigations: Aortocoronary bypass operations were followed by development of supraventricular tachycardia in 41 out of 428 operated cases. Atrial dilatation was detected in 37 cases, mostly before the appearance of atrial tachycardia. The data seem to prove that atrial dilatation has an important part in the pathogenesis of supraventricular tachyarrhythmias following reperfusion of myocardial ischemia.

Keywords: Atrial dilatation, supraventricular tachyarrhythmia, reperfusion, atrial irritability

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Abbreviations: EPS = electrophysiologic study; CL = cycle length; QT_C = corrected QT interval; ACT = intraatrial conduction time; AERP = atrial refractory period; VERP = ventricular refractory period; AVNRP = AV node Wenckebach point; CSNRT = corrected sinus node recovery time; ES = premature beat

Offprint requests should be sent to: F. Solti, H-1122 Budapest, Városmajor u. 68, Hungary

Akadémiai Kiadó, Budapest

Introduction

Reperfusion after myocardial ischemia is regularly followed by arrhythmia, mostly of ventricular origin. The effect of reperfusion on the genesis of arrhythmias has already been studied, but only the ventricular arrhythmias and the changes in the arrhythmic condition of the ventricle have so far been investigated /1, 7, 23, 24, 27, 39/. Although ventricular arrhythmias are more common after myocardial ischemia, but supraventricular arrhythmias may also occur. Therefore, the aim of this study was to investigate the effect of reperfusion on the arrhythmic condition of the atrium. In our former investigation the irritability of the atrium significantly increased on atrial stretching, and atrial tachycardia could be provoked on balloon dilatation of the atrium /34/. The second objective of our investigations was, therefore, to study the effect of reperfusion on the irritability of the atrium if it is combined with atrial stretching.

Materials and Methods

Experiments were performed in 26 mongrel dogs of both sexes, weighing 14 to 20 kg. The animals were anaesthetized with sodium pentabarbitone (30 mg kg^{-1} i.v.). A cuffed endotracheal tube was inserted into the trachea and ventilation was maintained with room air using volumen cycled respirator. The chest was entered through a horizontal sternotomy. For recording epicardial electrograms and electrical stimulation of the atria and ventricles, unipolar ring-shaped electrodes were fixed by atraumatic sutures to the epicardial surface of both atria and both ventricles. The left anterior coronary artery was freed proximal to its second major oblique branch and the thread loop was placed around it. For atrial stretching (dilatation) a balloon catheter was inserted via pulmonary vein into the left atrial appendix. The balloon was inflated until the left atrial pressure increased to 14-15 mmHg, generally 15-20 ml H_2O was necessary for atrial dilatation. For recording atrial and ventricular pressure curves Statham transducers were inserted through a catheter into both atria and the left ventricle. The force changes of the left ventricular wall were registered by a strain-resistance gauge of Walton—Brodie type. The cardiac output was measured by the thermodilution technique. A six-channel direct writing recorder — Hellige type — was used for recording ECG tracings, pressure curves and epicardial electrograms.

In course of electrophysiological study (EPS) for programmed electrical stimulation of the atria and ventricles Medtronic 5925 impulse generator was used. Electrical impulses (2.0 ms in duration) were delivered at twice diastolic myocardial threshold. The following electrical parameters were measured: (i) cardiac cycle length (CL); (ii) PQ interval (for AV conduction time); (iii) corrected QT time (QT_C), determined by using Bazett's formula /5/; (iv) intraatrial conduction time (ACT); (v) atrial effective refractory period (AERP); (vi) ventricular effective refractory period (VERP); (vii) AV node Wenckebach point (AVVRP); (viii) corrected sinus node recovery time (CSNRT).

For inducing atrial tachycardia early atrial single and double electrical stimuli were used; in normal atrial condition we failed to induce atrial tachycardia in dogs by this stimulation technique /33/. Myocardial ischemia was induced by temporary occlusion of the left anterior coronary artery. The EPS was carried out first in the basal — initial — state.

The EPS was repeated under atrial stretching. The reperfusion period started 15 min after coronary artery release and at last, the EPS was repeated during the reperfusion combined with atrial dilatation. The EPS lasted 2 to 4 min.

Clinical study: Four-hundred and twenty-eight aorto-coronary bypass operations were performed in the five-year period 1987 to 1991. The appearance of atrial tachycardia (tachyarrhythmia) was detected in 41 patients (study group) in the early postoperative period. A control group (387 patients) was matched for age, sex, distribution and number of bypass grafts.

Control group: age = 44.6 (20-72) year; sex: male = 271 (70%), female = 116 (30%); mean graft number = 2.3.

Study group: age = 45.5 (22-70) year; sex: male = 30 (73%), female = 11 (27%); mean graft number = 2.4.

Statistical analysis was performed by using Students's unpaired and paired t test and the variance analysis. Mean (SD) are demonstrated on the Tables.

Results

Under normal conditions — basal state — the electrophysiological variables were within the normal range of the dog heart /33/, and no supraventricular tachycardia could be induced. Repetitive atrial responses (couplet atrial ES) on early atrial extrastimuli could be detected only in three dogs. On the effect of atrial stretching the ACT significantly increased and the AERP markedly, and significantly shortened (Table I).

Table I

Effect of reperfusion and atrial stretching on cardiac electrical parameters. Data are mean (SD) expressed in ms

	CL	PQ	QRS	QT_C	ACT	CSNRT	AERP	VERP	AVNRP
Control (basal state)	555 (34)	105 (20)	65 (11)	290 (27)	40 (9)	40 (10)	130 (12)	140 (13)	285 (32)
Atrial balloon dilatation	565 (32)	110 (19)	68 (12)	300 (28)	60^{x} (12)	45 (11)	110^{x} (11)	140 (14)	295 (36)
Reperfusion	560 (34)	110 (19)	66 (11)	315^{x} (27)	45 (10)	50 (15)	120 (12)	120^{x} (13)	290 (34)
Reperfusion + atrial balloon dilatation	568 (34)	112 (18)	68 (12)	320^{x} (26)	64^{xx} (12)	50 (15)	100^{xx} (12)	120^{x} (14)	295 (34)

CL = Cardiac cycle length, PQ = AV conduction time, QT_C = Corrected QT time (electrical systolic length), ACT = Interatrial conduction time, CSNRT = Sinus node corrected recovery time, AERP = Atrial effective refractory period, VERP = Ventricular effective refractory period, AVNRP = AV node Wenckebach point

$^{x}P < 0.05$ vs control
$^{xx}P < 0.01$ vs control
reperfusion vs reperfusion + atrial stretching

Table II

Inducible atrial tachycardia on atrial stretching, with or without reperfusion (26 dogs)

No.	Balloon dilatation + reperfusion		Only balloon dilatation	
	Sustained	Non-sustained	Sustained	Non-sustained
1	1			1
2	1			1
3	1		1	
4		1		1
5	1			
6	1			1
7	1		1	
8	1		1	
9		1		1
10	1		1	
11		1		1
12		1		1
13	1			
14	1		1	
15	1			
16	1		1	
17		1		1
18	1			
19	1			
20	1		1	
21	1			1
22		1		1
23	1			
24	1			1
25	1			1
26	1		1	
	20	6	8	12

On reperfusion, the QT_C significantly prolonged, and the VERP significantly shortened. There were however only modest alterations in the electrophysiological parameters of the atrium; the AERP mostly slightly shortened on reperfusion, but this change was statistically not significant (Table I).

On reperfusion combined with atrial stretching the atrial electrical variables changed very remarkably, the AERP shortened and the ACT increased.

The decrease in AERP on reperfusion + atrial stretching proved significantly greater than on atrial dilatation without reperfusion. The QT_C prolonged and the VERP shortened very markedly on the effect of reperfusion + + atrial dilatation. The changes in the electrical parameters are summarized in Table I.

Table III

Appearance of spontaneous atrial tachycardia on atrial stretching with or without reperfusion (26 dogs)

No.	Balloon dilatation + reperfusion		Only balloon dilatation	
	Sustained	Non-sustained	Sustained	Non-sustained
1		1		
2	1		1	
3	1			1
4				
5		1	1	
6	1			
7	1			1
8	1		1	
9				
10		1		
11				
12				
13		1		
14	1		1	
15	1			
16	1		1	
17				
18	1			1
19	1			
20	1			
21	1			
22				
23	1		1	
24	1			
25				
26	1			
	15	4	6	3

The irritability of the atrium remarkably increased on atrial distension. In 20 dogs supraventricular tachycardias could be induced by early atrial extrastimuli; in 8 cases sustained and in 12 cases non-sustained atrial tachycardias were detected (Table II).

Spontaneous atrial tachycardia developed in 9 cases during atrial balloon dilatation; 6 sustained and 3 non-sustained ones (Table III).

On reperfusion the arrhythmic condition of the atrium increased only slightly, but in 3 dogs non-sustained atrial tachycardia could be induced by atrial extrastimuli. The repeated atrial responses were also more frequent than in the basal condition of the heart; repeated ES appeared in 7 dogs.

On reperfusion + atrial stretching the vulnerability and irritability of the atrium very markedly augmented. Supraventricular tachycardia was in-

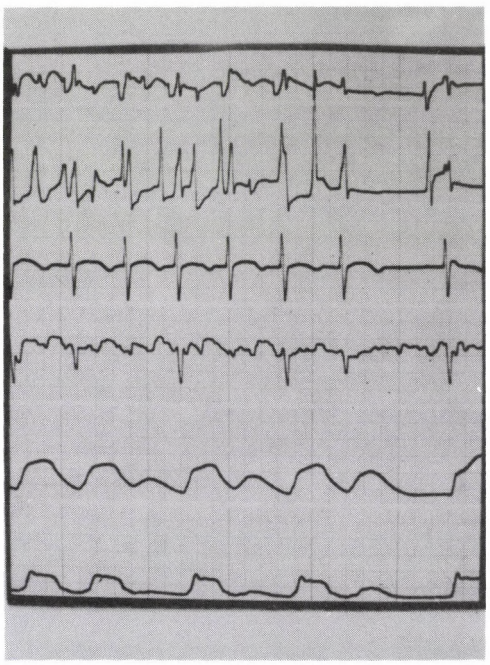

Fig. 1. Appearance of atrial flutter on reperfusion with atrial stretching
Paper recording speed: 50 $mm \cdot s^{-1}$, tracing from top: left atrial electrogram; right atrial electrogram; right ventricular electrogram; bipolar surface lead III, left ventricular strain gauge curve; left ventricular pressure curve.
Atrial flutter spontaneously developed and ceased spontaneously after 60 s. For the end of the tachyarrhythmia see the right side of the figure: the sinus rhythm restored

ducible in each dog; 20 sustained and 6 non-sustained atrial tachyarrhythmias were detected (Table II). Comparison of frequency of inducible atrial tachycardias on balloon dilatation with those of reperfusion + balloon dilatation showed a significant trend; more frequent and more serious (sustained) tachyarrhythmias were established in the latter group by variance analysis. Spontaneous tachycardia developed in 19 dogs (15 sustained and 4 non-sustained) during reperfusion with atrial stretching (Table III). A significant trend of more inducible and more serious atrial tachyarrhythmias on reperfusion + atrial stretching could be revealed by variance analysis. From

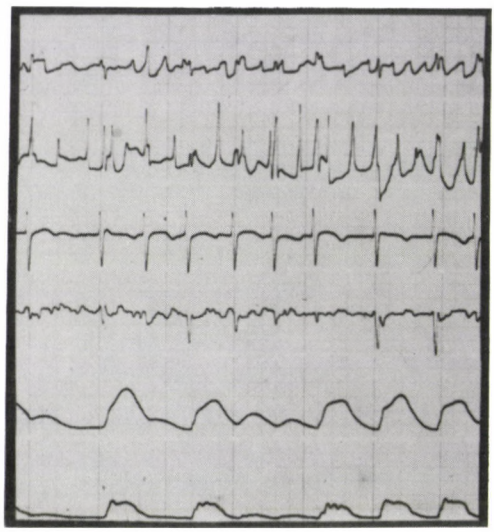

Fig. 2. Appearance of atrial fibrillation on reperfusion with atrial stretching
Paper speed: 50 mm·s^{-1}, tracing from top: left atrial electrogram; right atrial electrogram; right ventricular electrogram; bipolar surface lead III; left ventricular strain gauge curve; left ventricular pressure curve. The tachyarrhythmia developed spontaneously and it was interrupted after 80 s by balloon deflation

the supraventricular tachycardias 6 were atrial flutter, 4 atrial fibrillation. The appearance of atrial flutter and atrial fibrillation with supraventricular tachyarrhythmias is shown in Figs 1 and 2.

There were no important hemodynamic changes in the course of investigation. The left ventricular force markedly diminished on myocardial ischemia and gradually turned back to the initial level on reperfusion. The left ventricular systolic pressure mostly slightly increased on reperfusion. The cardiac output slightly (but not significantly) diminished in the reperfusion period. The left atrial pressure markedly increased on balloon dilatation (Table IV).

Appearance of atrial tachycardia following aortocoronary bypass grafting was detected in 41 patients (9.6%). The most important clinical data and electrophysiological characters of this study group are outlined in Table V.

Atrial dilatation was detected in 37 of the 41 cases. Mostly acute, transitory atrial dilatation was revealed prior to the development of tachy-

Table IV

Hemodynamic changes following atrial stretching and reperfusion. Data are mean (SD)

	LAP	LVSP	LVEDP	CO
Control (basal state)	5 (1)	105 (18)	8 (1)	2.0 (0.4)
Atrial stretching	15 (1)	104 (17)	9 (1)	1.8 (0.5)
Reperfusion	5 (1)	102 (17)	10 (2)	1.4 (0.6)
Reperfusion + atrial stretching	15 (1)	101 (18)	10 (3)	1.8 (0.7)

LAP = left atrial pressure (mm Hg)
LVSP = left ventricle peak (systolic) pressure (mm Hg)
LVEDP = left ventricle end diastolic pressure (mm Hg)
CO = cardiac output 1 min^{-1}

Table V

Appearance of aupraventricular tachycardia following aortocoronary bypass operation (41 cases)

Age (year)	Sex	Type of tachycardia	Atrial dilatation
45	male: 30	SVT: 28	37
(22—70)	female: 11	TPAF: 5	
		TAF: 8	

SVT = supraventricular — reentry — tachycardia
TPAF = tachycardia based on paroxysmal atrial flutter
TAF = tachycardia (tachyarrhythmia) based on paroxysmal atrial fibrillation

cardia. The supraventricular tachycardias appeared in the early postoperative period, 1-3 days following the operation. The tachycardia lasted 1-2 days. Permanent tachycardia (atrial fibrillation) developed only in one patients. The supraventricular tachycardia was based mainly on macroreentry mechanism (28 patients), in 5 patients on atrial flutter and in 8 patients on atrial fibrillation. The atrial dilatation proved the most important risk factor for the development of reperfusion atrial arrhythmia. We failed to

detect any close interrelationship between age, sex distribution, number of grafts, antecedent myocardial infarction and the development of atrial tachycardias following a coronary bypass operation.

Discussion

The arrhythmic condition of the heart is augmented on myocardial reperfusion, appearance of arrhythmias can regularly be detected. Mostly ventricular arrhythmias (ventricular ES, ventricular tachycardia, transient-ventricular fibrillation) develop, but in about 10% atrial arrhythmias can be observed. The irritability of the heart on reperfusion is based on a combined mechanism. Oxygen-derived free radicals can play a role in the genesis of reperfusion arrhythmias /3, 16, 19, 30/. The extent of prostacyclin release due to myocardial ischemia can also affect the arrhythmic condition of the heart during reperfusion /8, 37/. An increase of alpha-receptor density in the myocardium /9/, catecholamine discharge form the heart /18, 31/, an accumulation of myocardial calcium /36/ on reperfusion can also increase the vulnerability of the heart. Probably, the pathological changes in the action potential of the myocardial cells, due to myocardial hypoxia, represent the most important pathogenetic factor of reperfusion arrhythmias. On myocardial ischemia the action potential duration shortens, and the impulse conduction slows down; these changes will slowly regress during reperfusion /3, 12/. It is very important that the regression of the action potential duration will be therefore divergent in different regions of the heart in reperfusion; dispersion of the repolarization and refractoriness will develop. The effect of reperfusion on electrical property of the atrium has not yet been studied in detail; likely, similar but slighter action potential changes develop on reperfusion in the atrium. The data of the present study also suggest that first of all the irritability of the ventricle (marked shortening of the VERP and prolongation of QT_C) will increase during reperfusion. The arrhythmic condition of the atrium slightly augments on reperfusion, while atrial arrhythmias are manifested but rarely. The genesis of supraventricular arrhytmias is based on a complex mechanism. The atrial dilatation results in a very expressed increase in the irritability of the atrium. On the effect of marked and permanent atrial dilatation regularly atrial flutter or fibrillation will develop /7, 15, 25, 28/. On atrial stretching the vulnerability of the atrium is enhanced so that the

atrial balloon dilatation represents a very apt model for studying the pathomechanism of supraventricular tachyarrhythmias /34/. On atrial stretching, pathological alteration of the atrial action potential can be observed /21, 32, 35/, which may increase the arrhythmic condition of the atrium. A mechano-electrical feedback mechanism can also be involved in the development of atrial arrhythmias /13, 20/. The prolongation of intraatrial conduction facilitates the development of reentry-type supraventricular tachycardia /4, 22, 32/. It is worthy of mentioning that, according to our experimental data, the intraatrial conduction time significantly increases on atrial dilatation.

The results of the present study prove that reperfusion significantly facilitates the arrhythmic condition of the atrium. On atrial stretching + reperfusion the AERP more significantly shortened than on atrial stretching alone, and supraventricular tachyarrhytmias spontaneously appeared in most of the cases.

It is remarkable that after aortocoronary bypass grafting the supraventricular tachycardias appear more frequently than ventricular ones. In 5-10% of the cases supraventricular tachyarrhythmias will develop after myocardial revascularization /2, 10, 14, 26, 38/. We observed atrial tachyarrhythmias (paroxysmal atrial flutter, paroxysmal atrial fibrillation and supraventricular tachycardia) in 9.6% of the cases following aortocoronary bypass grafting. In our cases atrial dilatation regularly preceeded the development of atrial tachycardias. Dixon et al. /11/ also detected supraventricular tachycardia frequently after coronary heart operation if the left atrium was enlarged. Our data suggest that atrial dilatation has a decisive part in the manifestation of atrial tachycardias following coronary heart operation.

Conclusion: 1. On reperfusion, not only the ventricular irritability, but also the arrhythmic condition of the atrium augments. 2. Reperfusion markedly facilitates the atrial irritability during atrial dilatation. 3. In reperfusion the atrial tachycardias mostly will be manifested on atrial stretching.

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ENDOCRINOLOGY

CIRCADIAN PATTERN OF SERUM ANDROGENS IN WOMEN WITH CUSHING'S SYNDROME

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Circadian profiles of the serum levels of cortisol and five androgens were studied in 20 females including 8 controls, 7 patients with ACTH-dependent Cushing's syndrome and 5 with hypercortisolism due to adrenal adenoma. A significant 24-h periodicity was found for each steroid in all groups. Besides hypercortisolaemia, a significant increase of 11-hydroxyandrostenedione was shown in both forms of Cushing's syndrome. The most conspicuous finding was a decreased dehydroepiandrosterone and its sulphate in adrenal adenoma. The peaks for the studied steroids were shifted from the morning hours towards the noon in ACTH-dependent Cushing's syndrome and towards the evening (for cortisol) and night hours (for androgens) in adrenal adenoma.

Keywords: Serum androgens, cirdadian rhythm, Cushing's syndrome, women

Introduction

Unlike well-documented aberrations of the circadian rhythm of serum cortisol in Cushing's syndrome /5, 8/, the issue of circadian pattern of serum androgens in this syndrome has not been investigated so far. The aim of the present study was to fill this gap.

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Patients and Methods

Subjects and Blood Sampling

Seven women with ACTH-dependent Cushing's syndrome, aged 23-53 years, and five women with cortisol producing adrenal adenoma, aged 22-52 years, were examined. The diagnoses were based on surgical specimens and their histological examinations and/or on clinical and secretory remission after transsphenoidal microsurgery. The investigations were made prior to any therapy, in the follicular phase of the menstrual cycle, unless the patients were amenorrhoic. Eight volunteering nonobese healthy women, aged 16-40 years, served as controls. Consent was obtained from each of them.

The venous blood samples were withdrawn under standard ambulatory regimen at 8, 16, 20, 24, and again at 8 o'clock, for steroid analysis.

Steroid Analyses

In each serum sample, besides cortisol, the following androgens were measured: androstenedione, 11-hydroxyandrostenedione, dehydroepiandrosterone, dehydroepiandrosterone sulphate and testosterone. All six steroids were determined by radioimmunoassay (RIA), using antisera prepared in our laboratories, and tritiated radioligands. The reagents and procedures for determination of cortisol /13/, androstenedione /11/, 11-hydroxyandrostenedione /12/, dehydroepiandrosterone /3/ and testosterone /6/ were described elsewhere. Dehydroepiandrosterone sulphate was estimated by the method of Buster and Abraham /2/. Only chemicals of analytical grade were used. Radioactivity was measured on a Packard-Tricarb liquid scintillation spectrophotometer. The log-logit plot was routinely used for calculation of RIA results. The sensitivity (i.e. the lower limit of detectability) and coefficients of variation of the assays are presented in Table I. The list of all measured individual values is available on request at the authors.

Table I

Sensitivity and average intra- and interassay coefficients of variation for serum steroids

Serum steroid	Sensitivity	Intraassay (%)	Interassay (%)
Androstenedione	0.21 nmol/L	5.4	11.6
11-hydroxy-androstenedione	0.26 nmol/L	6.3	8.8
Dehydroepiandro-sterone	0.21 nmol/L	6.7	9.8
Dehydroepiandro-sterone sulphate	0.32 µmol/L	6.4	9.2
Testosterone	0.15 nmol/L	4.5	6.8
Cortisol	24 nmol/L	7.9	10.3

Biometrical Analyses

Each steroid diurnal profile was processed for each diagnostic group by Halberg's cosinor analysis /1/, using original programmes /10/.

The estimated means and their 95% confidence intervals are given for mesor (the rhythm-adjusted mean), amplitude (half of the 24-hour variability due to the rhythm) and acrophase (the estimated time of peak value). The significance of 24-hour rhythmicity was tested by the one-sided t test applied on the difference between amplitude and zero. The significance of differences between mesors, amplitudes and acrophases in controls, ACTH-dependent Cushing's syndrome and adrenal adenoma was tested with the aid of the Mann-Whitney test. The level of statistical significance for tests has been chosen at the probability alpha = 0.05.

Results

The inspection of Fig. 1 reveals an unequivocal finding: conspicuously low serum concentrations of dehydroepiandrosterone and particularly of its sulphate in patients with cortisol producing adrenal adenoma.

The biometrical analysis (Table II) detected the presence of a significant 24-hour periodicity for each steroid in all groups. Besides, some differences were ascertained both between the studied groups and between the steroids. A significant elevation of the rhythm-adjusted mean (mesor) was found for cortisol and 11-hydroxyandrostenedione in both forms of Cushing's syndrome. On the other hand, the mesor as well as the amplitude of dehydroepiandrosterone and its sulphate were significantly lowered in adrenal adenoma, compared to controls and to the ACTH-dependent Cushing's syndrome. A significantly decreased amplitude was observed also for androstenedione in adrenal adenoma versus controls.

Some significant differences concern also the acrophases (i.e. the estimated times of peak values) of serum androgens (Fig. 2).

The typical morning peaks observed in controls (with some delay for dehydroepiandrosterone sulphate), were lacking in adrenal adenoma, where the culmination was shifted towards the night hours. The time decomposition was less pronounced in ACTH-dependent Cushing's syndrome, where the steroid levels (except for testosterone) culminated around the noon. Statistically

→

Fig. 1. The 24-hour steroid profiles (full circles = measured values, dashed line = estimated cosine curve for the group) in controls (n=8), patients with ACTH-dependent Cushing's syndrome (n=7), and patients with Cushing's syndrome due to adrenal adenoma (n=5).
Vertical scales in nmol/L, for dehydroepiandrosterone sulphate in μmol/L. F = cortisol, AD = androstenedione, HA = 11-hydroxyandrostenedione, D = dehydroepiandrosterone, DS = dehydroepiandrosterone sulphate, T = testosterone. Asterisk denotes a missing measurement

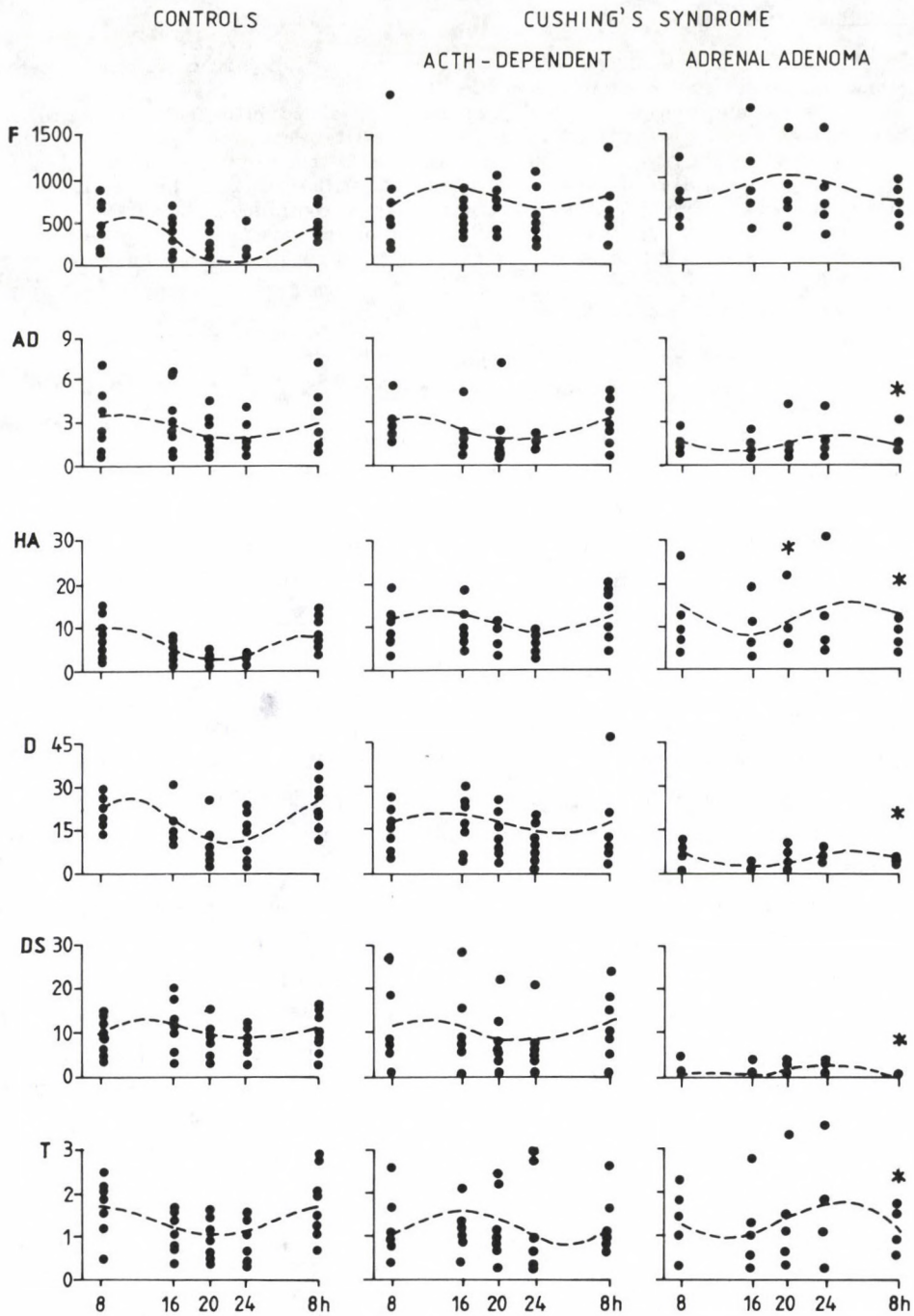
CONTROLS
CUSHING'S SYNDROME
ACTH-DEPENDENT
ADRENAL ADENOMA
F 1500
1000
500
0
AD 9
6
3
0
HA 30
20
10
0
D 45
30
15
0
DS 30
20
10
0
T 3
2
1
0
8
16
20
24
8h

Fig. 1

Table II

Rhythm characteristics for serum steroids in the three groups of serum donors

Serum steroid	Group of serum donors		Mesor (95% conf. limits)	Amplitude (95% conf. limits)	Acrophase (95% conf. limits)	Mean percentage rhythm (%)
Cortisol (F)	Controls*	#	308 (251—365)	220 (164—277)	10:47 (09:37—11:58)	87
	CS-ACTH^	#	654@ (496—811)	151 (79—222)	13:01 (09:11—16:52)	56
	CS-adenoma~	#	807@ (569—1046)	205 (-7—418)	19:38@ (15:50—23:26)	60
Androstene-dione (AD)	Controls	#	2.5 (1.6—3.4)	1.0 (0.7—1.3)	10:36 (07:53—13:19)	77
	CS-ACTH	#	2.5 (1.8—3.2)	0.7 (0.1—1.3)	11:13 (07:55—14:32)	40
	CS-adenoma	#	1.6 (0.8—2.5)	0.4@ (0.0—0.7)	03:45 (22:04—09:26)	42
11-hydroxy-androstene-dione (HA)	Controls	#	4.9 (3.6—6.2)	3.7 (2.5—5.0)	10:33 (09:21—11:44)	79
	CS-ACTH	#	9.5@ (7.9—11.0)	3.0 (1.4—4.6)	11:59 (09:59—13:59)	51
	CS-adenoma	#	11.2@ (4.4—18.0)	3.0 (1.2—4.9)	05:09 (00:53—09:26)	86
Dehydroepi-androsterone (D)	Controls	#	14.3 (10.8—17.8)	8.00 (5.9—10.0)	09:52 (08:27—11:17)	80
	CS-ACTH	#	13.7 (8.3—19.1)	4.9 (2.5—7.3)	11:44 (08:14—15:15)	49
	CS-adenoma	#	4.6 @,& (3.1—6.1)	1.0 @ ,& (0.6—1.5)	04:14 (22:56—09:32)	47
Dehydroepi-androsterone sulphate (DS)	Controls	#	8.1 (6.1—10.1)	2.7 (1.2—4.3)	13:44 (11:13—16:15)	45
	CS-ACTH	#	8.4 (3.3—13.5)	2.6 (0.9—4.3)	11:35 (07:30—15:40)	60
	CS-adenoma	#	1.0 @,& (-0.2—2.2)	0.2 @,& (0.0—0.4)	01:21 @ ,& (23:04—03:40)	61
Testosterone (T)	Controls	#	1.2 (0.9—1.5)	0.4 (0.2—0.6)	07:31 (06:04—08:59)	82
	CS-ACTH	#	1.2 (0.7—1.7)	0.4 (0.1—0.6)	15:36@ (10:18—20:54)	59
	CS-adenoma	#	1.2 (0.5—2.0)	0.4 (0.0—07)	00:35@ ,& (21:34—03:36)	56

Mesor (rhythm adjusted mean), nmol/l, for DS μmol/l
Amplitude (half of the 24-hour variability due to the rhythm), dtto
Acrophase (the estimated peak time), h:min
@ = significant difference against controls, & = dtto between CS-ACTH and CS-adenoma (two-sided Mann—Whitney—Wilcoxon test)
# = significance of the rhythm (one-sided t-test)
*n=8; ^Cushing's syndrome, ACTH-dependent (n=7); ~Cushing's syndrome, adrenal adenoma (n=5)

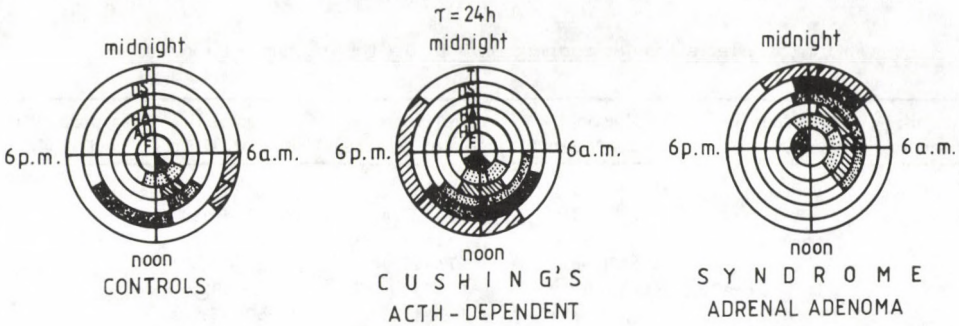

Fig. 2. Acrophases of circadian rhythm (bars), i.e. estimated times of peak values and their 95% confidence limits (shadowed) for six serum steroids in the three groups of serum donors. For abbreviations see Fig. 1

significant shifts of androgen peaks have been detected, however, only for dehydroepiandrosterone sulphate and testosterone in adrenal adenoma versus both controls and ACTH-dependent Cushing's syndrome (Table II).

The percentage rhythm, showing the quality of fitting of data, ranges between 40 and 87% of variance, explained by the regression. It is usually lower in Cushing's groups than in the controls.

Discussion

Our data add a chronobiological dimension to earlier descriptions of serum androgen levels in Cushing's syndrome. Thus, the finding of a significantly lower mesor of dehydroepiandrosterone and its sulphate in cortisol-producing adrenal adenoma extends the frequently-cited observation of Yamaji et al. /15/, who demonstrated that the morning values of dehydroepiandrosterone sulphate were normal or increased in ACTH-dependent Cushing's syndrome, while they were lower than those of normal subjects in all of their patients with hypercortisolism due to adrenal adenoma. In Cushing's syndrome due to adrenal adenoma, low serum dehydroepiandrosterone sulphate levels in the morning have been reported by some other authors, too /7, 14/. In the study of Levine et al. /9/ an increased level of serum dehydroepiandrosterone sulphate was ascertained in one out of six patients with cortisol-producing benign adrenal adenoma. On the other hand, extremely low serum levels of this androgen were encountered in one out of our seven patients with ACTH-dependent Cushing's syndrome (Fig. 1). Accordingly, low

serum dehydroepiandrosterone sulphate in cortisol-producing adrenal adenoma cannot be considered an absolute rule.

Our finding of increased 11-hydroxyandrostenedione mesor in both studied forms of Cushing's syndrome is in agreement with the only previous description /4/ of its increased levels in morning samples of one patient with ACTH-dependent Cushing's syndrome and two patients with cortisol-producing adrenal adenoma.

A pronounced difference between controls and both studied forms of Cushing's syndrome has been ascertained in the present study for mean acrophases of cortisol as well as for those of androgens. They were shifted from the physiological morning hours towards the noon in ACTH-dependent Cushing's syndrome and towards evening (cortisol) and night (androgens) hours in cortisol producing adrenal adenoma. Thus, a marked time dissociation between peak outputs of cortisol and androgens appears as a feature of adrenal adenoma, not yet described according to our best knowledge.

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COMBINED CYCLOSPORIN-A AND METHYLPREDNISOLONE TREATMENT OF GRAVES' OPHTHALMOPATHY

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Twelve patients with Graves' ophthalmopathy (grade 1-6, ATA classification) were treated with Cyclosporin-A, systemically in combination with methylprednisolone. We observed slight or moderate favourable effect in 9 cases. Our data suggest that the benefit was due to the methylprednisolone, the effectivity of which was enhanced by the cyclosporin even in the glucocorticoid-resistant cases.

Keywords: Graves' ophthalmopathy, Cyclosporin-A, methylprednisolone

Introduction

The unsolved treatment and severe side effects of immunosuppressive drugs led to the administration of Cyclosporin-A (a non-myelotoxic immunosuppressant used in organ transplantation) in some of the organ-specific autoimmune diseases. The first publication of successful treatment of Graves' ophthalmopathy with Cyclosporin-A appeared in 1983 /13/, and it was followed by reports on different success /1, 2, 5, 6, 11, 12, 15, 16/. There was no significant difference in efficacy between the prednisone and cyclosporin monotherapy in the study of Kahaly et al. /7/ and Prummel et al. /10/. The combination of prednisolone and cyclosporin seemed to be more efficacious than the monotherapy /4/. A reduced dose of corticosteroids in this combination may be followed by less serious side effects. Despite many effort there is no established protocol for administration of cyclosporin in the treatment of Graves' ophthalmopathy.

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Materials and Methods

Twelve patients suffering from Graves' ophthalmopathy (classes 1-6, grading the signs within each class: o, a, b, c, by American Thyroid Association /14/) were treated with Cyclosporin-A. The patients proved resistant to glucocorticoid monotherapy previously, which was performed for 12-16 weeks either by methylprednisolone or prednisolone. (Methylprednisolone was administered by the same dose and regimen as combined with cyclosporin (see later). The total dose of prednisolone varied between 2000 and 2500 mg.)

The patients, 10 females and 2 males aged between 35 and 58. All were euthyroid at the beginning of cyclosporin treatment. Earlier, they were given long-term methimazole treatment.

Ophthalmopathy had started 5 and 3 years earlier in 2 cases, 1 to 2 years earlier in 5 cases and 3 to 7 months earlier in 5 cases.

The following examinations were performed before treatment: FT4, sensitive TSH, T3-uptake, TRH stimulation test, tests for TRAb, and antibodies against thyroglobulin and microsomal antigen.

The patients were checked by an ophthalmologist: visual acuity, bulbar motility (Hess chart), and proptosis were measured (Hertel), visual field test (static and kinetic), slit-lamp examination; ophthalmoscopy, CT-scan or NMR and ultrasonographic examinations of the retrobulbar area were performed. The control of orbital lesions was performed by ultrasonography.

Relying on clinical observations, we applied cyclosporin in combination with glucocorticosteroid in the treatment of Graves' ophthalmopathy.

The daily dose of Cyclosporin-A was 4-10 mg/kg. The drug was administered orally, divided into two doses in each case. Besides cyclosporin, methylprednisolone was administered viz. 250 mg intravenously every 2nd or 3rd day (total amount = 1.5-2 g); after the i.v. administration every second day 32 mg doses were given orally and the above dose was decreased by 8 mg every two-week period.

The maintenance dose of methylprednisolone was 8-12 mg/day. Depot ACTH was given at the end of treatment.

The peripheral blood cyclosporin level was checked weekly during treatment. The cyclosporin whole-blood levels varied between 250 and 400 ng/ml (polyclonal antibodies, using the Sandoz diagnostic kit, analysed by Abbott TDX). Urine, blood urea nitrogen, serum creatinine, serum bilirubin and the hepatic transaminases were controlled, every two weeks.

Results

Among our patients one (No. 1) showed significant improvement; her diplopia, corneal ulcer and optic neuropathy ceased. She has been in permanent remission for 5 years (Table I).

We observed moderate improvement in two patients (Nos 2 and 9). Their diplopia improved, corneal and optic nerve involvement ceased.

Six patients (Nos 3, 4, 5, 6, 11, 12) showed only slight, not sufficiently impressive, improvement. Their corneal involvement decreased, diplopia diminished or was unchanged (Table II).

Ophthalmopathy worsened during the last period of treatment in 3 patients (Nos 7, 8, 10): their optic neuropathy and diplopia worsened.

First of all as a consequence of the decrease of soft-tissue signs and symptoms, all of our patients improved considerably in the first 2-4 weeks

Table I

Summarized effect of combined cyclosporin A and methylprednisolone treatment

Patient	Duration of G.O.	Cyclosporin therapy		Improved	Worsened	Remarks
		mg/kg/day	weeks			
1. G. J. 49 yr	6 m	5	4	remission		
2. K. J. 58 yr	2 yr	6.5	15	moderate		cataract, glaucoma
3. V. D. 48 yr	16 m	5	13	mild		recurrent thyrotoxicosis
4. D. V. 49 yr	17 m	10—5	24	mild		cataract, glaucoma
5. N. L. 49 yr	1 yr	5	24	mild		
6. F. J. 45 yr	5 yr	7.5	12	mild		glaucoma
7. K. J. 45 yr	6 m	6	16		+	diabetes m.
8. Sz. B. 44 yr	3 m	6.5	24		+	eye muscle surgery
9. N. J. 37 yr	3 yr	4	10	moderate		
10. Ny. G. 44 yr	5 m	4	20		+	chr. duodenal ulcer
11. K. G. 41 yr	7 m	7—5	12	mild		
12. Sz. E. 38 yr	1 yr	6—5	12	mild		

G.O.: Graves' ophthalmopathy, m: month, yr: years(s)

of treatment. Improvement slowed down or stopped later, even in 5 cases there was a relapse during the last period of the course.

The combined cyclosporin-methylprednisolone treatment ceased the corneal involvement in all 8 cases; the optic neuropathy improved in 5 cases, but it worsened in one (No. 10) (Table II).

Proptosis did not change significantly and the diplopia seemed resistant to the treatment. Diplopia showed some improvement in 6 cases, it did not change in 4 and it worsened in 2 cases.

Table II

Changes of ophthalmopathy according to classification of A.T.A.

Patient	Classification			
	Before treatment		After treatment	
1		2c 3c 4b 5b 6b		2a 3b 4o 5o 6o
2		2c 3c 4b 5a 6a		2b 3c 4a 5o 6o
3		2c 3b 4b 5a 6a		2b 3a 4b 5o 6o
4		2c 3c 4c 5b 6a		2b 3c 4b 5o 6a
5		2b 3b 4b 5a		2b 3b 4b 5o
6		2c 3c 4b 5b 6a		2c 3b 4b 5o 6a
7	r: l:	2b 3b 4c 2c 3b 4c	r: l:	2c 3b 4c 2c 3b 4b
8	r: l:	2b 3o 4a 2b 3a 4c	r: l:	2b 3o 4c 2b 3o 4b
9	r: l:	2c 3a 4b 1 2a	r: l:	2o 3a 4a 1 2o
10	r: l:	2c 3a 4b 5b 6a 2c 3a 4b	r: l:	2c 3a 4b 5o 6b 2c 3a 4b 5o 6a
11	r: l:	2b 3a 4c 2b 3o 4c	r: l:	2a 3o 4b 2a 3o 4b
12	r: l:	2b 3b 4b 5a 2b 3a 4b 5a	r: l:	2a 3a 4b 5o 2a 3o 4b 5o

r: right, l: left

We failed to detect any significant change in the volume of extraocular muscles at the end of treatment (Table II). It was unchanged in 3 cases (Nos 3, 4, 5), tended to decrease in 5 (Nos 6, 7, 8, 9, 11) and increased in 1 (No. 10); it decreased in the left eye and increased in the right one in 1 case (No. 2). None of these changes were significant.

Visual acuity improved in 4 cases, worsened in 4 and did not change in 4. One (No. 4) of four patients whose vision deteriorated had cataract which progressed during the 6 months of treatment; in contrast, her soft-tissue involvement showed a mild improvement and the corneal ulcer healed.

Side effects were observed in 6 cases. Mild nephropathy and hepatopathy occurred in the 3rd week of treatment in 1 case (No. 1), which worsened in the 4th. Cyclosporin treatment was stopped in this case. The urine, renal function tests, serum bilirubin and hepatic transaminase enzymes of

Table III

Side effects of combined cyclosporin A and methylprednisolone treatment

Patients	Side effects
1	Transitory mild nephropathy and hepatopathy, hyperesthesia, dysgeusia, hypertrichosis, swelling of the breasts
2	Hyperesthesia, dysgeusia, hypertrichosis
3	Ø
4	Paresthesia, hypertrichosis
5	Ø
6	Paresthesia, headache
7	Ø
8	Oral candidiasis
9	Ø
10	Hypertrichosis (slight moustache growth)
11	Ø
12	Ø

this patient normalized (Table III) within 2 weeks. Mild hirsutism appeared in 4 cases, and 4 patients had neurological complaints: paresthesia, hyperesthesia and/or a disturbance in the sense of taste.

Discussion

Despite its use since 1983 /13/ the treatment of Graves' ophthalmopathy with cyclosporin is controversial /3, 8, 10/. The interpretation of our results is difficult and ambiguous. We found that Cyclosporin A has not had a sufficiently convincing effect on our patients in the treatment of Graves' ophthalmopathy. The observed slight or moderately favourable effect in 9 cases out of 12 can be far more attributed to the methylprednisolone than cyclosporin. The sudden improvement during the first period of treatment may be explained by the intravenously administered high dose of methylprednisolone. Namely, cyclosporin needs a longer time to develop its effect. The dose of methylprednisolone was increased in some cases if the activity of infiltrative symptoms had reappeared or increased in the course of treatment. In these cases we observed a sudden beneficial effect. But we have to

consider that the previously administered glucocorticoid monotherapy was unsuccessful in these cases. Therefore, we think it likely that some additional immunosuppressive and/or immunomodulating qualities of cyclosporin have enhanced — under certain conditions — the effectivity of glucocorticosteroid treatment.

During the 3rd week of treatment the hyperthyroidism relapsed in one patient (No. 3), in spite of the improvement of her ophthalmopathy. Because of her serious thyrotoxic condition she was operated on. After operation the cyclosporin treatment was continued. From pathogenetical point of view, it seems to be worthwhile to mention that, while the ophthalmopathy significantly improved, a severe relapse occurred in her thyroid hyperfunction. Earlier we had another patient suffering from severe thyrotoxicosis and diabetes mellitus. This patient was successfully treated with antilymphocytic globulin but her ophthalmopathy became active and progressed at the same time. These observations confirm the supposition that Graves' ophthalmopathy had a distinct pathogenetic entity than Graves' disease itself.

Our data are in agreement with findings of the Prummel's group, who found that prednisolone monotherapy is more effective than cyclosporin alone, and the combination of the two drugs can be effective in patients who do not respond to either treatment alone.

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TREATMENT OF POLYGLANDULAR AUTOIMMUNE SYNDROME WITH CYCLOSPORIN-A

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The case history of a 30-year-old female patient is reported. Following an unknown viral infection that had occurred four years earlier, insulin-dependent diabetes mellitus vitiligo, Addison's disease, amenorrhoea, hyperthyreosis and, finally, severe pancytopenia with dominant thrombocytopenia developed. On the basis of clinical aspects and laboratory findings, an infrequent polyglandular autoimmune syndrome (type II) was verified. Substituent therapy and steroid stoss therapy also was introduced, without any sign of improvement. For the lack of therapeutic effect and owing to serious thrombocytopenic bleeding, treatment with Cyclosporin-A was indicated, which produced total remission of the illness. Nowadays the patient being on follow-up, has no sign of disease activity.

Keywords: Polyglandular autoimmune syndrome type II, clinical symptoms, Cyclosporin-A

Introduction

Polyglandular autoimmune syndrome (PGAS) is based on an autoimmune mechanism resulting in malfunction of two or more endocrine organs. On the basis of the presence or absence of adrenal cortex insufficiency, two major groups of the disease are distinguished. If the symptom complex is, concomitant with Addison's disease, a further well-determined twofold distinction can be made. The two types are differentiated on the basis of age, beginning of disease and co-occurrence of certain endocrine diseases /1, 5—8/.

PGAS type II is characterised by the presence of primary adrenal cortex insufficiency, autoimmune thyropathy and/or insulin-dependent diabetes mellitus. The key element of the syndrome is adrenal cortex insufficiency, thought in the past it was attributed to tuberculotic origin, nowadays it

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is frequently believed to be a result of an autoimmune process, and is often accompanied by an autoimmune damage of other endocrine glands or nonendocrine organs /6/.

The present study, through the case history of a young female patient, is intended to draw attention to this rare and manywise manifested disease, its pathomechanism and possible treatment. Because this syndrome is very rare diagnosed and the Cyclosporin-A treatment had dramatic effect, makes this case conspicious. It should be noted that our effort to find similar therapeutic strategy for PGAS in international medical literature has failed.

Case report

On April 1, 1992, I. Sz. female, aged 30, was admitted to our hospital in severe condition. Her grandparents died old, her father died of lung cancer. The patient's mother is alive, in good health. She has had no siblings. She is the mother of a boy being in good health.

In 1982 she had a normal delivery. In 1988, on indication of dysmenorrhea following some viral infection, she had her intrauterine contraceptive device removed, and because of non-severe thrombocytopenia and splenomegaly, she was taken over from the gynaecological ward to our section. The patient was free from complaints, we did not observe bleeding tendency. Her platelet count was $110 \times 10^9/l$, and sternum smear negative. As an autoimmune origin could not be excluded, she was summoned for control inspection but she did not present. In 1989, she was admitted to the medical ward of the local hospital with asthenia, weight loss, polyuria, polydipsia and hypotonia. Insulin-dependent diabetes mellitus (IDDM) with accompanying vitiligo was diagnosed. On the basis of clinical symptoms and hypotension, hypadrenia was suspected but not verified. The patient was treated with a 4 I.U. Insulin Actrapid MC (Novo Nordisk) and 16 I.U. Insulin Ultralente MC (Novo Nordisk). In 1990, she was readmitted to medical ward with cumulative hypoglycaemic episodes. There was brownish pigmentation on her face and vitiligo scattered on her body; hepar: 2 cm, lien: 3 cm under costal arch; blood pressure: 90/70 mmHg, tachycardia (120/min) and amenorrhea. She was remarkably thin. High sensitivity to insulin and low level of plasma cortisol which did not increase on ACTH, hyponatraemia, hypochloraemia, hyperpotassaemia, low level of urine cortisol and 17-ketosteroid values justified the diagnosis of Addison's disease. Since the alleged cause of earlier diagnosed IDDM and adrenal cortex insufficiency were of autoimmune origin, the patient was submitted to immunochemical tests. In that time besides insufficiency of the two endocrine glands, hyperthyrosis was diagnosed. Laboratory findings: E.S.R.: 2 mm/h, WBC: $3.4 \times 10^9/l$, Hgb: 124 g/l, platelet count: $70 \times 10^9/l$, serum cholesterol: 2.5 mmol/l, plasma cortisol: 3.2 µg/dl, 6 h after ACTH: 5.0 µg/dl, 24 h after ACTH: 5.3 µg/dl, urine cortisol: 8 µg/24 h, antinuclear antibodies: negative complement fixation with hepatic antigen: negative, with WBC antigen: negative, circulation immunocomplex (PEG-precipitate): 340 µg/ml, IgG: 7.6 g/l (slightly lower than normal), IgA: 1.5 g/l, IgM: 1.0 g/l (normal), C3: 72 mg/dl (normal), C4: 12 mg/dl (subnormal), antinuclear antibody (with indirect immuno-fluorescent method): negative. The patient was discharged with 2 I.U. Insulin Actrapid MC, 2 I.U. Insulin Ultralente MC, 3 x 10 mg thiamazole, 2 x 0.1 mg fluorocortisone daily medication.

After re-admission with disturbance of carbohydrate metabolism, in February and March, 1991, she was treated in medical ward and intensive therapy unit, when, following a 13-month treatment with thiamazole, thyroid stimulating hormone (TSH) level normalized, therefore, the thiamazole medication was abandoned. With 2 I.U. Insulin Actrapid MC 6 I.U. Insulin Monotard MC

(Novo Nordisk), 2 x 0.1 mg fluorocortisone daily substitute medication the patient was free from complaints. Then she was, once again, admitted to medical ward. On admission she had the following complaints: asthenia (appeared a month before and was constantly increasing), gingival haemorrhage, petechias and suffusions on the legs. Hypoplasia of the bone marrow causing pancytopenia was diagnosed. The patient was taken over to our section with suspicion of systemic autoimmune disease. From among the symptoms showed on admission we wish to lay emphasis on purpuras, suffusions, vitiligo all over the body, facial pigmentation, anaemic mucosas and severe gingival haemorrhage. Liver and spleen palpable 2 cm and 3 cm under the costal arch, respectively. Blood pressure: 120/80 mmHg, body weight: 50 kg. Laboratory findings: E.S.R.: 9 mm/h, WBC: 2.1 x 10^9/l, hgb: 88 g/l, platelet count: 50 x 10^9/l, periferial blood smear: seg: 56%, mo: 2%, ly: 42%, bleeding time (with Duke's method): > 20 min, prothrombin rate: 1.08, thrombin time (TT): 27 sec, control: 20 sec, partial thromboplastin time (PTT): 43 sec, control: 39 sec, total serum protein: 45 g/l, albumin: 32 g/l, Na: 145 mmol/l, K: 4.2 mmol/l urin protein: opalescent, Donne's test: negative, glucosuria was present, acetonuria was present too. Urine sed.: negative. Direct and indirect Coombs test: negative. LE-test: negative. Antinuclear antibody: negative, anti-DNA (with ELISA method): negative, smooth muscle antibody (HRPO immunohistology): negative, antithrombocyte antibodies (with direct immunofluorescent method): positive. On the basis of the identified and treated Addison's disease, IDDM, vitiligo and concomitant immunothrombocytopenic purpura, we diagnosed PGAS type II. Against immunothrombocytopenia causing severe bleeding, we applied stoss therapy with methylprednisolon natirum succinate (1 g daily for 3 days, thereafter 250 mg daily for 4 days). From the 8th day we applied 1 mg/kg b.w. methylprednisolon orally. However, on the 3rd day, gastrointestinal diffuse mucosal bleeding developed, we added cimetidine, etamsylate, conserved erythrocyte mass, and from the 6th day fresh frozen plasma to the treatment. Because of refractory thrombocytopenia, on the 8th day we introduced 1 mg Vincristin sulfate intravenously. The poor therapeutic effect and unceasing severe bleeding causing grave shock necessitated the introduction of Cyclosporin-A therapy on the 11th day (dose: 5 mg/kg b.w.). A few days later bleedings discontinued and the blood counts normalized. On the 20th day the patient was discharged, she was free from complaints. Since then, she has been under follow-up. Maintenance therapy: diet with 140 g carbohydrate content, mornings 16 I.U. Insulin Actrapid MC + 20 I.U. Insulin Monotard MC, evenings 8 I.U. Insulin Actrapid MC + 8 I.U. Insulin Monotard MC, 3 x 0.1 mg fluorocortisone, 16 mg methylprednisolon, 2 mg/kg b.w. Cyclosporin-A (1.25 ml Sandimmun Sandoz) orally, potassium citricum, calcium lactogluconate, aluminium-magnesium-carbonate daily. Control inspection findings: WBC: 5.5 x 10^9/l, Hgb: 141 g/l, platelet count: 10.4 x 10^9/l, total serum protein: 62 g/l, albumin: 32 g/l, cholesterol: 3.9 mmol/l, plasma cortisol: 6 h: 580 nmol/l, 24 h: 540 nmol/l. Immunological tests: lymphocyte marker tests (ORTHO-monoclonal antibodies): total T count near minimum of normal range (CD 3: 52%), subnormal T helper count (CD 4: 20%), T suppressor population: higher than normal (CD 8: 32%), total B count: normal (CD 19: 12%). Surface Ig expression of B cells: polyclonal. Adrenal cortex antibody, thyroglobulin antibody, thyroid microsome antibody, antinuclear antibody, anti-DNA: negative. Examinations were carried out after complete therapy. Soluble interleukin-2: 40 pg/0.1 ml (normal). HLA-status: HLA-B 8, DR-2 and -3 are positive. For clinical course see Table I.

Table I

Clinical course
I. Sz., female (born 1962)

Year	Diagnosis
1982	normal delivery
1988	unidentified viral infection dysmenorrhoea splenomegaly
1989	diabetes mellitus type I vitiligo latent Addison's disease
1990	manifest Addison's disease amenorrhoea hyperthyrosis
1992	immune thrombocytopenia

Discussion

Polyglandular ailment was first reported in 1926 by Schmidt /11/, who had observed idiopathic adrenal cortex insufficiency and thyropathy in two patients. He emphasized the identity of lymphocytic infiltration in both organs /11/. The range of polyglandular diseases broadened when association of hyperthyrosis, diabetes mellitus and hypoparathyrosis with Addison's disease was recognized /3/. In 1954 Bloodworth et al. /2/ attributed polyglandular diseases to an immune mechanism. Later, in 1956, Roitt et al. /10/ were the first to find anti-thyroid antibodies and circulating adrenal antibodies in patients who suffered from Addison's disease concomitant with Hashimoto's thyroiditis. The term "polyglandular autoimmune syndrome" was created by Neufeld et al. /8/ in 1981. Two major groups of the disease have been distinguished on the basis of presence vs absence of adrenal cortex insufficiency. A further twofold distinction of the syndrome co-occurent with Addison's disease was proposed by Leshin /5/ in 1985 (Table II). Clinical aspects of PGAS type I and II were systematized by Neufeld et al. /7, 8/. PGAS I begins in the childhood, hypoparathyrosis, Addison's disease, chronic mucocutaneous candidiasis frequently co-occur. PGAS II, also named Schmidt's syndrome, is frequently accompanied by concomitant or subsequent adrenal cortex insufficiency and autoimmune thyropathy and/or insulin-dependent diabetes mellitus. Nowadays, nonetheless, the term covers the combi-

Table II

Division of polyglandular autoimmune diseases (Leshin /5/)

A. Presence of Addison's disease	
type I	hypoparathyrosis chronic mucocutaneous candidiasis Addison's disease
type II	Addison's disease autoimmune thyropathy diabetes mellitus type I
B. Absence of Addison's disease	
type III	autoimmune thyropathy diabetes mellitus type I and/or anaemia perniciosa myasthenia gravis primary biliary cirrhosis

nation of an autoimmune thyroiditis and/or diabetes mellitus type I with presence of anti-adrenal antibodies and/or positive familial history, and it also covers primary adrenal cortex insufficiency with hypogonadism. Females are at double risk compared to males. Approximately 70% and 50% of the patients display autoimmune thyropathy and IDDM, respectively. Autoimmune thyropathy is either Graves's disease or, just as likely, Hashimoto's thyroiditis. In a half of the cases, Addison's disease appears first, predominantly after 20 years of age. In 20% of the cases, thyropathy or diabetes mellitus occurs co-temporaneously with Addison's disease, and in 30% hypadrenia follows other diseases. In some cases, gonad insufficiency is the first symptom and it is accompanied by ovarial overweight. The association of non-endocrine diseases, among them vitiligo, anaemia perniciosa, myasthenia gravis and immunothrombocytopenic purpura have been diagnosed very rare by /1, 5, 6, 8, 9, 13/.

PGAS II is a relative rare disease. Its estimated occurrence is 15 to 20/one million population /12/. According to recent findings, it is inherited on by dominant autosomes way, often through generations. In its pathogenesis, genetic as well as environmental (viral) and other (e.g. hormonal) factors play an important role. The fact that females are much more concerned suggests the importance of hormonal factors. As most authors agree, the pathophysiology of PGAS II is based on malfunction or insufficiency of suppressor T cells, and on the development of autoreactive clones /7, 9, 13, 14/.

Organ-specific antibodies may help in verifying autoimmune disease /13/. In a high proportion of PGAS II patients (60 to 90%), anti-adrenal antibodies have been found. Parietal cell antibodies have been found in 40% of the cases, islet cell antibodies in 25% /1, 19, 14/.

Clinicians have a choice of two possible therapies. Most of the patients can be successfully treated with hormonal substitution, and immunosuppressive therapy can also be applied /1, 7, 9/. Cyclosporin-A has proved to be the most promising immunosuppressive drug. Cyclosporin-A (Sandimmun) is effective at the early stage of antigen perception, it hinders the production of interleukin-2, the T and B cell function, and, also, it bars autoimmune mechanism. Side effects of Cyclosporin-A (hepatotoxicity, renal toxicity, gingival hyperplasia, lymphoma induction) can be reduced or avoided by applying a low dose /4/.

In the above case, an autoimmune process affected several organs. A year after the initial thrombocytopenia (causing dysmenorrhoea) and splenomegaly, vitiligo, insulin-dependent diabetes mellitus, then manifest Addison's disease developed concomitantly with hyperthyrosis and amenorrhoea. Owing to substituent therapy, the patient became partially free form complaints; nevertheless clinical treatment was necessary because of labile diabetes mellitus. Two years after the polyglandular disease, a grave general bleeding developed, resulting from immune thrombocytopenia. Continuing substituent therapy and resorting to all therapeutic possibilities, administration of Cyclosporin-A was necessary because of unsatisfactory therapeutic effects. Within a short time we observed remission of the disease. The patient having been under follow-up, is now in good condition. She is taking 3 x 2 mg/kg. b.w. Cyclosporin-A weekly.

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THE SIGNIFICANCE OF BIRTH WEIGHT DISCORDANCE IN TWINS

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The author found, among 329 twin pairs, 50 (15.2%) cases of weight discordancy reaching or exceeding 22%. Among the 50 twin pairs, there were 65 boys and 35 girls, a sex ratio of 185.7. This degree of weight discordancy appears to be unrelated to maternal age, parity and gestational length. Growth retardation of one or both fetuses was significantly more frequent (80%) among weight-discordant than among concordant one (11.1%). There were more perinatal deaths between discordant than concordant twins. Among the twins who were born with evidence of growth discordancy, there was slightly increased incidence of abnormal presentation, delivery by cesarean section, and low Apgar score as compared to the concordants.

Keywords: Twin pregnancy, discordant growth

Introduction

Theoretically, the birth weights of twins should be closely identical, taking into account the fact that they develop in the same uterine milieu. Nevertheless, the birth weights of twins often differ significantly, usually as a result of discordant growth rate. Since marked difference in the birth weights of twins is of considerable frequency, it appeared a matter of interest to study the frequency of discordancy among twins and its impact upon the circumstances of delivery and the neonatal outcome.

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Patients and Methods

The charts of childbearing patients of our institution from January 1, 1975, to December 31, 1992, were reviewed. Utilizing the data of the delivery books and the medical records in order to determine the frequency of twin births and the weight differences between the twins, the latter was determined by multiplying the birth weight expressed in grams, of the smaller of the twins by 100 and dividing the result with the birth weight of the larger twin /11/. The result was expressed in per cent. The results being $\leq$ 78%, i.e. differences $\geq$ 22%, were considered significant. A weight difference of $\geq$ 21.26% (11.51 $\pm$ SD 9.75%) is within the range of normal in Hungary /20/.

We investigated the correlations between significantly discordant ($\geq$ 22%) and concordant twins in terms of maternal age, parity, gestational length, and sex of the twins. We also evaluated the frequency of abnormal lie immediately before delivery, the method applied at delivery, the 5 minute Apgar score and relationship of weight to calculated gestation length in the investigated cases along with the respective rates of neonatal mortality. The twin neonate was considered growth-retarded if his or her birth weight was under the 10th percentile applicable for singletons on the basis of the Deter et al. /6/ standards. Sex ratio was calculated as the number of boys in relation to 100 girls. The statistical significance was calculated on the basis of the chi-square test.

Results

During 18 years 30 671 women gave birth in the obstetric unit of our hospital. Of these, 329 (1.1%) delivered twins. From these there was evidence of significant discordancy in birth weights ($\geq$ 22%) in 50 instances (15.2%).

In relation to maternal age, the highest rate of weight discordancy occurred in the 21-35 year old age group. In relation to birth order, 1-3 parity were found to be associated with the highest rate of weight discordancy. Relevant to the length of gestation, the highest frequency of weight discordancy was found between the 37th and 38th weeks.

In 29 (58%) of the 50 cases, the first-born twin in the remaining 21 (42%) the second-born were larger.

In relation to gestational length, one or both of the weight-discordant twins were growth-retarded in 40 (80%) instances. Among the concordant, growth retardation prevailed only in 31 cases (11.1%). This difference is significant at the $P < 0.001$ level.

In 34 (68%) of 50 twin pairs, the smaller of the weight-discordant twins was growth-retarded. Both twins were growth-retarded in 6 (12%) cases. In 10 (20%) instances of weight-discordancy both twins were above the lowermost 10th percentile. However, in this group, it was only in one instance that of both twins exceeded 2500 g in weight.

In the group of significantly weight-discordant and concordant twins one or both of the fetuses showed abnormal (breech or transverse) presentation in 30 cases (60%) and 145 (52%), respectively.

In those cases where there was a significant weight-discordancy between the twins, in 30 (60%) instances was delivery effected by cesarean section. In the concordant group abdominal delivery was recorded in 97 (34.7%) cases ($P < 0.005$).

An unfavourable 5-min Apgar score (< 7) was noted in 22.7% of the weight-discordant twins. In the concordant group, the frequency was 14.1% (an insignificant difference).

The sex distribution of weight-discordant twin pairs were the following:

Both boys: 25 Boy — girl: 5

Both girls: 10 Girl — boy: 10

The sex ratio for twins belonging to the same sex was 250. The sex distribution of all weight-discordant twins was 65 boys and 35 girls (sex ratio 185.7). For the 279 concordant twin pairs, the sex ratio was 108.6. The difference between the two was significant at $P < 0.001$. The sex ratio of all newborn in our department during the 18 years of the study was 106. As compared to this, the sex ratio of 185.7 represented a very significant difference ($P < 0.001$). In 15 cases of weight-discordancy between neonates of different sexes, the boy was larger than the girl in 7 instances.

Of the 100 newborn deriving from weight-discordant twin pregnancies 17 (17%) whereas of the 558 concordant twins 60 (10.8%) died perinatally. The twin pair with birth weight > 2500 g both survived.

The forms of placentation were: 31 dichorionic diamniotic, 16 monochorionic diamniotic and in 3 cases monochorionic monoamniotic.

Discussion

The difference between the body weights of twins is generally expressed in percentage of the weight of the larger twins. There is no clear agreement with regard to the definition of significant weight discordancy. Some authors /4, 13/, consider a difference exceeding 15% significant, others draw the borderline at 20% /16, 17, 19/ or even at 25% /8, 15/. One study used the weight of the larger twins as 100% and considered the difference of 36% as significant /1/.

Naturally, the frequency of weight-discordancy will depend upon the criterion utilized for definition of this entity. In this material, the use of a 15% weight difference would have allowed the identification of 83 (25.2%) discordant twin pairs. The use of 20% weight difference would have define 54 (16.4%) twin pairs as discordant. A total of 27 (8.2%) pairs would have been discordant if the criterion of significance had been considered at 25% weight difference.

Weight discordancy associated with twin gestation may result from placental insufficiency, inappropriate implantation site ("crowding"), developmental defect caused by chromosomal or anatomic abnormality, unequal supply with blood, difference in utilization of nutrients, and fetofetal transfusion. In principle, most cases of weight-discordancy derive either from inadequate utilization, or difference in delivery of fetuses of the available nutrients. The high sex ratio raises the possibility that a sex-related component may influence the growth rates. This interpretation is supported by the fact that the average weight of boys in singleton pregnancies is higher than of girls /5, 9/. It was found an average weight difference of 81-142 g in favour of boys /2, 3/. Naturally, this difference would not amount, in itself, to a significant weight discordancy.

According to several reports /7, 10, 14/ weight discordancy predisposes to increased perinatal mortality. Some studies could not, however, find a significantly increased risk when the weight difference reached 20-24% /8, 18/. In our material, among discordant twins, the frequency of growth retardation and perinatal mortality increased and involved one or both members of the pair. In general, the pregnancy outcome was less favourable in association with weight discordancy than in its absence.

It should be emphasized that the growth of the smaller twin may be an expression of some underlying pathological condition. For this reason alone, intensive fetal surveillance is necessary, once sonographic evidence of significant weight discordancy has been demonstrated. Aorta and umbilical artery Doppler velocity waveforms allow recognition of fetal discordant weight /16, 17/.

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IMMUNOLOGY

ANTICARDIOLIPIN ANTIBODIES: ASSOCIATION WITH ANTI-DNA ANTIBODIES, DISEASE ACTIVITY, RENAL INVOLVEMENT AND A HISTORY OF THROMBOSIS IN SYSTEMIC LUPUS ERYTHEMATOSUS

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A one-year study was conducted to evaluate the clinical significance of anticardiolipin antibody (ACA) whether it was a reliable predictor for thromboembolic events and related diseases in systemic lupus erythematosus (SLE) patients. The correlation between ACA and anti-ds-DNA antibodies and disease activity was also studied. Of particular importance was the question if any association could be found between ACA positivity and renal disorders in SLE patients. One hundred and eighty-seven serum samples from 88 SLE patients were assayed for ACA. Clinical records of these patients were reviewed for a history of thromboembolic events, related diseases and renal disorders, 80.7% of the 88 SLE patients were positive for ACA. The incidence of thrombosis and related diseases within this group was 35.1%. Since the correlation was not significant, it does not seem to be advisable to use elevated ACA values as predictive for thromboembolic events and related diseases. On the other hand, an apparent association between ACA levels, anti-DNA antibody levels and disease activity was found.

Keywords: Cardiolipin antibody, anti-DNA antibody, systemic lupus erythematosus

Introduction

Antiphospholipid antibodies in SLE have received considerable attention. There is an association between antiphospholipid (or anticardiolipin) antibodies, biological false positive test for syphilis and lupus anticoagulant activity. There are characteristic clinical conditions associated with ACA: arterial and venous thrombosis, thrombocytopenia and recurrent fetal loss. These associations are also termed antiphospholipid syndrome /5/.

Abbreviations: ACA: anticardiolipin antibody, SLE: systemic lupus erythematosus, anti(-ds)-DNA: anti(-double-stranded) DNA antibody

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Our study aimed at evaluating the clinical significance of anticardiolipin antibody (ACA): the association between ACA and thromboembolic events and renal involvement, and the correlation between ACA, anti-ds-DNA antibodies and disease activity.

Patients and Methods

The study was based on a total of 187 serum samples from 88 patients (84 females and 4 males; median age 38 years ranging from 20 to 59 years). All patients satisfied the classification criteria of the American Rheumatism Association (ARA).

All samples were tested for the presence of ACA, anti-ds-DNA among other blood parameters for a one year period in 1991 and 1992.

ACA assay was performed by a modification of the method of Loizou et al. /8/.

In brief, microtiter plates were coated with cardiolipin (Sigma, USA, lot ≠ 60H8377) diluted 1:500, washed once with Tween-PBS and then blocked by the addition of 100 µl PBS-BSA to prevent non-specific binding of immunoglobulin. Fifty µl serum in standard dilution was added, incubated overnight at 4 °C then washed. Fifty µl 1:2000 diluted conjugate (anti-human-IgG, -IgM, -IgA, -lambda and -kappa) and horseradish peroxidase enzyme were added and the mixture was incubated for 1.5 h at 37 °C. Thereafter, 50 µl ELISA buffer was added and the reaction was terminated with 50 µl 8 M sulphuric acid. Absorbance was read at 405 and 495 nm. The values of ACA were expressed as units in comparison with the standard serum. The result was considered to be positive if it exceeded by 3 SD the mean value obtained with control serum of healthy volunteers. Clinical records were reviewed for a history of thromboembolic events, thrombocytopenia, spontaneous abortion, seizures, psychosis, migraine and renal disorders.

Mean values are shown in the tables. For statistical analysis the chi-square test with Yates' correction and regression analysis were used. P values < 0.05 were regarded as significant.

Results

Correlation of ACA levels with history of thromboembolic events, thrombocytopenia, fetal loss, seizures, psychosis and migraine

The mean value plus 3 SD of ACA obtained with control serum was 28 U/ml ($= 8 \pm 3 \times 6.6$). Additionally, we subdivided ACA values into low, high positive and negative ones as done by Alving et al. /1/. Although this subdivision is arbitrary, it is, in our opinion, useful to estimate the major ranges of ACA levels and the occurrences of thromboembolic complications and related diseases within these ranges.

Positive ACA was detected in 71 (80.7%) of the 88 patients, the remaining 17 were found negative (Table I).

The correlation between ACA levels and a history of thromboembolic events and related diseases was not significant.

Table I

Comparison of ACA levels with a history of thrombosis and related diseases

ACA	History of thrombosis	
	positive (n=29)	negative (n=59)
low positive (29-90 U/ml)	17	28
high positive (> 90 U/ml)	6	14
negative	6	17

From these results one can only state that positive ACA remains a special marker in SLE patients and that the probability to develop thromboembolic complications and related diseases is higher than 50%.

The majority of the 71 patients with positive ASA display low ACA levels. Therefore, it seems to be of no benefit using the ACA value in itself as a parameter predictive for developing thromboembolic events or related disease.

Correlation of ACA levels with anti-DNA antibody levels

The same procedure and subdivision of ACA levels was used as described above. The comparison of anti-ds-DNA antibody levels, subdivided in low, high positive and negative values is shown in Table II. There was a significant correlation between ACA levels and anti-ds-DNA levels (correlation coefficient $r = 0.292$, $P < 0.002$).

Table II

Comparison of ACA levels with anti-DNA antibody levels

ACA	anti-DNA level		
	low (18-92 U/ml) (n=44)	high (> 92 U/ml) (n=19)	negative (n=25)
low positive	25	7	13
high positive	8	9	3
negative	11	3	9

These results are at variance with the observations of Loizou et al. /8/, Harris et al. /5/ and Colaco et al. /2/ who failed to find any correlation between these two parameters.

Furthermore, there were marked fluctuations in ACA levels and anti-ds-DNA levels in patients who were monitored more than once. Twenty-five patients were controlled in this regard, but the sample was too small for calculation.

Association between ACA levels, renal disorders and thromboembolic events and related diseases

This problem is another subject of controversy in the literature available /3, 6, 9/. Though there was no significant association between the parameters investigated (Table III), it seems to be likely that the possibility to develop renal disorders is as high as to develop thromboembolic complications (or related diseases) or both. This is partly in accordance with the findings of Cooper et al. /3/, who found a strong correlation between ACA levels and renal involvement.

Table III

Comparison of ACA levels with a history of renal disorders and thromboembolic events or related diseases

ACA	Renal disease (n=30)	No renal disease (n=58)	Thrombosis positive (n=29)	Thrombosis negative (n=59)
positive	28	49	27	50
negative	2	9	2	9

Discussion

In this study the most important finding was the close relationship between ACA levels and anti-ds-DNA levels. The question why this relationship exists remains still unclear and the background of this phenomenon represents a wide field for further investigations /4, 5, 7, 10/.

Since there was a positive correlation between anti-ds-DNA and ACA levels, the rise and/or fluctuation in ACA levels might be a good marker for disease activity /6/. On the other hand, we conclude that the probability to

develop renal involvement and/or thromboembolic complications or related disease in case of moderate or high ACA in connection with moderate or high anti-DNA levels is more than 50%.

However, it does not seem to be advisable to use elevated ACA value as predictive parameter for thromboembolic events and related diseases.

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LIPID ABNORMALITIES IN URAEMIC PATIENTS ON CHRONIC HAEMODIALYSIS

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Patients kept on haemodialysis because of chronic renal insufficiency were investigated for lipid profiles. The cholesterol level did not differ as compared to the age-matched control, while the triglyceride level was elevated. The correlation was found between the lipid parameters, period spent in dialysis programme and level of serum creatinine and urea. In renal failures of different origin the lipid levels are in relationship with the underlying disorders.

Keywords: Renal insufficiency, cholesterol, triglyceride, HDL-C, apoproteins, lipoproteins

Introduction

Recently, an increasing attention has been focussed on lipid and lipoprotein changes developing from secondary hyperlipoproteinaemias in diabetes mellitus and chronic renal disorders, diseases affecting a considerable part of the population. The incidence of atherosclerosis is significantly increasing in both cases /35, 42, 44/. The lifetime of patients with chronic renal disorder had been determined as distributed by their basic disease, but in the past few years -- as a result of the widespread haemodialysis programme and its well-developed methods as well as the spreading use of kidney transplantation -- atherosclerotic complications tend to become the main cause of death /25, 29/. In a 13-year follow-up study, Lindner et al.

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/35/ observed a mortality rate of 56% in permanently haemodialysed patients, and more than 50% of fatal cases was considered a result of some atherosclerotic complications. Data reported by other authors show that the number of patients with chronic renal disorders who die of atherosclerotic complications is almost identical with that of the infections common in patients with uraemia /34/. Ritz et al. /44/ claim that regarding fatal myocardial infarction the chronic dialysed patients are in danger 9-17-fold more than normal population. On the other hand, some studies present an increased plasma lipid level of dialysed patients less important than hypertension as far as atherosclerosis is concerned /31, 45, 47/. These data call attention to the fact that every factor that may accelerate the development of atherosclerotic complications in renal disorders may have special significance later from the aspect of therapy. There have been studies recently to analyse the development of hyperlipoproteinaemia noticeable in patients with chronic renal disorder. That is why we investigated the lipid profile in patients with chronic renal failure.

Patients and Methods

Fifty renal patients (36 males, 14 females, 48.6 years old on the average) from a chronic haemodialysis programme were studied. The average period of haemodialysis was 34.7 weeks (range 8 to 181 weeks) (Fig. 1). Twenty-five patients suffered from chronic glomerulonephritis, 7 from chronic pyelonephritis, 6 from diabetic nephropathy, 12 patients formed a separate group including those with polycystic renal diseases, Alport-syndrome, Schönlein—Henoch nephropathy and toxic renal disorders. Forty-six patients formed a control group, which included 20 females (40.5 years old on the average) and 26 males (mean age 52.8 years). These did not suffer from diabetes mellitus or any other metabolic disorder, liver or kidney dysfunction.

Each of the patients was tested for serum creatinine, urea, glucose, uric acid, total protein, electrolite levels and liver enzymes. Serum cholesterol, triglyceride, HDL-C, LDL, apo A1, apo B100 and apo (a) levels were measured, and lipoprotein electrophoresis was performed.

Serum cholesterol and triglyceride were assayed with a Boehringer enzyme kit, HDL cholesterol with the phospho-wolframate-magnesium precipitation method. LDL cholesterol was calculated by the Friedewald formula when the triglyceride concentration was below 4.5 mmol/l /21/.

Apoprotein examination was performed with radioimmunoassay (RIA) in which the Pharmacia kit was used, normal values: apo A1: 0.94—2.06 g/l, apo B: 0.38—1.32 g/l.

Serum lipoprotein (a) levels were determined by Pharmacia RIA, normal values: up to 450 U/l.

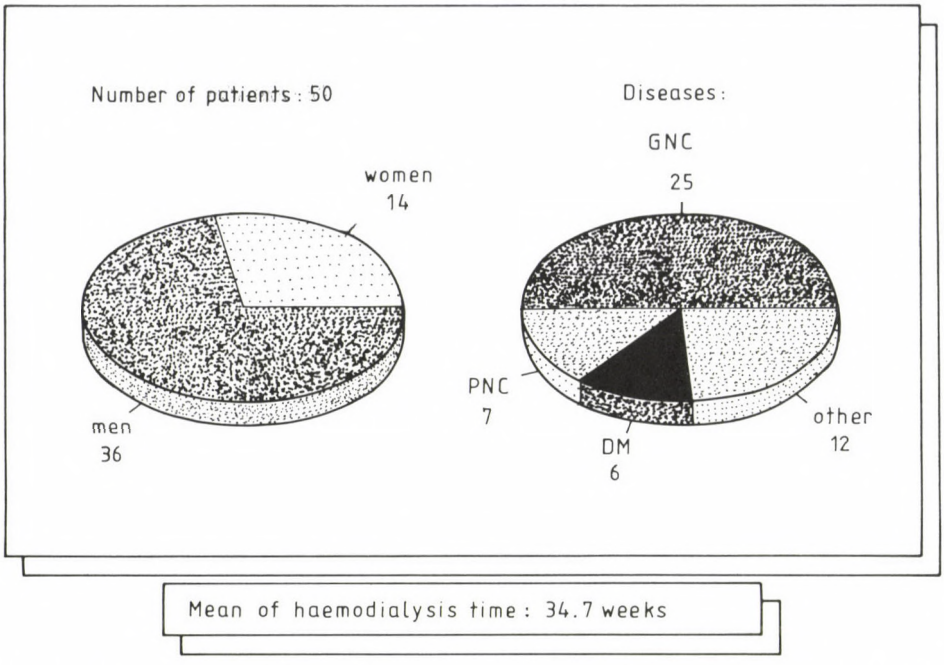

Fig. 1. Distribution of patients by sex and underlying diseases. GNC (n=25), PNC (n=7), DM (n=6), Other (n=12)

Abbreviations: GNC = glomerulonephritis, PNC = pyelonephritis, DM = diabetes mellitus

Results

The triglyceride level was significantly elevated ($P < 0.001$) in patients with chronic renal insufficiency, compared to the age-matched healthy control group, whereas HDL cholesterol level was slightly decreased (Fig. 2). The relationship between the period of haemodialysis programme and serum lipid level was also analysed (Table I). The triglyceride level positively correlated ($P < 0.015$) with haemodialysis time and increased significantly ($P < 0.001$) in patients with chronic glomerulonephritis compared to chronic renal failure of other origin (Fig. 3). The apo B 100 and apo A1 levels were not elevated. The lipoprotein (a) level was elevated in 6 patients with diabetic nephropathy (Fig. 4). HDL-cholesterol level slightly decreased as compared to the control. We failed to recognize correlation between serum urea, creatinine and lipid levels. The serum total protein level showed a strong positive correlation with the triglyceride level, while it had a weaker negative correlation with the HDL-cholesterol level (Table II).

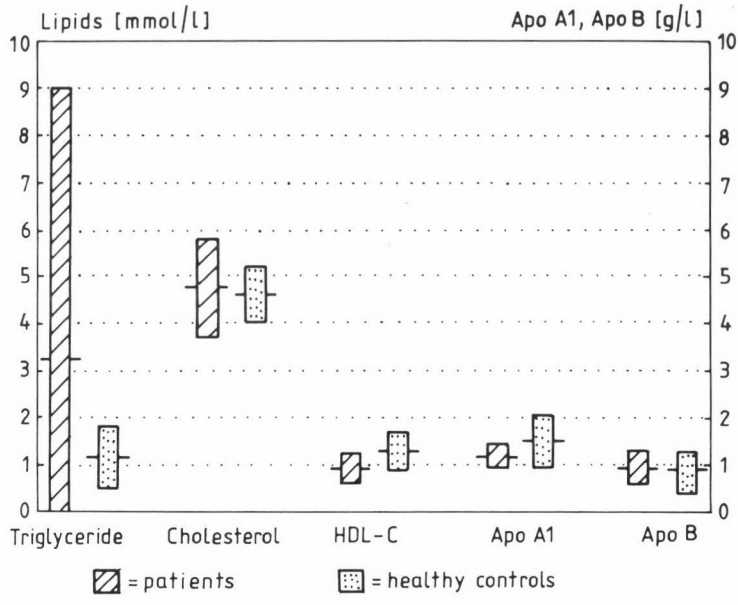

Fig. 2. The serum lipid parameters of patients with chronic renal failure compared to healthy controls (means ± SD), patients (n=50), healthy controls (n=46)

Table I

The connection between length of haemodialysis and serum lipid levels in chronic renal failure. Patients (n=50)

	HD-time		
	r	P	s.
Cholesterol	-0.1963	0.109	
HDL-C	-0.1772	0.134	
Triglyceride	0.3396	0.015	*
Apo B	-0.0649	0.343	
Apo (a)	0.0052	0.487	
Apo A1	0.0634	0.347	

r = correlation coefficient, P = probability,
s. = signification
*P< = 0.05,

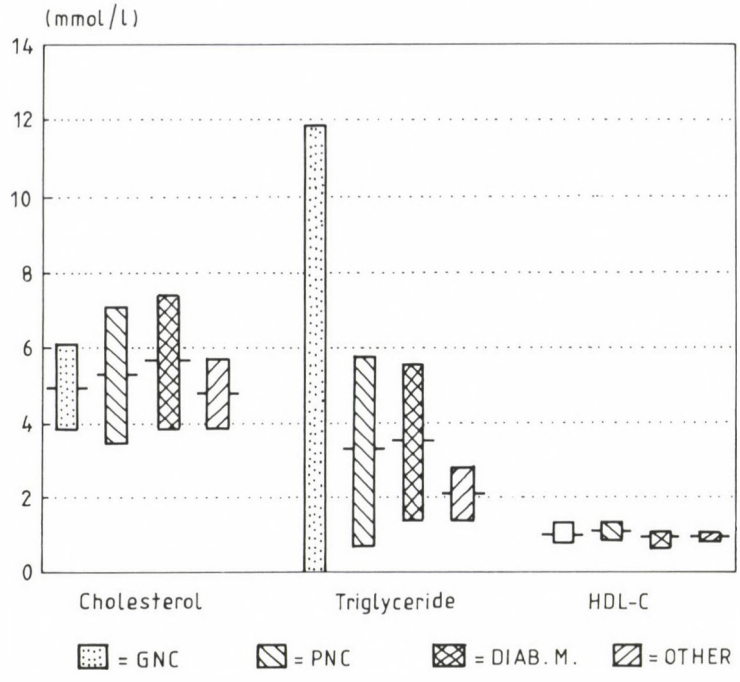

Fig. 3. The lipid parameters in renal failures of different origin. GNC (n=25), PNC (n=7), DIAB. M. (n=6), Other (n=12)
Abbreviations: GNC = glomerulonephritis, PNC = pyelonephritis, DIAB. M. = diabetes mellitus
(means ± SD)

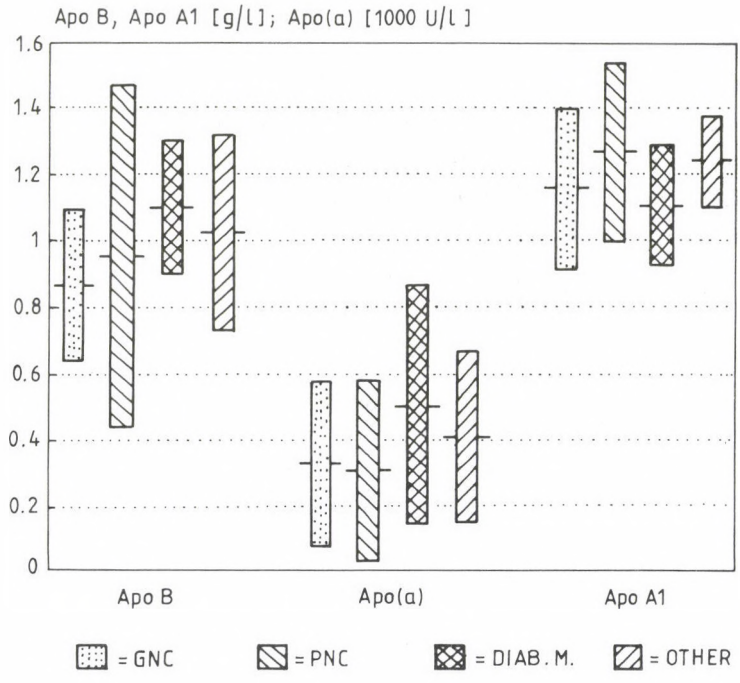

Fig. 4. The apolipoprotein levels in renal failure patients with different aetiology. GNC (n=25), PNC (n=7), DIAB. M. (n=6), Other (n=12)

Abbreviations: GNC = glomerulonephritis, PNC = pyelonephritis, DIAB. M. = diabetes mellitus C means ± SD)

Table II

The connection between the renal function and lipid parameters. Patients (n=50)

	Total Protein			Creatinin			Urea		
	r	P	s.	r	P	s.	r	P	s.
Cholesterol	0.16	0.14		0.21	0.08		-0.06	0.34	
HDL-C	-0.32	0.015	*	0.20	0.09		-0.09	0.26	
Triglyceride	0.42	0.002	**	-0.01	0.47		0.07	0.32	
Apo B	0.06	0.35		0.09	0.26		-0.08	0.30	
Apo (a)	0.01	0.48		-0.10	0.25		0.04	0.39	
Apo A1	0.29	0.026	*	-0.27	0.036	*	-0.01	0.47	

r = correlation coefficient, P = probability, s. = signification

*$P < 0.05$, **$P < 0.01$

Discussion

It has been observed /2, 24, 36/ that in the background of hyperlipoproteinaemia characteristic of the disease the lipoprotein synthesis, especially the apo B synthesis of the liver is likely to increase to compensate the protein loss through the kidneys. Some authors /23, 24/ found an increase in the activity of the hydroxy-methyl-glutaryl-CoA reductase, which is the decisive key enzyme in the intracellular cholesterol synthesis in experimental nephrosis of the rat. Others /13/ think that the stimulus of increased lipoprotein production may be the hypoalbuminaemia and the consequence of decreased colloidosmotic pressure. In addition, patients suffering from nephrosis lose -- through the kidneys -- lecithin-cholesterol-acyltransferase (LCAT), an enzyme having an important role in lipid metabolism, which is necessary for the efficient activity of HDL and cholesterol transport /17, 23, 27/.

Another part of research was focussed on the differences existing in renal disorders of different origin. Serum triglyceride level was found to increase in chronic renal insufficiency /14, 15, 28, 42, 43/. Serum cholesterol level usually remained in the normal range /11, 39, 42/, although it may slightly increase, beside hypertriglyceridaemia dominance /5, 12, 14, 30/. HDL cholesterol values show a 50-75% decrease in non-dialysed, and haemodialysed patients with chronic renal failure /11, 28, 39/, and their C/HDL-C and LDL-C/HDL-C ratios are high /16, 43/.

Vascular disease is the most important cause of mortality in chronic renal insufficiency. Cardiovascular disease is usually associated with high concentrations of plasma cholesterol, LDL-cholesterol and apo-B, and reduced concentrations of HDL-cholesterol. According to a Cholesterol Lowering Atherosclerosis Study (CLAS) the progression of atherosclerotic lesions in patients who had undergone coronary by-pass surgery may be linked to increased concentrations of triglyceride-rich lipoproteins. Earlier studies /28/ revealed that patients with renal failure had increased levels of plasma triglyceride and VLDL-cholesterol. It has recently been reported /40, 41/ that patients with chronic renal failure have increased concentrations of lipoprotein(a).

A non-dialysable, so-called inhibitor factor starts to spread in the plasma /18, 37/, resulting in a decrease of the apo CII/apo CIII ratio. Apo C II is necessary for the activation of the lipoprotein lipase whereas apo C III acts as an inhibitor of the lipoprotein lipase enzyme /19, 46/.

Thus, the decrease of apo C II/apo C III ratio results in a decrease of the lipoprotein lipase activity, leading to an accumulation of lipoproteins rich in triglycerides in the plasma /7, 8, 10, 41/. The analysis of the particular lipoprotein fraction compositions showed that in cases of uraemia it is the triglyceride component that increases within certain lipoproteins /38/. In HDL, the apo A I and apo A II concentration decreased /7, 8, 26, 33/. The apo B and apo C I concentrations were found normal or slightly decreased, whereas apo E level showed no change, or decreased, especially in males /1, 6, 8, 26, 33/. The level of apo C III increased significantly /6, 8, 26, 48/.

In accordance with literary data we found that triglyceride level increased in patients with chronic renal insufficiency, whereas the HDL-C level showed a slight decrease, the inverse relation between triglyceride and HDL-C being probably responsible for the latter /4, 32/. Lipoprotein lipase activity correlated with serum triglyceride levels inversely and with HDL cholesterol levels directly /8, 9, 32/. No significant changes were found in the cholesterol and apo B levels, suggesting that it is the damage of triglyceride-catabolism that has a primary role in the lipid abnormalities of uraemic patients. It is rather controversial to consider lipoproteins rich in triglyceride as risk factors /9/. When compared to literary data /3, 9/, the lipid parameters in different basic diseases leading to chronic renal insufficiency, were surprising, by differing from the literature.

Differences found in chronic glomerulonephritis group might be explained partly by immunosuppressive treatment of the diseases that increase lipid abnormalities, however, our patients did not get any immunosuppressive drugs. There may be metabolical disorders caused by diabetes, increased VLDL and apo B production in the background of the serum trigylceride level increase in diabetes mellitus patients. The high lipoprotein (a) level noticed in these patients can be explained by the small number of patients on the one hand, considering, it as an accidental feature, but there is another possibility, i.e. type-2 diabetes mellitus defined polygenic disorder may accompany with the alteration of other genes, thus of lipoprotein (a) resulting in the increase of lipoprotein (a).

The main renal complication of diabetes mellitus is the Kimmelstiel--Wilson syndrome, which leads to macroalbuminuria. Some earlier studies revealed that macroalbuminuria might be accompanied by increased lipoprotein (a) concentration in the serum, however, the literature is controversial in this respect /22, 49/.

Some earlier studies /20/ suggest that triglyceride level decreases at the beginning of haemodialysis, then it increases. So we also examined the change of serum triglyceride level as related to the length of time being on haemodialysis. The length of haemodialysis time was found not to influence the different lipid levels, but serum triglyceride levels showed significant positive correlation with the haemodialysis time. Earlier, Ponticelli et al. /42/ failed to find any connection between plasma triglyceride level, age, sex, dialysis time, blood sugar level after an overnight fast and insulin reaction to intravenous glucose.

Our results suggest that the lipid profile changes in chronic renal insufficiency may be one of the causes acclerating cardiovascular disease in these patients. If we want to be successful in preventing this complication, we should begin the lipid-lowering treatment and start the haemodialysis programme at the same time.

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PORPHYRIN STUDIES IN CHRONIC RENAL FAILURE AND RENAL TRANSPLANTATION

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Haemoglobin (Hb), free erythrocyte porphyrins (FEPs), protoporphyrin and haem contents as well as delta-aminolevulinic acid (ALA)-dehydrase activity were estimated in blood samples from patients with chronic renal failure (CRF), from those with renal transplantation, and from healthy control subjects. In CRF patients a highly elevated FEPs level and a significantly increased protoporphyrin concentration were found. A well-defined decrease was observed in the mean value of ALA-dehydrase activity, Hb and haem contents when compared to the control values. However, in patients with renal transplantation significant decreases were observed in Hb and haem concentrations while the ALA-dehydrase activity and the FEPs and protoporphyrin concentrations were approximately at the control levels.

Keywords: Haemoglobin, free erythrocyte porphyrins, protoporphyrin, haem, delta-aminolevulinic acid-dehydrase, chronic renal failure, renal transplantation

Introduction

The literature is poor in studies on porphyrin biosynthesis in uraemic patients, despite the evidence showing the important role of porphyrin in haemoglobin biosynthesis. Most of patients on haemodialysis for chronic renal failure were reported to be within normal limits when examined for abnormal porphyrins /6, 10/. On the other hand, several such patients have been reported to have high abnormal accumulation of porphyrins in plasma, urine and faeces /7, 11/. Plasma porphyrins and free erythrocyte protoporphyrin (FEP) were found to be elevated in some uraemic patients on maintenance haemodialysis /11/. Serum delta-aminolevulinic acid (ALA), erythro-

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Abbreviations: Hb = Haemoglobin, FEPs = free erythrocyte porphyrins, ALA = delta-aminolevulinic acid, CRF = chronic renal failure, RT = renal transplantation.

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cyte coproporphyrin and protoporphyrin were found significantly higher in non-dialysed patients than in controls while ALA-dehydrase and uroporphyrinogen-l-synthetase activities were inhibited in these patients /16/.

The present study was conducted to show whether the abnormalities of porphyrin biosynthesis in uraemic patients are or are not stopped after renal transplantation.

Materials and Methods

This study was performed on (i) 20 adult patients, from both sexes, suffering from chronic renal failure. The patients were on maintenance haemodialysis at the Urology and Nephrology center, Mansoura University, Egypt, (ii) another group consisting of 16 adult patients of both sexes who had undergone successful renal transplantation at least one year before and had been treated with cyclosporin A and azathioprine or some other immunosuppressive agents. A group of 20 healthy adult subjects, from both sexes, was used as a control group. Heparinized blood samples were obtained from all patients and control subjects.

Haemoglobin concentration was determined in whole blood by Drabkin's method /3/ while free erythrocyte porphyrins as well as blood protoporhyrin and haem contents were estimated according to the methods described by Piomelli /9/ and Labbe et al. /8/, respectively. The assay of activity ALA-dehydrase was done in whole blood samples, in which haematocrit values were determined, according to the method of Weissberg et al. /15/. For statistical analysis, the standard methods of Hill /5/ were used.

Results

It is apparent from Table I that the mean Hb value was highly significantly ($P < 0.001$) lower in patients with chronic renal failure (CRF) than that for control subjects. Similar, but less expressed, difference was observed in patients with renal transplantation.

ALA-dehydrase activity was highly significantly inhibited in CRF patients while in the group of transplanted patients the activity of this enzyme was practically in the range of be control value (Table I). On the other hand, there are highly significant and significant ($P < 0.05$) elevations in concentrations of free erythrocyte porphyrins and protoporphyrin, respectively, in blood of CRF patient while in transplanted patients the mean values of these porphyrins do not significantly differ from the control values. In contrast, haem content value was highly significantly decreased in patients with CRF and a similar but less expressed difference was found in patients with renal transplantation.

Table I

Mean values ± S.S. for haemoglobin (Hb), free erythrocyte porphyrins (FEPs), protoporphyrin (PP) and haem concentration as well as delta-aminolevulinic acid dehydrase (ALA-D) activity in blood of controls and of patients with chronic renal failure (CRF) and with renal transplantation (R.T.)

Item		Controls (n)	CRF (n)	R.T. (n)
Hb	(g/100 ml)	12.0 ± 72 (20)	$6.3 \pm 2.56^{**}$ (20)	$8.4 \pm 5.17^{*}$ (16)
FEPs	(µg/100 ml)	14.4 ± 7.66 (20)	$20.4 \pm 3.27^{**}$ (18)	11.2 ± 4.32 (15)
PP	(µmol)	$(8.9 \pm 2.93)\cdot10^{-3}$ (14)	$(12.1 \pm 3.76)\cdot10^{-3*}$ (18)	$(7.9 \pm 2.43)\cdot10^{-3}$ (15)
Haem	(mol)	$(11.3 \pm 1.68)\cdot10^{-3}$ (14)	$(3.5 \pm 1.12)\cdot10^{-3**}$ (18)	$(5.8 \pm 3.75)\cdot10^{-3*}$ (15)
ALA-D	(Units)^{o}	11.2 ± 5.56 (20)	$7.2 \pm 2.25^{**}$ (18)	15.7 ± 9.05 (15)

$^{*}P < 0.05$, $^{**}P < 0.001$

^{o} = µmol delta-aminolevulinic acid utilized/ml/min

() = number of subjects

Discussion

As shown from the present results, the decrease Hb concentration in patients with CRF indicates characteristic anaemia of these patients. Hence, renal anaemia, which increases with progression of renal insufficiency /4/ indicates an insufficient compensatory increase in red cell production in response to blood loss and haemolysis. Erythropoietin deficiency /12/ and uraemic bone marrow intoxication /13/ have been implicated as major causative factors for the development of renal anaemia. This concept is in agreement with the observation that renal anaemia usually disappears several months after transplantation /2/. This view may be supported by the elevated Hb concentration in our patients of renal transplantation which in higher than the CRF value (Table I).

On the other hand, as indicated from the present results, one can say the Hb deficiency in these two-patients, undergoing this sutdy, may be related to the diminished haem synthesis observed in these patients. In this concept, there are increases in free erythrocyte porphyrins in patients with CRF and these changes may be attributed to abnormalities of some enzymes of

the biosynthetic haem pathway. Such increases support an other previous finding in which FEPs were elevated in 70% of uraemic patients on maintenance haemodialysis and plasma porphyrins were increased in 58% of the same patients /1/.

As shown in our results a high elevation was observed in protoporphyrin concentration associated with a high decrease in haem content in CRF patients. These abnormalities may be attributed to the inhibition of ferrochelatase and/or deficiency of iron, which is lost from the blood of these patients into the dialyzer during maintenance hemodialysis. Furthermore, in similar findings in other disease, in which porphyrin prevalent, it was found a direct inhibitory effect of protoporphyrin on the enzyme system of the haem pathway /14/. This inhibition had resulted in the accumulation of FEPs. Moreover, a highly significant inhibition was observed in ALA-dehydrase activity in CRF patients with progressing anaemia. In a previous study, erythrocyte ALA-dehydrase activity was found to be highly inhibited in non-dialysed uraemic patients, and marked inhibition in other dialysed uraemic patients; and a similar pattern, but less significant, was observed in erythrocyte uroporphyrinogen-l-synthetase activity. However, addition of zinc to the practically zinc-free haemolysates of uraemic patients caused an induction of ALA-dehydrase /16/. This latter finding proves that ALA-dehydrase activity is zinc-dependent.

The observations described here do not support the evidence of dermatologic manifestations of these studied patients that resembled either the bullous dermatosis of haemodialysis or porphyria cutanea tarda.

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THE EFFECT OF TSH AND TSI ON THE THYROGLOBULIN EXPRESSION OF CULTURED HUMAN THYROID CELLS

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Human thyroid cells in culture stimulated by TSH and TSI were used in order to detect thyroglobulin expression. After three days stimulation the cells were incubated with monoclonal thyroglobulin antibody and FITC-conjugated antiglobulin. Fluorescent index (the intensity of fluorescence related to hundred analysed cells) was estimated for each experimental group. The most effective stimulation of the thyroglobulin expression was detected after TSH stimulation at the concentration of 0.1 mU/ml. TSI from active Graves' patients provoked the highest expression of thyroglobulin at concentration of 1.0 mg/ml, but the fluorescence index was lower than after TSH stimulation. The thyroglobulin expression was intracellular, large, partly confluent granules were detectable mainly in the perinuclear area. Antigen expression on the surface of cultured thyroid cells could not be detected. The morphology of thyroglobulin expression as detected by immunofluorescence, was the same after TSH and TSI stimulation. It is concluded, that both stimulating factors, i.e. TSH and TSI, are involved in the thyroglobulin expression of human thyroid cells.

Keywords: Human thyroid cells, TSH, TSI, thyroglobulin expression, immunofluorescence

Introduction

Thyroglobulin (TG), a 660 kD glycoprotein which plays an important role in the synthesis and storage of thyroid hormones /6, 7, 13/, is synthesized in the thyroid follicular epithelial cells and stored in the follicular lumina. Thyroid-stimulating hormone (TSH) increases TG synthesis in the thyroid cells and, at the same time, stimulates resorption of the TG by

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the thyrocytes through endocytosis. Thyroid hormones are released from the TG and secreted through proteolysis /1, 4, 5, 13/. TG determination is of great significance in the diagnosis and follow-up of different thyroid patients /6, 7, 9, 14/. The microsomal antigen of the thyroid -- another autoantigen which is also a target of immunological processes in different thyroid diseases -- and the factors regulating its expression and synthesis have been widely studied /3, 4, 5, 8, 10, 11, 12/. According to the results of Chiovato et al. /3/, TSH and thyroid stimulating immunoglobulin (TSI) stimulate the expression and synthesis of microsomal autoantigen in thyroid cells. In the present study we examined the TG expression in cultured human thyroid cells after TSH and TSI stimulation, using immunofluorescent method. To the best of our knowledge, examinations concerning the effect of both TSH and TSI on thyroglobulin expression of cultured human thyroid cells have not been published, so far.

Materials and Methods

Human thyroid cells in culture

Surgically removed human euthyroid adenomas and normal human thyroid tissue surrounding thyroid cysts were used. Thyroid tissue was minced, digested in Collagenase Type IV (SIGMA) for two hours at 37 ^{o}C and passed through 200 µm nylon mesh to eliminate clumps. Cell viability was checked and was more than 80% in all preparations used. The cells were seeded at 2×10^{5}/ml concentration onto round coverslips plated in wells of a 24-well plate. The thyroid cells were cultured in 5% CO_2 and 95/air at 37 ^{o}C in RPMI supplemented with 10% FCS and 1% antibiotics (penicillin and streptomycin).

Sera and TSH concentrations

Sera from five patients with Graves' disease were used. All sera were positive for TSI as measured by the generation of cAMP /2/ and negative for anti-TG as well as anti-microsomal antibodies using Thymmune-M and Thymmune-T kits (Wellcome). IgG was prepared by DEA Sephadex separation and used at different concentrations: 0.5; 1.0; 2.0 and 5.0 mg/ml. Active Graves' disease was diagnosed by conventional clinical and laboratory criteria (history taking, physical examination, TRH test, sTSH-RIA, FT4-RIA, FT3-RIA and thyroid scintigraphy. TSH (SIGMA, Thyrotrop hormone) was used at the following concentrations: 0.01; 0.1 and 1.0 mU/ml.

Experimental procedure

TSH and TSI were added at the time of seeding the cells on coverslips. The cells were cultured for 3 days. Immunofluorescence investigation was initiated by washing the thyroid cells growing on coverslips. After fixation in paraformaldehyde cells were treated with chilled acetone and then incubated in the sequence with: (i) monoclonal mouse anti-human thyroglobulin (Dakopatts) at the dilution of 1:50; (ii) FITC-labelled anti-mouse IgG and IgM produced in sheep (RUB 2nd STEP); (iii) cells were mounted in glycerol and examined with a fluorescence

microscope. The incubations were carried out at room temperature for 15 min. All incubations were made in triplicate.

Evaluation of immunofluorescence

300 cells of each coverslip were estimated. The fluorescence intensity of positive cells was graded and marked as follows: light +; medium ++; and strong +++. The results were referred to 100 cells as fluorescence index.

Controls

1. The same procedure was carried out using sera from healthy volunteers. 2. Cells without adding either TSH or sera were incubated with the same monoclonal antibody and then with FITC-conjugated anti-mouse immunoglobulin. 3. Cells stimulated with TSH and TSI from Graves' patients were incubated with FITC-labelled anti-mouse immunoglobulin alone.

Results

Effect of different TSH concentrations

TSH at the concentration of 0.1 mU/ml caused the most expressed thyroglobulin expression in cultured thyroid cells (fluorescence index: 168 $\pm$ 35). At higher concentration -- 1.0 mU/ml -- the thyroglobulin expression was less (122 $\pm$ 22) and the lowest fluorescence index was calculated after stimulation with 0.01 mU/ml TSH (68 $\pm$ 9). Results are shown in Fig. 1.

Effect of different TSI concentrations

TSI was the most effective at the concentration of 1.0 mg/ml, fluorescence index: 84 $\pm$ 14. At higher concentration the TSI was less effective, fluorescence index: 73 $\pm$ 24 at 2 mg/ml and 71 $\pm$ 12 at 5.0 mg/ml. The least effect on thyroglobulin expression was caused at the lowest concentration (0.5 mg/ml) of TSI (32 $\pm$ 10). Thyroid-stimulating immunoglobulins from different patients resulted in very similar changes on thyroglobulin expression. Differences in fluorescence indices were mainly the consequences of different TSI concentrations. Results are demonstrated in Fig. 1.

Controls

All controls -- except incubation of stimulated cells only with FITC-conjugated anti-mouse immunoglobulin -- showed thyroglobulin expression of

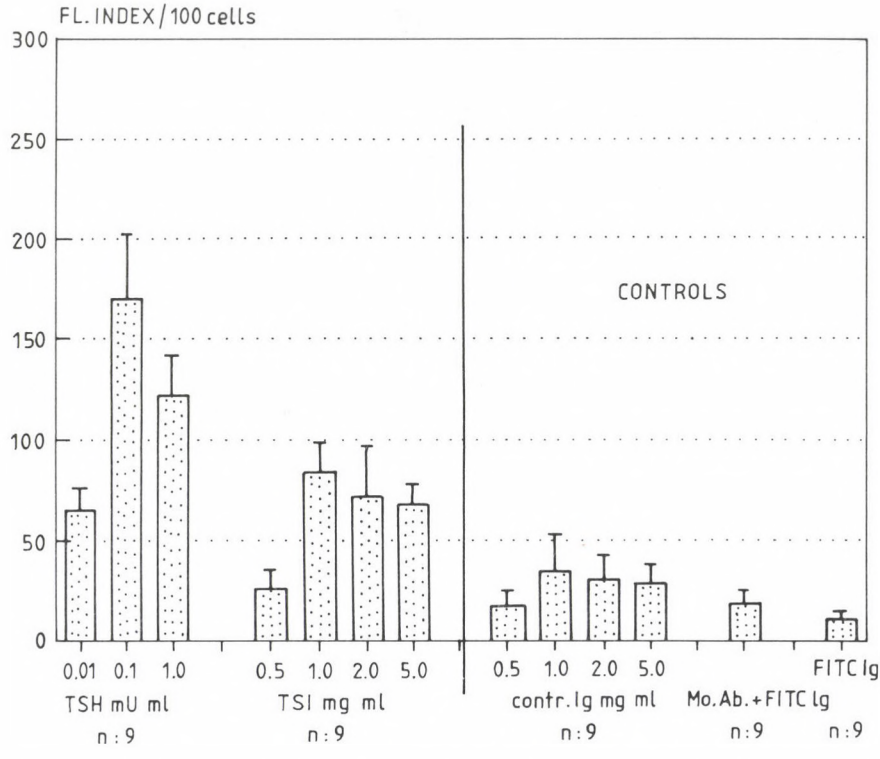

Fig. 1. The effect of different TSH and TSI concentrations on the thyroglobulin expression of cultured human thyroid cells, evaluated as fluorescence index/100 cells. The results of control examinations are demonstrated, too. Mo.Ab + FITC Ig means results without stimulation (no TSH or TSI added). FITC Ig means stimulation with TSH and TSI followed by FITC-conjugated anti-mouse Ig incubation alone. Mean + S.D. are demonstrated

the same low range. Their results are comparable with the effect of the lowest concentration (0.5 mg/ml) of TSI applied, but are significantly lower than the effect of TSH at 0.01 mU/ml concentration. (Results are shown in Fig. 1).

Fig. 2. Immunofluorescence after TSH stimulation at the concentration of 0.1 mU/ml. Small fluorescent granules distributed in the cytoplasm of thyroid cells and large, partly confluent, granules in the perinuclear area. Original magnification x 400

Fig. 3. Immunofluorescence after stimulation with TSI at 1.0 mg/ml concentration. Small granules distributed in the cytoplasm of the thyroid cells and large granules with intensive fluorescence. Original magnification x 400

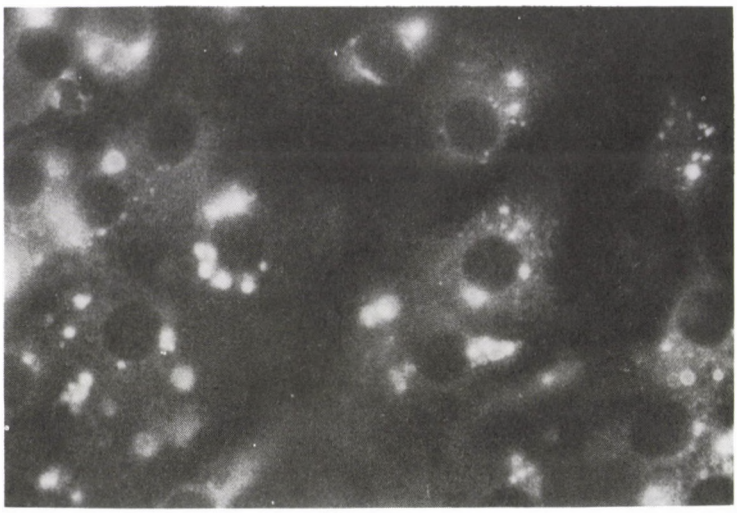

Fig. 2

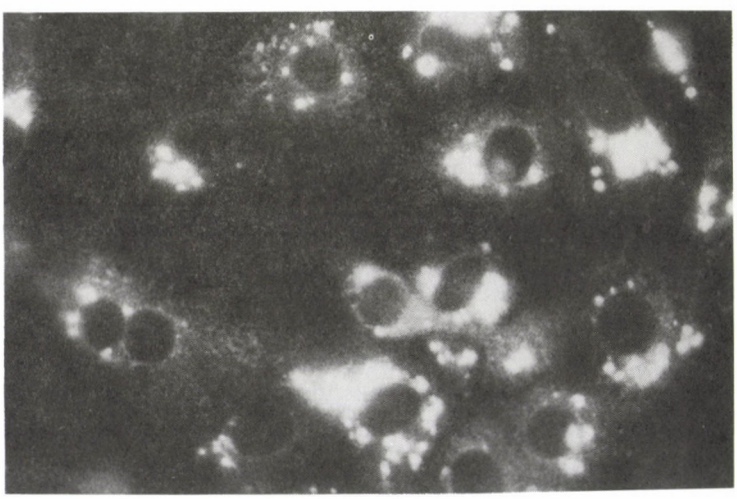

Fig. 3

Morphological aspects of thyroglobulin expression

The thyroglobulin expression was restricted to the cytoplasm of cultured thyroid cells. The nuclei were negative. Granules of different size and fluorescent intensity distributed in the cytoplasm (Fig. 2) and large, here and there confluent, granules located perinuclearly were also detectable (Fig. 3). There was no difference in the morphology of fluorescent positivity caused either by TSH or TSI.

Discussion

Thyroglobulin and antibodies directed against it as well as the microsomal antigen and its antibodies play an important pathogenetical role in thyroid diseases /5, 7, 10, 12, 15/. Both antigen-antibody systems have been widely investigated. Chiovato et al. /3/ demonstrated the effect of TSH and TSI on the expression of microsomal antigen, they used human thyroid cells in culture. We investigated the effect of TSH and TSI on the expression of thyroglobulin antigen. Our results indicate a tight correlation between the presence of TSH in the culture medium and the appearance of thyroglobulin antigen in the cultured thyroid cells. The most effective concentration of TSH was 0.1 mU/ml. TSI from active Graves' patients also provoked an increased thyroglobulin expression in the thyroid cells, but its effectivity was lower than that of TSH. A similar difference was described by Chiovato et al. /3/ regarding the microsomal antigen expression. The morphological characteristics of thyroglobulin are different from the microsomal antigen, as compared our results with the data of Chiovato et al. /3/. Thyroglobulin was detected only in form of large granules, frequently confluent and not so finely dispersed as the microsomal antigen. We failed to detect thyroglobulin antigen on the surface of thyroid cells.

In conclusion, we may say that both TSH and TSI are involved in the expression of thyroglobulin, but TSI seems to be less effective according to our results. Factors other than TSH and TSI (IFN gamma, GF, TNF and others) are probably also involved in the thyroglobulin expression, but their precise effect and their connections to the TSH and TSI should be topics of further examinations.

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IS THE INCIDENCE OF ACUTE MOUNTAIN SICKNESS (AMS) AT MEDIUM ALTITUDE IN THE AUSTRIAN ALPS INFLUENCED BY THE HEIGHT OF HOME RESIDENCE OF THE ALPINIST?

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In previous studies the incidence of acute mountain sickness (AMS) at medium altitude was examined in the Austrian Alps, where many tourists come from low parts of Europe. This study assesses the influence of the height of home residence on the incidence of AMS at medium altitude. The severity of high-altitude adaptation disorder was quantified by using a scoring system after an interview and a clinical examination in 84 lowlanders, mainly those from Hungary. Forty-two alpinists with a home residence of 800 to 1000 m served as control. The incidence of AMS was 1.4% at 2000 m and 7.4% in 3000 m. The most frequent symptoms were slight headache and peripheral or periorbital oedema. The AMS-score of the Hungarian alpinists did not differ significantly from that of the alpinists with a home residence of height 800 to 1000 m. Conclusion: in contrast to the situation at high altitude, at medium height tourists from lowlands are not at higher risk of AMS than other alpinists.

Keywords: Acute mountain sickness, medium altitude, lowland dwellers

Introduction

Lowland dwellers who rapidly reach high altitude may develop one or more symptoms such as headache, anorexia and insomnia. The condition characterized by symptoms like progressive vomiting, shortness of breath, severe headache and ataxia is called acute mountain sickness (AMS). Physical examination of these patients may disclose tachypnoea, pulmonary rales, and periorbital as well as peripheral oedema.

The incidence of AMS at high altitude was first reported by Hackett et al. /3/ who found 53% of 278 unacclimatized hikers to suffer from AMS at

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4243 m altitude in the Himalayas of Nepal. AMS is not quite uncommon at medium altitude either. An incidence of 3.1% in 2000 m mounting to 53% at 4559 m has been reported from the Austrian /9/ and Swiss /5/ Alps.

Lowland dwellers are at particular AMS-risk at high altitude, for instance in the Himalaya, but up to now no research has been done on AMS-prevalence at medium altitude. The aim of our study was to evaluate the influence of residential height at home on the incidence of AMS in the Austrian Alps.

Methods

This study was performed in the Austrian Alps. On two levels of altitude (at 2000 m Rax/Schneeberg area and at 3000 m Hohe Tauern) lowland dwellers mainly those from Hungary were examined for AMS after reaching the respective summits. The AMS-evaluating system established by Hackett et al. /3/ was used throughout; this consists of a short structured interview and a physical examination. Each tourist was asked for headaches (light: one score point; severe: two score points), nausea (one point), vomiting (two points), dizziness (one point). As part of the alpinists had not slept in alpine huts, insomnia was not recorded. The physical examination put emphasis on periorbital or peripheral oedema (one site: one point; more than one site: two points) respiratory rate (more than 25 breaths/min: two points), pulmonary rales (slight: one point; severe: two points) and ataxia (Romberg-test, finger-nose-test; two points). Presence of AMS signs was always revised by a second examiner. Severity of the height adaptation disorder was quantified by adding up the score points. Subjects without any sign and symptom of AMS were considered healthy, those with one or two score points were considered slightly to moderately affected, and those with three or more score points were regarded as suffering from AMS. Alpinists living at an altitude of 800 to 1000 m in Austria were assumed as a control group.

Chi-square analysis of contingency tables and unpaired t test. $P < 0.05$ was considered significant.

Results

128 alpinists, 94 men and 34 women, were examined: 72 probands at 2000 m altitude and 56 at 3000 m altitude. At 2000 m altitude 52 and at 3000 m altitude 32 of the probands were lowland dwellers. The other examined alpinists were residents at 800 to 1000 m altitude. Age ranged from 19 to 64 years. Age and sex did not vary significantly between the groups. The examination was performed on summits, that had to be reached by foot without aid of a cable car.

At 2000 m, one alpinist (1.4% of all probands) had an AMS score of ≥ 3 indicating that he suffered from AMS, 7 alpinists (9.7%) showed slight to moderate signs of height adaptation disorders (score 1 or 2) and 64 probands

Table I

Distribution of score (mean ± SD) in lowland dwellers (A) and probands with home residence at 800 to 1000 m altitude (B), examined at 2000 and 3000 m altitude. Numbers (percentages) of alpinists

Score	2000 m A n = 52	2000 m B n = 20	3000 m A n = 32	3000 m B n = 24
0	46 (88.5)	18 (90)	22 (68.7)	18 (75)
1-2	5 (9.6)	2 (10)	7 (21.9)	5 (20.8)
≥ 3	1 (1.9)	0 (0)	3 (9.4)	1 (4.2)
mean	0.23	0.15	0.75	0.54
SD	0.73	0.49	1.37	1.06

(88.9%) were without any sign of AMS (score 0). At 3000 m altitude, significantly more alpinists showed signs of AMS: four probands (7.2%) had an AMS score of ≥ 3, 12 probands (21.4%) of 1 or 2 and 40 probands (71.4%) of 0 ($P < 0.05$).

The mean AMS-score of both the lowland dwellers and alpinists with home residence at 800 to 1000 m altitude rose significantly, the percentage of unaffected subjects dropped with increasing altitude ($P < 0.05$). The incidence of AMS signs did not differ significantly between lowland dwellers and the other alpinists at either heights (Table I).

Table II shows the incidence of the AMS signs and symptoms. Slight headache and periorbital or peripheral oedema occurred most frequently. In the lowland dwellers group, ataxia was shown by two tourists. None of the alpinists suffered from severe headache or vomiting, only slight pulmonary rales were documented in 2 probands.

Table II

Numbers (percentages) of probands with symptoms and signs of AMS (A = lowland dwellers, B = tourists with home residence from 800 to 1000 m)

Symptom or sign	2000 m A n = 52	2000 m B n = 20	3000 m A n = 32	3000 m B n = 24
Headache:				
none	48 (92.2)	18 (90)	27 (84.4)	21 (87.5)
light	4 (7.7)	2 (10)	5 (15.6)	3 (12.5)
severe	0 (0)	0 (0)	0 (0)	0 (0)
Nausea:				
no	50 (96.2)	20 (100)	30 (93.7)	23 (95.8)
yes	2 (3.8)	0 (0)	2 (6.3)	1 (4.2)
Vomiting:				
no	52 (100)	20 (100)	32 (100)	24 (100)
Dizziness:				
no	52 (100)	20 (100)	30 (93.7)	23 (95.8)
yes	0 (0)	0 (0)	2 (6.3)	1 (4.2)
Tachypnoea:				
no	51 (98.1)	20 (100)	30 (93.7)	23 (95.8)
yes	1 (1.9)	0 (0)	2 (6.3)	1 (4.2)
Oedema:				
none	48 (92.3)	19 (95)	28 (87.5)	20 (83.3)
1 site	4 (7.7)	1 (5)	4 (12.5)	4 (16.7)
2 sites	0 (0)	0 (0)	0 (0)	0 (0)
Pulmonary rales:				
none	52 (100)	20 (100)	30 (93.7)	24 (100)
+	0 (0)	0 (0)	2 (6.3)	0 (0)
+ +	0 (0)	0 (0)	0 (0)	0 (0)
Ataxia:				
no	52 (100)	20 (100)	30 (93.7)	23 (100)
yes	0 (0)	0 (0)	2 (6.3)	1 (4.2)

Discussion

A considerable increase in the number of Hungarian mountaineers in the Austrian Alps has taken place since the opening of the border. Most of the mountain tours performed in Austria reach summits below 3000 m.

The incidence of AMS in high altitude has been well defined since many years, where as for the situation in medium altitude a wide range from 1.4

to 2.5% was published in the literature /4, 6—8, 10, 11/. Reliable results were achieved after the now established AMS-scoring system was introduced /3/. In our study all examinations and evaluations were performed after the alpinists had rested for one hour, so that signs and symptoms with no regard to AMS (effort-induced tachypnoea and oedema of fingers caused by tight rucksack-straps) could be excluded. All alpinists examined in this study were just on one-day tours, therefore possible sleeping disorders as sign of AMS could not be evaluated. AMS scores correlate inversely with the arterial oxygen saturation /1/.

AMS scores rose significantly both in lowland dwellers and in alpinists with residence at home of 800 to 1000 m increasing altitude without significant difference between the two groups. Although 5 alpinists reached an AMS score of ≥ 3, neither full blown high-altitude pulmonary oedema nor high-altitude cerebral oedema was observed. In medium altitude the less dramatical forms of maladaptation are clearly much more common.

The experience from the Himalaya shows, that careful adaptation to height is necessary in high altitude. Lowlanders are at much higher risk to suffer from AMS, especially after rapid ascent. On the contrary in medium altitude, none of our probands had undergone an adaptation time, all the same no significant difference in the incidence of AMS between mostly Hungarian mountaineers and the presumably better adapted mountaineers from places between 800 and 1000 m showed. As the number of investigated alpinists was not very large in this study, the investigation of a larger sample is necessary to confirm the results. Furthermore, the investigation of a larger sample of hikers at increasing height levels will show from which altitude on lowlanders and highlanders differ in the respective AMS incidence.

Outcome: our study confirms that adaptation disorders occur at medium altitude not rarely. Although AMS is not mentioned in the alpine accident statistics in Austria /2/, maladaptation to height may be of importance for mountain emergencies. Lowlanders are at no higher AMS risk than all other alpinists in our study.

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EFFECT OF CHORIOCARCINOMA SUPERNATANT ON NATURAL KILLER AND LYMPHOKINE-ACTIVATED KILLER CELL ACTIVITY

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The effect of JEG-3 choriocarcinoma supernatant on human natural killer cell and lymphokine-activated killer cell activity was investigated. Choriocarcinoma supernatants from JEG-3 cell lines were obtained at the time of their optimal growth. K562 erythroblastoid cells were used as target cells for natural killer and lymphokine-activated killer cell mediated lysis in a ^{51}Cr release assay. The choriocarcinoma supernatants had a significant dose-dependent suppressive effect on natural killer and lymphokine-activated killer cell activity. This suppression was more expressed at high effector:target cell ratios. Therefore, choriocarcinoma supernatants appear to have potent inhibitory effects on many aspects of cellular immunity, although, additional studies should be performed to futher characterize and identify immunoregulatory molecule(s) in choriocarcinoma supernatants.

Keywords: Choriocarcinoma supernatant, natural killer cell, lymphokine-activated killer cell, immunosuppression

Introduction

Many experiments have shown that endocrine manipulation of animals alters the size and activity of the lymphoid organs /28/. It has also been claimed that sex hormones have a direct effect on levels of immune respon-

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Abbreviations: CCA: choriocarcinoma, CPM: count per minute, ^{51}Cr: sodium ^{51}chromate, E/T: effector:target ratio, FCS: fetal calf serum, hCG: human chorionic gonadotrophin, IL-2: interleukin-2, IL-2-R: interleukin-2 receptor, LAK: lymphokine-activated killer, MHC: major histocompatibility complex, mRNA: messenger RNA, nK: natural killer, PBLs: peripheral blood lymphocytes, S.D.: standard deviation, TCM: tissue culture medium

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siveness measured in vitro /26/ and they are responsible, at least in part, for the retention of the fetal allograft /6/.

A variety of effector cells, including cytotoxic T lymphocytes, natural killer (NK) cells and natural cytotoxic cells accumulate at the site of graft rejection. These cell populations might also be activated and involved at the feto-maternal interface because of recognition of semi-allogeneic antigens on trophoblast surface. Recent studies /5/ have demonstrated that human placental syncytiotrophoblast synthesizes mRNA for IL-2, which potentially induces lymphokine-activated killer (LAK) cells by producing IL-2 protein. The NK cell and LAK cell system is claimed to be of importance in host defense against viruses, parasites and neoplasia, as well as in regulating the bone marrow /29/. The effector cells concerned are capable of recognizing tumour tissues and are reduced in activity in pre-eclampsia, where increased levels of circulating trophoblast cells occur. Drake et al. /9/ have reported that trophoblast cells are also susceptible to LAK-cell-mediated killing. In a previous publication, relating to breast cancer /30/, chance observations implicated sex hormones in the regulation of the NK and LAK effector systems.

JEG-3 choriocarcinoma (CCA) cells are considered a pure cell model for human trophoblast and for their steroidogenic potential. Human chorionic gonadotrophin (hCG) is also a constant and predictable secretory product of trophoblast cells. Matsuzaki et al. /19/ reported the biochemical characterization of a choriocarcinoma-derived factor and determined its immunosuppressive activity in human T cell responses. NK and LAK cells, unlike cytotoxic T lymphocytes, are not dependent on the major histocompatibility complex (MHC) class I antigen for target cell recognition /21/. Because trophoblast cells do not express class I MHC antigens, NK and LAK cells may be the principal maternal immune defense cells capable of mounting and immunologic response to trophoblast tissue /3, 4/. In our early experiments /12, 13/ molar villous fluid, which is the concentrated product of molar trophoblast cells, suppressed lytic activity of mononuclear cells. In the present study we investigated the direct effect of cultured human JEG-3 CCA supernatant on the NK and LAK effector systems in vitro.

Materials and Methods

Cell lines and growth conditions. K562 and JEG-3 CCA cells were kindly supplied from the Japanese Cancer Research Resources Bank, Tokyo and from Chiba University, Chibacity, Tokyo (Dr. M. Sekiya). JEG-3 cell line was maintained as continuous monolayer cultures in 75 cm^2 flasks (Corning, Iwaki Glass, Japan) containing 35 ml or 20 ml of a tissue culture medium (TCM) consisting of RPMI 1640 medium, 15% fetal calf serum (FCS), penicillin G sodium (100 U/ml), streptomycin sulfate (100 µg/ml) and 2 mM L-glutamine (Scientific Product Laboratories Co. LTD., Ekimaebiru, Ojiya-city, Niigata, Japan) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Culture supernatants were collected from CCA cell lines immediately after the cultures reached confluence. K562 cells were maintained in suspension in upright flasks in TCM under the same conditions.

Preparation of effector cells. Human peripheral blood lymphocytes (PBLs) containing the NK cell population were separated from freshly drawn heparinized blood by centrifugation on Ficoll-Paque (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). For NK experiments, the interface containing PBLs was collected, washed twice in RPMI, and resuspended in TCM. Macrophages, which can have an inhibitory effect on the NK cell activity, were removed by allowing them to adhere to FCS-coated petri dishes for 1 h at 37 °C. After this step the concentration of the cell suspension was adjusted to 1×10^7 cells/ml in TCM. For LAK assays, PBLs were resuspended at a concentration of 1.5×10^6 cells/ml in complete medium consisting of RPMI 1640 supplemented with 10% heat-inactivated human AB serum (INC Biomedicals, Inc., Costa Mesa, CA 92626), penicillin G sodium (100 U/ml), streptomycin sulfate (100 ug/ml), 2 mM L-glutamine (this medium is subsequently referred to as RPMI-10 AB medium) and 1000 U/ml recombinant human IL-2 (Takeda Medical Co., Osaka, Japan). PBLs were activated to generate LAK cells by adding 10 ml from this cell suspension to 25 cm^2 flasks (Corning, Iwaki Glass, Japan) and incubating horizontally for 4 to 5 days at 37 °C in a humidified atmosphere of 5% CO_2 in 95% air. The cells were then washed twice and resuspended in the same culture medium without IL-2. Prior to cytotoxic assays LAK cells were adjusted to a concentration of 1×10^7 cells/ml in TCM.

Assay of NK cell and LAK cell activity on K562 cells. K562 cell suspension cultures were routinely split into TCM in a ratio of 1:4 one day prior to the NK assay. Cells were then washed twice in RPMI and labeled with 200 µCi sodium ^{51}chromate (^{51}Cr; New England Nuclear, Boston, MA) in 1 ml cell suspension. After incubation for 1.5 h at 37 °C, cells were layered over 3 ml of undiluted FCS and centrifuged for 10 min at 1500 rpm, washed twice in TCM, adjusted to 3.33×10^5 cells/ml TCM and kept on ice until used. Assays were performed in 96-well round-bottomed plates (Sumilon, Japan). One hundred microliters of less or more concentrated CCA supernatant or TCM was added first, followed by 100 µl of NK cell preparation or LAK cell preparation (effector cells) and 50 µl of radiolabeled target cells (K562) in effector:target (E/T) ratios of 15:1, 30:1, and 60:1. Each test was conducted in triplicate. Plates were centrifuged at 500 rpm for 4 min, and then incubated at 37 °C for 6 h. Plates were centrifuged again immediately before harvesting, and 100 µl of supernatant was collected in glass counting tubes from each well and assessed for released ^{51}Cr in a gamma counter (Aloka; Auto Well Gamma System, ARC-300).

In both NK and LAK experiments, spontaneous ^{51}Cr-release was measured in wells containing target cells alone. Total incorporation was determined by counting the total radioactivity of the wells containing labeled target cells. Each assay was set up in triplicate and the percentage of specific lysis (^{51}Cr release) was calculated as follows:

$$\% \text{ specific lysis} = \frac{\text{CPM } ^{51}\text{Cr (experimental)} - \text{CPM } ^{51}\text{Cr (spontaneous)}}{\text{CPM } ^{51}\text{Cr (total)} - \text{CPM } ^{51}\text{CR (spontaneous)}} \times 100$$

The data were expressed as mean ± standard deviation of the percentage of specific lysis.

Viability of NK and LAK cells in presence of TCM or CCA supernatants. NK and LAK cells were incubated at 37 °C for 6 h in the presence of either TCM, RPMI-10 AB, or CCA supernatants, and viability was assessed by the trypan blue exclusion test.

Statistical analysis. For NK and LAK assays, data were analysed with Statview II statistical software on a Macintosh II/ci computer. Differences among groups were determined by

two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) post-hoc testing.

Results

Spontaneous release of ^{51}Cr was less than 5% for cytotoxicity assays using K562 cells as targets. Experiments of NK and LAK cell activity with K562 cells as targets were performed at E/T ratios of 15:1, 30:1, and 60:1, and all experiments showed optimal cytotoxicity at the E/T ratio 60:1. For NK assays the percentage cytotoxicity was 28, 39, and 47% for E/T ratios of 15:1, 30:1, and 60:1, respectively (Fig. 1). In case of LAK assays the percentage of cytotoxicity was 69, 77, and 85%, respectively, under the same conditions (Fig. 2). CCA supernatants (less and more concentrated) were studied in the NK and LAK assays at E/T ratios of 15:1, 30:1, and 60:1 and optimal suppression of killing activity was observed at an E/T ratio of 60:1. Supernatants from two kinds of JEG-3 CCA cell cultures (containing

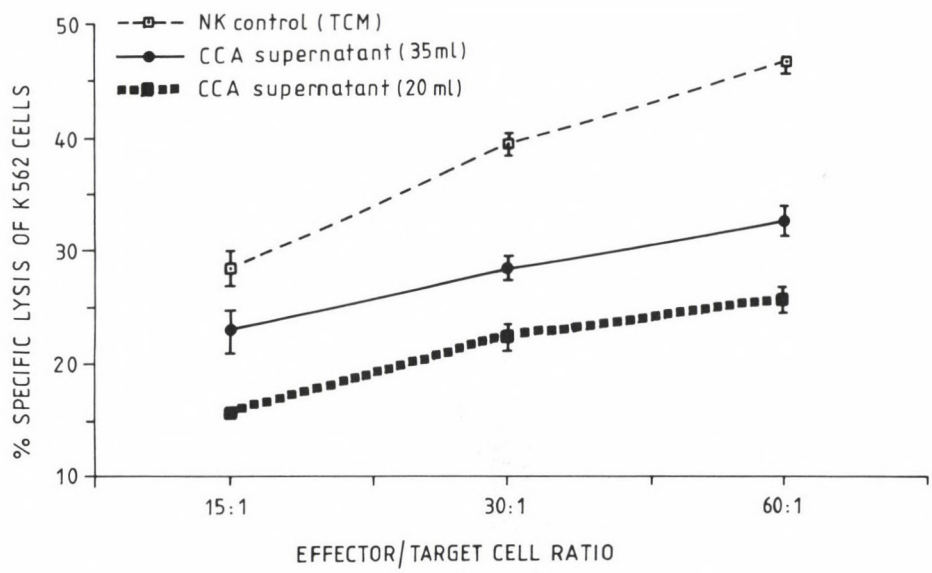

Fig. 1. Compared to control (TCM), JEG-3 choriocarcinoma (CCA) supernatants (less concentrated = 35 ml, more concentrated = 20 ml) significantly suppressed natural killer cell cytotoxicity in the K562 target cell assay at each effector : target ratio ($P < 0.05$). The suppression was more expressed at effector : target ratios 30:1 and 60:1 than at 15:1. The final concentration of CCA supernatant in the cultures was 40%. The difference in the suppression of NK cell activity between the two different concentrations of CCA supernatant was significant ($P < 0.05$). Mean $\pm$ S.D. values for triplicate samples from a representative experiment

35 ml = less concentrated and 20 ml = more concentrated supernatant) were added undiluted to the NK and LAK assays. The less concentrated supernatant contains less concentration of sex hormones and other factors produced by CCA cells than the more concentrated one. Figures 1 and 2 show that the suppression of both CCA supernatants was dose dependent and at E/T ratios of 30:1 and 60:1 their suppression was more significant than at E/T ratio of 15:1 ($P < 0.05$). The suppression of the less and more concentrated CCA supernatants reached its maximum at E/T ratios of 60:1 in all experiments. The differences in the suppression of NK and LAK activity of the less and more concentrated CCA supernatants were significant at each E/T ratio ($P < 0.05$). In NK assays the percentage of cytotoxicity in the presence of the less concentrated CCA supernatant was 23, 28, and 33% and for the more concentrated CCA supernatant was 16, 22, and 26% at E/T ratios of 15:1, 30:1, and 60:1, respectively. In LAK experiments the percentage of cytotoxicity was 49, 54, and 54% for the less concentrated and 38, 40, and 44% for

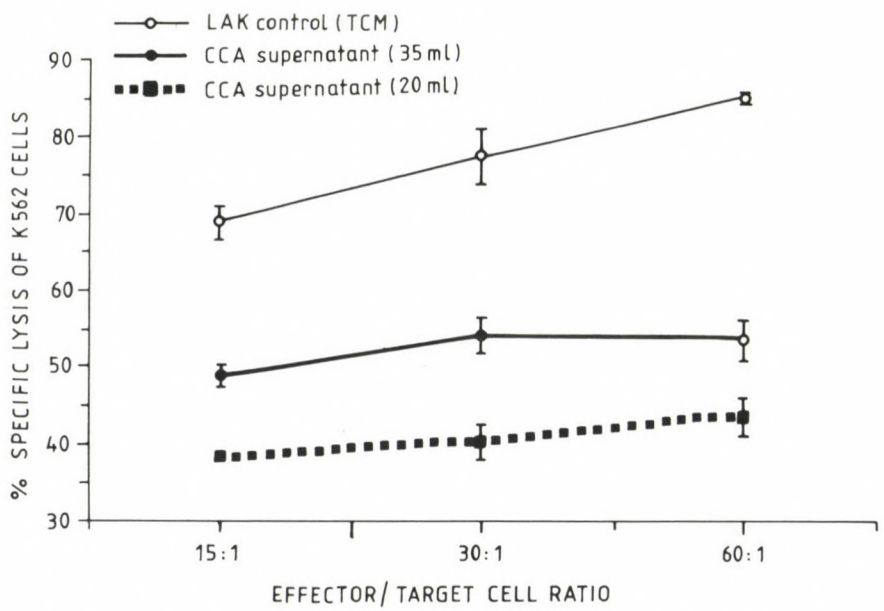

Fig. 2. Compared to control (TCM), JEG-3 choriocarcinoma (CCA) supernatants (see Fig. 1) significantly suppressed lymphokine-activated killer (LAK) cell cytotoxicity in the K562 target cell assay at each effector : target ratio ($P < 0.05$). The suppression was more expressed at effector : target ratios 30:1 and 60:1 than at 15:1. The final concentration of CCA supernatant in the cultures was 40%. The difference in the suppression of LAK cell activity between the two different concentrations of CCA supernatant was significant ($P < 0.05$). Mean $\pm$ S.D. values for triplicate samples from a representative experiment

the more concentrated JEG-3 CCA supernatant at E/T ratios of 15:1, 30:1, and 60:1. Viability of NK and LAK cells after culture in CCA supernatants was greater than 95% and did not differ significantly from TCM and RPMI-10 AB controls.

Discussion

Reportedly, a CCA-derived factor has immunosuppressive activity in human T cell responses /19/. Possible identity of a CCA-derived factor with the one obtained from normal trophoblast has also been published /18/. It suppresses IL-2-dependent T cell mediated responses, acting specifically on the events in the proliferation phase, but not those in the activation phase of IL-2 and IL-2-R mediated T cell responses. T cells activated by recognition of paternal alloantigens, however, may stimulate the IL-2-dependent as well as IL-2-independent pathways. It is, therefore, of importance to understand the factor-mediated immunoregulation of feto-maternal interaction in order to examine whether the pregnancy-associated factors suppress T cell immune responses as well as NK and LAK cell activity. The implantation site in molar pregnancy is infiltrated by several types of immunocompetent cells including cytotoxic T lymphocytes, helper/inducer T cells, NK cells /17/ and, potentially, LAK cells /5, 20, 22/. These cells may influence trophoblast proliferation, viability and function through direct cytotoxicity or release of cytokines. The current study demonstrated that CCA supernatants significantly suppress both NK-cell and LAK-cell cytotoxic functions and may play an important role in the immunobiology of normal and molar pregnancy and gestational trophoblastic tumours.

NK cells lyse tumour cells by recognition of an unknown target structure and activation of unique cytoplasmatic signal transduction pathways, subsequently releasing cytolytic (perforin) granules /16, 25/. LAK activity may be mediated by members of both T and NK cell lineage, the effector cells responsible for malignant trophoblast lysis are predominantly lymphokine-activated NK cells. These LAK cells and NK cells recognize the same target molecule; their cytotoxic effects are quantitatively, but not qualitatively, different. After IL-2 stimulation, these LAK cells release a greater quantity of natural killer cytotoxic factor against target cells and therefore exhibit a higher level of cytotoxicity than NK cells /15/.

Similarly to our previous results /27/, in this study we demonstrated a significant inhibitory effect of two kinds of JEG-3 CCA supernatants on NK and LAK cell-mediated lysis of classical K562 target cells. Under the same culture conditions equal number of JEG-3 CCA cells were grown and maintained in 20 ml and 35 ml TCM in the same type of flasks for four days. The intensity of CCA cell proliferation was approximately the same under the 20 ml and 35 ml volume of TCM. Therefore, the concentration of sex hormones and other factors produced by CCA cells was obviously higher in the 20 ml TCM than in the 35 ml one. We found that both the less and the more concentrated CCA supernatants at a 40% final concentration in culture markedly inhibited the lytic activity of NK and LAK cells. This suppression was more expressed at higher E/T ratios than at lower ones. Furthermore, in case of higher concentrations of CCA cell-derived factors the suppression was also significantly greater. These data suggest that JEG-3 CCA supernatant contains factor(s) that suppress both NK- and LAK-cell-mediated lysis.

Studies have been performed to characterize the suppressive factors(s) in CCA supernatants. The hCG present in culture at physiological levels has been shown to depress a range of proliferative responses /14/ and also induces regulatory T-cell activity /11/. Careful studies have reported however, that relatively high progesterone and estrogen concentrations are necessary for suppression of NK and LAK cell activity /8, 10, 24/. The data presented in this paper suggest that hCG, which is known to act via a common receptor, markedly suppresses lysis of K562 target cells when present in the culture at a wide range of concentrations.

Several studies indicate that the mechanism of immunosuppression by CCA supernatants involves inhibition of the IL-2 signal transduction pathway /1, 2, 7, 23/. Although we did not study this mechanism directly, our results support this hypothesis, for IL-2 is an initiation signal for effector functions of both NK cells and LAK cells /5/.

CCA supernatants appear to have potent suppressive effects on many aspects of cellular immunity. Although we have demonstrated that CCA supernatants significantly inhibit NK and LAK cytotoxicity in vitro, additional studies should be performed to further characterize and identify immunoregulatory molecule(s) in CCA supernatants.

Several pregnancy-associated factors which are produced by either trophoblast or decidual cells might regulate a variety of maternal immunocompetent cells, including NK and LAK cells, at the feto-maternal interface by different mechanisms. In combination with decidual suppressor cells they

may contribute to the establishment and maintenance of pregnancy. Presumably, the JEG-3 factor(s) may also act on the immune system of patients with choriocarcinoma, preventing tumour rejection by the host.

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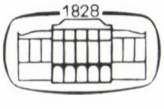

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