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*Acta Microbiologica Hungarica 39 (1), pp. 3—11 (1992)*

# EFFECT OF MANNOZYM ON THE COURSE OF LCMV INFECTION IN MICE WITH UNDEVELOPED AND NORMAL IMMUNE SYSTEM

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(Received November 1, 1990)

Adult germfree (Gf) mice with undeveloped immune system due to antigen deficient environment, conventional (Cv) mice with normal immune system and Cv suckling mice with undeveloped immune system due to age were treated intraperitoneally with Mannozym (M, 0.1% zymosan suspension) 4 days or 4 days and 1 day before the intracerebral inoculation with lymphocytic choriomeningitis virus (LCMV). One dose of M was equal to 40 mg/kg of zymosan. In suckling mice, both applied doses of M contributed the development of fatal lymphocytic choriomeningitis after infection with 100 $LD_{50}$ dose of LCMV, thus M pretreatment increased the cellular immune response to LCMV infection. M pretreatments had no influence on the course of LCMV infection either in adult Gf or in Cv mice. Spleen hypertrophy was caused by applied doses of M both in adult (Gf and Cv) and Cv suckling mice, but modulating effect on the cellular immune response manifested simultaneously only in Cv sucklings.

Zymosan is a polymannan-polyglucan complex, first isolated from the cell wall of baker's yeast by Pillemer and Echer [1]. Mannozym (M) a zymosan containing cell wall derivate of *Saccharomyces cerevisiae* is used in human therapy for enhancement of non specific immunity and to prevent unwanted side effects of X-ray treatment [2–4]. Its mode of action is not exactly known yet, but it has been demonstrated in animal experiments that Zymosan influencing through different aspecific mechanisms the function of the mononuclear phagocytic system, can increase the phagocytosis [5–12] and it enhances both the humoral and cellular specific immune responses, too [13]. In our present experiments the effect of M on the cellular immune response to lymphocytic choriomeningitis virus (LCMV) infection was studied in mice with normal or undeveloped immune system.

It is known that the fatal lymphocytic choriomeningitis followed by intracerebral (i. cer.) LCMV infection is the consequence of the cytotoxic reaction of LCMV antigen specific T lymphocytes to cells expressing viral antigens on the leptomeninx [14–16]. The course of virus infection thus greatly depends on the cellular immune responsiveness of the animal. Acute lymphocytic choriomeningitis develops in adult mice with intact developed immune system

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and the animals die on the 6–8th day after virus infection. In mice with insufficient T lymphocyte function, like in adult germfree (Gf) [17] and in conventional (Cv) suckling mice [18, 19] both with undeveloped immune system, lymphocytic choriomeningitis fails to develop and the mice surviving the infection become virus carriers. Manifestation of LCMV infection in the form of fatal meningitis is hindered by immunosuppressive effects [19–23] and enhanced by immunostimulatory treatments; in suckling and Gf mice by accelerating the development of their cellular immune response [24–29]. In the present experiment it was examined whether M pretreatment can influence the cellular immune response to LCMV infection in Gf adult mice with undeveloped immune system due to antigen-deficient environment, in Cv suckling mice with undeveloped immune system due to age, and in Cv adult mice with intact developed immune system.

## Materials and methods

*Experimental animals.* Gf and Cv 6-week-old C3H and Cv 6-week-old young adult and 2-week-old suckling CFLP mice (LATI, Gödöllő, Hungary) of both sexes were used. Germfree state was maintained and controlled as published earlier [30]. In the experiment with suckling mice, the number of mice was decreased to 10 per litter.

*Mannozym.* The preparation (Institute for Serobacteriological Production and Research, Human, Budapest) contains 1 mg/ml water-insoluble glucomannan polysaccharide purified from *S. cerevisiae.* It is suspended in isotonic saline without any preservative [4].

*LCMV infection.* The strain WE maintained in our laboratory by serial intracerebral passages in mice was used. For brain suspensions and virus dilutions PBS solution was used. For titration 0.03 ml amounts of LCMV diluted 1:10 were inoculated i.cer. into 6 week-old mice. $LD_{50}$ value of the virus was determined by Reed and Muench's method [31].

*Recovery of LCMV.* Young adult mice were inoculated i. cer. with 1:10 dilutions of brain suspensions prepared from mice surviving LCMV infection and sacrificed on the 21st day of the experiment. Presence of LCMV was confirmed by the tipical neurological symptoms and deaths of mice.

*Examinations of the lymphoid system.* Lymphocyte count was determined from blood taken from the caudal vein under standardized conditions. For determining relative lymphoid organ weight, body weight and after sacrifice spleen and thymus weights were determined and relative organ weight was calculated.

$$\text{Relative organ weigth} = \frac{\text{organ weight (mg)}}{\text{body weight (g)}}$$

*Statistical evaluation.* Student's *t* test was applied. The significance level was $p = 0.05$.

## Experiments and results

The effect of pretreatments with a single or double doses of M on the course of i. cer. LCMV infection was examined in adult Gf and Cv mice (Experiments I and II) and in Cv sucklings (Experiment III). In virus infected groups the development of neurological symptoms (tremor, spasm) characteristic of lymphocytic choriomeningitis were registered. The experiments ended on the 21st day after virus infection.

In the experiments untreated mice (groups C) and mice pretreated with one or two doses of M (groups M and MM, respectively) but not infected with LCM virus were included as well. No deaths were recorded in any experiment among these mice not infected with LCMV.

*Experiment I.* Gf and Cv C3H mice were treated intraperitoneally (i. p.) with 1 ml of M four days or 4 days and 1 day before the LCMV infection. One ml dose of M is equal to 40 mg/kg of zymosan in case of 25 g body weight. On the day after the second M treatment half of the animals in each group of treated and untreated mice were infected with 100 $LD_{50}$ previously titrated LCMV. The other half of the animals were inoculated i. cer. with virus free normal mice brain suspension. In groups infected with LCMV deaths occurred with neurological symptoms characteristic of lymphocytic choriomeningitis. Treatments and number of mice, rate and time of deaths are shown in Table I.

**Table I**

*Number and treatments of Gf and Cv mice infected with 100 $LD_{50}$ of LCMW; ratio and time of deaths in Experiment I*

| Mice | Inoculation | | | Mortality rate % | Time of death |
|---|---|---|---|---|---|
| | i. p. | i. cer. | No. of mice | | |
| Gf | M 1 ml | LCMV | 15 | 80 | 8–10th day |
| | M 2×1 ml | LCMV | 16 | 78 | 8–10th day |
| | untreated | LCMV | 16 | 81 | 8–10th day |
| Cv | M 1 ml | LCMV | 16 | 100 | 7–9th day |
| | M 2×1 ml | LCMV | 16 | 100 | 7–9th day |
| | untreated | LCMV | 16 | 100 | 7–9th day |

In the Gf groups death occurred on the 8–10th day after virus infection and part of animals (20%; 22%; 19%; respectively) survived the infection. In all the three groups of Cv mice all animals died on the 7–9th day after virus infection. In case of both Gf and Cv mice the ratio and time of deaths were similar in M pretreated and untreated animals. Thus, the applied treatments with M did not influence the course of LCM virus infection either in Gf or in Cv mice.

*Experiment II.* Conventional adult CFLP mice were inoculated i. p. with 1 ml of M 4 days or 4 days and 1 day before the LCMV infection. On the day after the treatments, three parallel virus titrations were performed at the same time and under similar circumstances using M pretreated and untreated mice. Titration was carried out by using tenfold dilutions ($10^{-2}$–$10^{-6}$) of LCMV. Ten male and female mice were inoculated with each virus dilution. There were 20

mice in all the three groups that were inoculated with virus free mouse brain suspension. In groups infected with LCMV deaths occurred with neurological symptoms characteristic of lymphocytic choriomeningitis on the 7–10th day after infection. The ratio of deaths observed during the three parallel virus titrations and the $LD_{50}$ values of the LCMV calculated on the basis of death rate are shown in Table II. The $LD_{50}$ value regarding LCMV displays no difference during the three parallel titrations, which means, that the applied pretreatments with M do not influence the course of LCMV infection.

**Table II**

*Rate of death in LCMV infected conventional adult CFLP mice and calculated $LD_{50}$ values in Experiment II*

| LCM-virus dilutions | Number of deaths/Number of infected mice | | |
|---|---|---|---|
| | Mannozym pretreatments i.p. | | Untreated |
| | 1 ml/mouse | 2×1 ml/mouse | |
| $10^{-2}$ | 9/10 | 10/10 | 10/10 |
| $10^{-3}$ | 7/10 | 9/10 | 7/10 |
| $10^{-4}$ | 8/10 | 7/10 | 10/10 |
| $10^{-5}$ | 1/10 | 1/10 | 1/10 |
| $10^{-6}$ | 0/10 | 0/10 | 0/10 |
| LCMV $LD_{50}$ value | $10^{-4,19}$ | $10^{-4,28}$ | $10^{-4,4}$ |

*Experiment III.* CFLP Cv 2-week-old suckling mice were treated i. p. with 0.2 ml of M 4 days or 4 days and 1 day before the LCMV infection. In case of suckling mice with 5 g body weight, 0.2 ml of M is equal to 40 mg/kg zymosan. Twentyfour litters of suckling mice were included in this experiment. Half of the mice were treated i. p. with a single 0.2 ml dose and 2×0.2 ml dose of M in each of 12 litters. On the day after treatments, in each of 6 litters mice that had been pretreated with different doses of M were infected i.cer. with 100 $LD_{50}$ of previously titrated LCMV and mice in each of the remaining 6 litters were inoculated in a similar way with virus-free mouse brain suspension. Mouse groups, number of mice in each group and treatments are shown in Table III. Rate and time of death in the virus infected groups are shown in Fig. 1.

In groups infected with LCMV death occurred with neurological symptoms characteristic of lymphocytic choriomeningitis. Rate and time of deaths were different in the three virus-infected groups. Mice not treated with M died in 25% (LCM group) on the 9–10th day, while mice pretreated with a single dose of M died in 56% (M–LCM group), those treated with two doses of M died in 66% (MM–LCM group). In groups M–LCM and MM–LCM deaths started on the

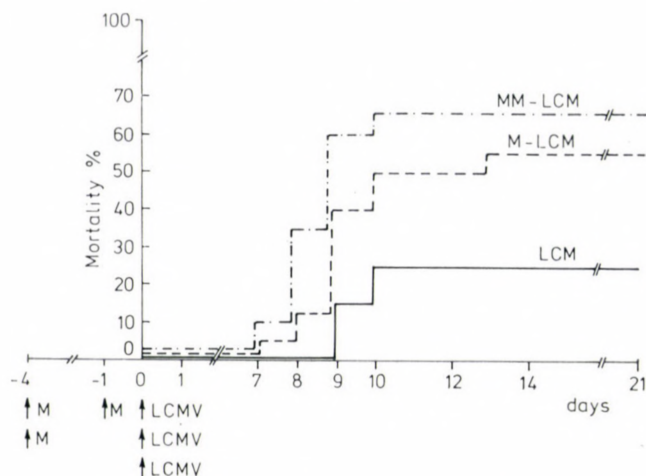


*Fig. 1.* Rate and time of deaths after LCMV infection in suckling mice

7th day after inoculation. Thus in groups pretreated with M deaths occurred in larger proportion and earlier than in virus infected but not pretreated mice. The results show that pretreatment with M enhanced the development of fatal lymphocytic choriomeningitis in suckling mice.

*Recovery of LCMV.* LCMV could be isolated from the brain of mice surviving the virus infection in each experiment thus these animals were symptom-free virus-carriers.

**Table III**

*Conventional suckling mouse groups and their treatments in Experiment III*

| Groups | Inoculation | | No. of mice |
|---|---|---|---|
| | i.p. | i.cer. | |
| M — LCM | M 0.2 ml | LCMV | 30 |
| MM — LCM | M 2×0.2 ml | LCMV | 30 |
| LCM | untreated | LCMV | 60 |
| M | M 0.2 ml | X | 30 |
| MM | M 2×0.2 ml | X | 30 |
| C | untreated | X | 60 |

X Virus-free mouse brain suspension

*Examination of the lymphoid system* was carried out in each experiment on the 7–11th days after M treatment. Lymphocyte count and relative spleen and thymus weight was determined in half of the animals in groups of untreated

mice (groups C) and in groups treated with one dose of M (groups M) or two doses of M (groups MM) but not infected with LCMV.

Peripheral blood lymphocyte counts and relative thymus weights of M treated mice did not differ from the values of untreated controls in any experiments. Relative spleen weights of mice are shown in Fig. 2.

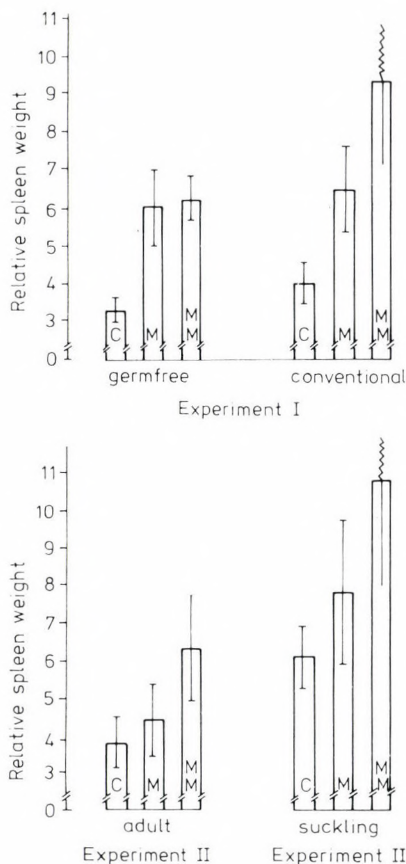


*Fig. 2.* Average of relative spleen weights on 7–11th days after treatments

In Experiment I the mean relative spleen weight was significantly higher than those of untreated controls already after a single dose of M, while it was significantly higher only after the double treatment (group MM) in Experiments II and III.

The results show that in mice of M and MM groups spleen hypertrophy occurred on the 7–11th days after the M treatment, at the same time when the mice in virus-infected groups died.

## Discussion

*In Experiment I* death followed by infection with 100 $LD_{50}$ of LCMV occurred in the same ratio and time in mice treated or not treated with M both in Gf and Cv groups. The result that deaths in the Gf groups ensued later and in smaller proportion than in Cv groups, is in accordance with our earlier results [17] and it can be explained by the undeveloped immune system of Gf mice due to antigen-deficient environment.

*In Experiment II* there was no valuable difference between the $LD_{50}$ values determined in the three parallel virus titrations using M pretreated and untreated adult Cv animals.

The results of Experiments I and II show that pretreatments with M had no effect on the course of i.cer. LCMV infection. It means that the applied doses of M did not influence the cellular immune response to LCMV either in adult Gf mice with undeveloped or in Cv mice with developed immune systems.

*In Experiment III* suckling mice pretreated with M died earlier and in larger proportion than virus infected but not pretreated mice and mortality rate was higher in the double dose treated mice than in the single dose treated ones. Thus M enhanced the development of fatal lymphocytic choriomeningitis i.e. contributed the cellular immune response to LCMV. This stimulating effect of M manifested in higher degree in the double dose than in the single dose treated mice, thus it was dose-dependent.

The 2-week-old M pretreated mice in this experiment reacted similarly as the 3-week old untreated animals [25, 26], thus the M accelerated the development of cell-mediated immunological capacity in suckling mice.

*Spleen hypertrophy* was caused by M pretreatment both in adult (Gf and Cv) and Cv suckling mice, but the stimulating effect of M on the specific cellular immune response manifested simultaneously only in Cv sucklings. This experience is not contrary to our earlier results attained in similar system with pretreatment with different microbial immunomodulants. Previously it was found that the effect of treatments causing spleen hypertrophy and their immunomodulatory effect were not related to each other. Cellular immune response of adult mice was stimulated by irradiated endotoxin preparation [29] and suppressed by *Bordetella pertussis* vaccine [20, 23] with spleen hypertrophy, while it was stimulated by *Brucella* suspension without spleen hypertrophy [28]. Regarding to our earlier experiences it was also concluded that the direction of immunomodulatory effect of the same treatment can be different depending on the age of the experimental animals. Our earlier results which allowed us such conclusions and our present results are compared in Table IV.

The cellular immune response of Cv suckling mice with undeveloped immune system due to age was stimulated alike by *B. pertussis* vaccine, parent

**Table IV**

*The effect of microbial immunomodulants on the cellular immune response to LCMV*

| Mice | Immune system | | Immunmodulatory effect | | | |
|---|---|---|---|---|---|---|
| | State | Function | *B. pertussis* vaccine | Endotoxin preparations | NDV vaccine | Mannozym |
| Conventional suckling | undeveloped | insufficient | stimulation [25] | stimulation [26] | stimulation [32] | stimulation |
| Conventional adult | developed | normal | suppression (delaying) [20, 23] | stimulation [29] | no effect [35] | no effect |
| Germfree adult | undeveloped | insufficient | suppression (inhibition) [23] | stimulation [29] | no effect [33] | no effect |

or radiodetoxified endotoxin preparations, Newcastle Disease Virus (NDV) vaccine, and Mannozym, thus by treatments with immunomodulants of bacterial, virus and yeast origin. The cellular immune response of adult mice, both in Cv mice with developed and Gf mice with undeveloped immune system due to antigen deficient environment was stimulated only by endotoxin preparation, it was suppressed by *B. pertussis* vaccine (but not in the same degree), and it was not influenced by NDV vaccine or Mannozym.

The present results reinforced our earlier observations that the direction of immunomodulatory effect can be influenced by age and are in accordance with results according to which M enhances the specific immune response [13]. It can be concluded from Mannozym's effect of stimulating cellular immune response in suckling mice that M may be used for accelerating the development of cell mediated immunological capacity in organisms with undeveloped immune system.

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# EFFECT OF MICROBIAL IMMUNOMODULANTS ON THE COURSE OF LCMV INFECTION IN OLD MICE WITH THYMUS INVOLUTION

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(Received January 17, 1991)

Old mice with thymus involution were treated intraperitoneally with a live vaccine containing a mesogenic strain of attenuated Newcastle Disease Virus or with Mannozym (M, 1% zymosan suspension). One day after the treatments mice were infected with lymphocytic choriomeningitis virus (LCMV) intracerebrally. The fatal course of the consequent LCMV infection was stimulated by each of the pretreatments, indicating that the cellular immune response was stimulated. The results are compared with results of experiments carried out on suckling, young adult and old mice in similar experimental systems. The authors' previous publication suggesting that the direction and degree of the immunomodulant effect may be influenced by the actual age-dependent condition of the lymphoid system, have been confirmed.

The neurological symptoms of intracerebral (i.cer.) lymphocytic choriomeningitis virus (LCMV) infections are based on the animal's cellular immune response capacity. T lymphocytes play the basic role in the fatal illness [1–3]. If the immune system is intact, the disease will be fatal in 6 to 9 days in 100%, whereas neonatal mice, having immature immune system do not develop meningitis, thus, survive as persistent virus carriers [4].

In previous studies [5], we found low cellular immune-response capacity to LCMV in mice with physiological thymus involution. Only few of these old animals developed the symptoms and histological signs characteristic of LCM. The remaining survived as asymptomatic virus carriers [6].

In further experiments, we succeeded in stimulating — by pretreatment with an endotoxin (LPS) preparation — the cellular immune response to LCMV of old mice with thymus involution. In a similar experimental system pretreatment of suckling mice with endotoxin [7], NDV vaccine or Mannozym also proved to have an immunostimulant effect [8, 9]. The above observations prompted us to study whether the two latter microbial immunomodulants studied by us influenced the course of LCM in old mice having physiological thymus involution.

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## Materials and methods

*Experimental animals.* CFLP and C3H mice of both sexes and various ages (LATI, Gödöllő, Hungary) were used.

*NDV vaccine.* The vaccine (Phylaxia, Budapest) contained live attenuated Newcastle Disease Virus (NDV), a mesogenic strain (Strain H) grown in embryonated hen's eggs.

*Mannozym preparation.* (Institute of Serobacterial Production and Research Human, Budapest). Composition: 1 mg of *Saccharomyces cerevisiae* polysaccharide per ml, suspended in saline (0.1% zymosan suspension), without preservative. The water-insoluble active substance (glucomannan) is built up mainly of glucose and mannose molecules [10].

*LCMV infection.* The WE virus strain was maintained in this Institute in serial mouse-brain passages and pretitrated i.cer. in 6-week-old mice. The i.cer. inoculum was 100 $LD_{50}$. Control mice were given brain suspension from noninfected mice, i.cer. The inoculated mice were examined daily twice for neurological symptoms (tremor, convulsions) characteristic of LCM; deaths were registered.

*Re-isolation of LCMV.* Groups of 6-week-old mice were inoculated i.cer. with individual brain suspensions (1 : 10 dilution) prepared from mice that had survived the observation period. Re-isolation was considered positive if the inoculated animals had died with symptoms characteristic of LCM.

*Examination of the lymphatic system.* The relative spleen and thymus weights and the spleen and thymus indices of mice were determined as follows:

$$\text{Relative lymphoid organ weight} = \frac{\text{lymphoid organ weight (mg)}}{\text{body weight (g)}}$$

$$\text{Lymph organ index} = \frac{\text{average of relative lymph organ weight in the experimental group}}{\text{average of relative lymph organ weight in the control group}}$$

*Statistical evaluation.* Student's two-sample $t$ test; significance: $P < 0.05$.

## Experiments and results

Two experiments were performed.

In *Experiment I* CFLP mice, 18 to 20 months of age, were treated with 0.1 ml of NDV vaccine or PBS, i.p. One half of the pretreated mice were inoculated with LCMV i.cer., the other half (controls) with a suspension prepared from noninfected mouse brains, also i.cer. The experimental groups, the numbers of mice and the treatments are shown in Table I.

**Table I**

*Experiment I*

| Mouse groups | Route of inoculation | | |
|---|---|---|---|
| | i.p. | i.cer. | n* |
| NDV–LCM | NDV vaccine | LCM virus | 20 |
| LCM | PBS | LCM virus | 20 |
| NDV | NDV vaccine | X | 20 |
| Control | PBS | X | 20 |

X = Normal brain suspension
* No. of mice/group

In the groups infected with LCMV all deaths were preceded by neurological symptoms characteristic of LCM. The cumulative mortalities are shown in Fig. 1.

In the LCM group the first mice died on day 9, mortality reached 70%; in the NDV–LCM group, on the other hand, deaths already began on day 6 and mortality reached 90%.

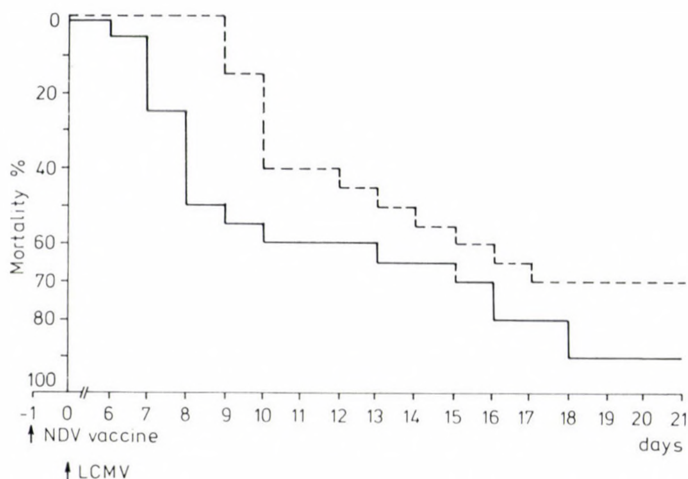


*Fig. 1.* Cumulative mortality per cent in Experiment I. ——— NDV–LCM; – – – – LCM

In *Experiment II*, C3H mice 12 to 18 months of age were treated twice with 1 ml of M or PBS 4 days and 1 day before LCMV infection. One ml zymosan dose corresponded to 40 mg/kg body weight as calculated for 25 g average body weight. Half of the pretreated mice were infected with LCMV, the others (control group) received normal brain suspension. The injections were given i.cer. The groups of mice, the numbers of mice per group and the treatments applied are shown in Table II. All the mice that died in the LCMV-inoculated groups had shown the symptoms characteristic of LCM. Cumulative mortality data are shown in Fig. 2.

The first deaths occurred in both groups on the 8th day postinoculation. In the LCM group 90%, in the M–LCM group 100% of the animals had died by the 14th day. The daily mortality for the latter group exceeded that calculated for the LCM group by 10% or more. There were no deaths in the nonifected groups during the 21-day observation period.

The virus inoculum was titrated in 6-week-old mice in both experiments. All the young mice infected with 100 $LD_{50}$ of LCMV died between post-infection days 6 and 9, showing the symptoms of LCM.

**Table II**

*Experiment II*

| Mouse groups | Route of inoculation | | |
|---|---|---|---|
| | i.p. | i.cer. | n* |
| M-LCM | Mannozym | LCM virus | 20 |
| LCM | PBS | LCM virus | 20 |
| M | Mannozym | X | 20 |
| Control | PBS | X | 20 |

X = Normal brain suspension
* No. of mice/group

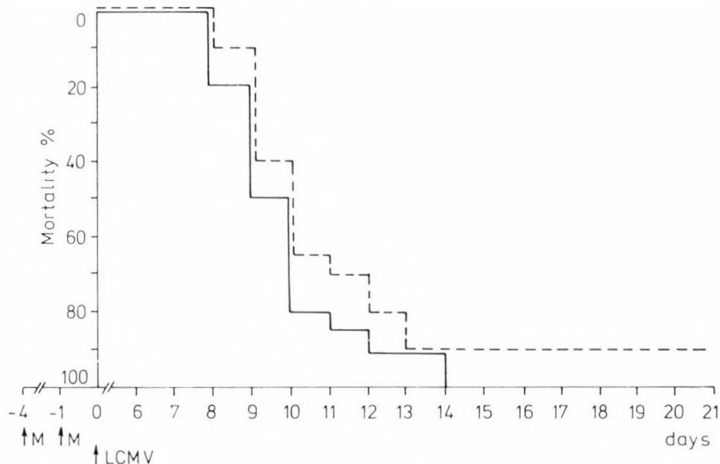


*Fig. 2.* Cumulative mortality per cent in Experiment II. ——— M–LCM; – – – – LCM

*Re-isolation of LCMV.* We succeeded in re-isolating LCMV from the brain of each of the mice that had been killed at the end of the observation period, indicating that the survivors had been asymptomatic virus carriers.

*Examination of the lymphatic system.* On the 9th day after infection half of the mice in groups NDV and M and in the control groups were sacrificed. The relative thymus and spleen weights and indices were calculated. The relative weights in the pretreated groups did not differ from those of the control significantly. The same was indicated by the thymus and spleen indices, which were $< 1.3$.

To characterize the degree of thymus involution in old mice, we determined the relative thymus weight of ten 6-week-old young C3H mice and the same number of CFLP mice of the same age. Related to the average values obtained, the thymus indices were calculated. For the C3H mice 12 to 18 and the CFLP mice 18 to 20 months of age, the thymus index was 0.35 and 0.21, respectively.

It can be concluded that there existed a considerable thymus involution in the old mice applied by us; in accordance with the respective mice, the involution was more pronounced in the CFLP mice than in the C3H ones.

## Discussion

The old mice with striking thymus involution used in the present experiments had decreased cellular immune-response capacity. In contrast with the 100% mortality reached by the young mice, six to nine days after i.cer. LCMV infection, the old mice died later and at a higher rate. Senescent mice developed fatal LCM in both experiments the slower the more pronounced the thymus involution was. In C3H mice 12 to 18 months of age (thymus index, 0.35) mortality was 90% between days 8 and 13 (Fig. 2), whereas in CFLP mice 18 to 20 months of age (thymus index, 0.21) it was 70% between days 9 and 16 (Fig. 1). These results are in accordance with our previous experiments [5, 11].

Literary data have shown that the fatal course of the illness following i.cer. LCMV infection is supported by effects stimulating cellular immune response, whereas LCM fatality is hindered by immunosuppressing effects [6, 7, 12–20]. Accordingly, effects stimulating cellular immune response increase whereas suppressing effects decrease the death rate accompanying infections induced by a constant inoculum from LCMV.

In the present experiments, we examined the effect of pretreatment with two microbial immunomodulant preparations of Hungarian origin (NDV vaccine and M) on the course of i.cer. LCMV infection in old mice.

On the effect of pretreatment with NDV vaccine (Experiment I, Fig. 1) the old mice died sooner and at a higher rate than those having not been pretreated with the vaccine. The postinfection mortality of the former began on the 6th day, which is the time characteristic of the death of young LCMV-infected mice. Their daily mortality was higher by 20% than that of the old mice not pretreated with NDV vaccine.

In the M-pretreated group (M–LCM) of Experiment II (Fig. 2) the time of the death was about the same as of those which had not received M treatment (LCM group), the daily mortality, on the other hand, was by 10% higher, and 100% of the mice died, in contrast with the 90% death rate observed in the LCM group.

There is no doubt that pretreatment with NDV vaccine or M stimulates the fatal course of i.cer. LCMV infection, i.e. the cellular immune response capacity of the old mice is increased in this way. The stimulation appeared to be more intense in the CFLP mice, which were older and showed more pronounced thymus involution than the younger C3H mice.

In previous experiments, we examind the immunomodulant effect of two bacterial preparations in a similar experimental system, namely, mice of various ages of life. The results of our present and previous experiments are comparatively presented in Table III.

**Table III**

*Cellular-immune-response influencing effect of microbial immunomodulants on LCMV infection in mice of various ages*

| Age of the mouse | Immune system | | Immunomodulant effect | | | |
|---|---|---|---|---|---|---|
| | Condition | Function | *B.pertussis* vaccine | Endotoxin preparations | NDV vaccine | Mannozym |
| Suckling | immature | insufficient | stimulation [16] | stimulation [7] | stimulation [8] | stimulation [9] |
| Adult | mature | normal | suppression [13, 18] | stimulation [20] | no influence [21] | no influence [9] |
| Old | regressed | insufficient | no influence [11] | stimulation [6] | stimulation | stimulation |

Our results make it clear that in suckling mice with physiologically immature immune system the cellular immune response was stimulated by all the microbial preparations examined by us and used also in the practice. Among them, *Bordetella pertussis* vaccine and the radiodetoxified endotoxin (rdLPS) were bacterial preparations whereas NDV vaccine contained live attenuated virus and Mannozym was of fungal origin. In adult mice of mature immune system only the rdLPS showed a stimulating effect; *B. pertussis* suppressed, whereas NDV vaccine and M did not influence the cellular immune response. In old mice with regressing immune system and insufficient immune function, *B. pertussis* did not influence, whereas the NDV vaccine and M — both examined in the present study — similarly to rdLPS, did stimulate cellular ime munresponse.

The present results agree with our previous publications suggesting that the direction and degree of immunomodulant effects may be influenced by the actual condition of the immune system, which, on the other hand, is age-dependent [21]. One should reckon with this experience during clinical administration of the corresponding preparations.

The preparations examined in our present experiments are being widely used in Hungary, viz. the NDV vaccine for preventive immunization in the veterinary practice, Mannozym in the human therapy to stimulate nonspecific defensive capacity of the organism and to protect the organism against harmful side-effects of radiotherapy. In the course of their administration their

effect stimulating cellular immunity may also be active and may widen the practical value of the preparations.

The beneficient effect of NDV infection in tumourous patients [22] and of NDV vaccination in some virus infections [23] may be in connection with the immune response stimulating capacity of the NDV vaccine. The results with Mannozym are in accordance with the experiences indicating the specific defence capacity of the organism is increased by administering of Mannozym [4]. Our experiences suggest that the preparations under study may be suitable for stimulation of the weak immune function of old patients.

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# MORPHOLOGICAL CHANGES OF *NEISSERIA GONORRHOEAE* IN AMNIOTIC FLUID OF PREGNANT WOMEN IN THEIR MIDTRIMESTER

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Ten selected samples of amniotic fluid obtained through transabdominal amniocentesis from 10 pregnant women in their 17th–19th weeks of pregnancy were investigated for the survival of *Neisseria gonorrhoeae* in amniotic fluid. In 8 cases an antibacterial effect was observed with morphological changes comparable to the effect of benzylpenicillin. When *N. gonorrhoeae* was inoculated in amniotic fluid at $10^5$–$10^7$ cells/ml, it survived as an average 2 h longer than after inoculation of $10^2$–$10^4$ cells/ml. Electron microscopic pictures of gonococci taken after 2, 6 and 24 h incubation in amniotic fluid correlated with the growth curves. Electron microscopically there were marked morphologic changes of *N. gonorrhoeae*, viz. vacuolar degeneration of their cytoplasm with a damage to the bacterial wall up to its complete destruction and lysis of the cell.

Pregnancy does not protect the women against gonorrhoea. However, the neurohumoral and the somatic changes during pregnancy act to some extent as a barrier against the penetration of bacteria, including gonococci, into the upper sections of genital organs of pregnant woman.

Protection against infection during the period of gestation is a joint action of several defense mechanisms, as e.g. lysozyme and IgA in cervical mucus, chorioamniotic membranes, placental barrier, transplacental IgG antibodies, and the gradually developing self immune system of the fetus [1, 2].

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An integral part of these mechanisms is also the amniotic fluid containing a complex of components with antibacterial effect: zinc and peptides, immunoglobulins, lysozyme, complement, peroxidases, alpha-1-antitrypsin, transferrin etc. [3–7]. However, the humoral changes taking place in the organism during pregnancy cannot be neglected, either.

The etiopathogenesis of penetration of gonococci into the upper part of genital organs of the pregnant woman, and thus also the elucidation of the origin of some complications, were studied by several authors. Galask et al. [8] and Swanson [9], had studied the adherence of *Neisseria gonorrhoeae* to amniotic cells, while Morse and Fitzerald [10] had described an antibacterial effect of progesterone.

Since the current literature brings only few data on antibacterial effect of amniotic fluid upon *N. gonorrhoeae* we attempted to contribute to the elucidation of etiopathogenesis of gonorrhoeic infection in pregnant women.

## Patients and methods

*Samples.* The effect of amniotic fluid on the growth of *N.gonorrhoeae* was studied on 10 selected samples of amniotic fluid obtained through transabdominal amniocentesis from 10 pregnant women between the 17th–19th week of their pregnancy. In all cases under the study of amniotic fluid was indicated by a geneticist and a gynaecologist in the frame of prenatal genetic counselling. No fetal malformation was detected in any of the examined women and later all of them delivered in term healthy, eutrophic babies.

The samples of amniotic fluid were neither centrifuged nor filtrated in order to avoid removal of the natural components. They did not contain any blood or meconium. Their sterility was tested by inoculation on solid Endo medium and blood agar (base No.4, Imuna, Šarišské Michal'any, Czechoslovakia). The samples had been frozen to —30 °C within 30 min from their taking, and stored at this temperature for maximum 7 days.

As a control was used the liquid culture medium G77L. G77L was prepared exactly according to the formula of MacFarlane and Elias-Jones [11].

*Technique of transabdominal amniocentesis.* An essential attribute of every transabdominal amniocentesis is a precise sonographic examination carried out before the procedure itself. The pregnant women must void the urine spontaneously before the procedure. The 3.0 MHz sound of ultrasound apparatus (MEDATA AB, MK 600 HQ, ATL, USA) was fixed to the abdominal skin and the surrounding area was disinfected. The sound together with the physician's hand were covered by a sterile textile cover with an opening for amniocentesis. The penetration of punction needle (disposable 0.9 mm x 90 mm needle, WYGON, France) to the abdominal cavity was continuously monitored on the display of sonographic apparatus. The place suitable for punction — a "lake" of amniotic fluid free of any umbilical cord loop — was selected sonographically. After the puncture of amniotic cavity the mandrin was removed and the punction needle was connected to a disposable syringe by a sterile connective tube (type HS 3.6×150 RR, Logarex 66201 or HS 1.8×450 RR, Logarex 66203 Koh-i-noor Hardtmuth), thus eliminating the transfer of movements of the physician's hand to the punction needle. Then the amniotic fluid was aspirated.

*Preparation of inoculum of N.gonorrhoeae.* Ten different strains of *N.gonorrhoeae* obtained from the first culture taken from 10 women with uncomplicated gonorrhoeic cervicitis were used. The culture of *N.gonorrhoeae* was made on the modified blood agar (base No.3, Imuna Šarišské Michal'any, Czechoslovakia) with 5 μg/ml lincomycin hydrochloride (Medexport) and 20 μg/ml colimycin (Laboratoire Roger Bellon) at 36 °C and 10% $CO_2$. All strains were tested for susceptibility to antibiotics by the plate dilution method [12] and were screened for production of penicillinase by a rapid iodometric test [13]. In this study were used strains of *N.gonorrhoeae* MT7560/87, MT7776/87, ZA7956/87, MT8004/87, MT8281/87, MT8452/87, MT8603/87, ZA8617/87, MT8824/87, MT9824/87 with MICs penicillin 0.016–0.125 μg/ml,

tetracycline 0.032–0.25 μg/ml, chloramphenicol 0.125–0.25 μg/ml and erythromycin 0.016–0.125 μg/ml. None of them produced penicillinase. As a control was used the strain *N. gonorrhoeae* MAU 76/71 non-penicillinase producing (Czechoslovak State Collection).

For the experiment an 18-h culture (modified blood agar base No. 3) of *N. gonorrhoeae* was used. Gonococci were taken from Petri dishes with a sterile bacteriological loop, suspended in 1 ml amniotic fluid and in 1 ml liquid culture medium G77L serving as a control and immediately poured into 9 ml amniotic fluid and into 9 ml G77L.

*The follow-up of survival of N. gonorrhoeae in amniotic fluid.* From each woman 20 ml of amniotic fluid was taken through transabdominal amniocentesis. Frozen samples were let to melt at room temperature, then adjusted to 36 °C in thermostat. The 20 ml samples were then divided into 2 test tubes by 10 ml each, and to both gonococci were inoculated at $10^2$–$10^4$ c.f.u./ml (first series) and $10^5$–$10^7$ c.f.u./ml (second series).

After homogenizing by shaking the inoculated samples were further divided into 2 equal aliquots. One test-tube from each series served for the follow-up of the growth curves of *N. gonorrhoeae* in amniotic fluid and the other one for the study of the morphological changes of *N. gonorrhoeae* observed under electron microscope during the survival of the bacteria in amniotic fluid.

For constructing the growth curves, the tubes were incubated at 36 °C. Samples were taken at 0, 2, 4, 6, 8, 10 and 24 h. After a thorough shaking of each test-tube, 0.5 ml sample was transferred to 4.5 ml phosphate buffered saline, shaken, and further diluted from $10^{-1}$ to $10^{-7}$. Aliquots of 0.1 ml were poured over the surface of modified blood agar No. 3, were incubated at 36 °C and 10% $CO_2$ for 48 h, then colonies of *N. gonorrhoeae* were counted. The results were recorded graphically.

Suspensions of 1 ml G77L with *N. gonorrhoeae* were also inoculated in 9 ml G77L. Growth curves were constructed using the method described above.

*Electron microscopy of gonococci in amniotic fluid.* The content of each test-tube of the second series, after thorough shaking, was divided into 3 test tubes, so that two tubes contained 1.6 ml, and the third one 1.8 ml of amniotic fluid inoculated with *N. gonorrhoeae*. All the test-tubes were then incubated at 36 °C. Electron microscopic examination was performed from the first tube after 2 h, from the second one after 6 h and from the third one after 24 h.

Samples taken from 5 pregnant women were allowed for spontaneous sedimentation during incubation and then the supernatant (the amniotic fluid over the sediment) was carefully removed. Samples from another 5 women were elaborated in the same way, only the spontaneous sedimentation was replaced by centrifugation at 2000 g for 10 min, at the same time intervals.

The sediment obtained was fixed with 0.258 $mol^{-1}$ glutaraldehyde with Sörensen phosphate buffer (pH 7.2–7.3) with the subsequent osmification by osmium tetroxide ($OsO_4$) as described by Millonig [14]. For dehydration, increasing concentrations of acetone were used (50, 70, 90 snd 100%). The material was then embedded in Durcupan ACM (Fluka, Switzerland). Polymerization of the material took place at 60 °C for 72 h. Sections of pyramides and specimens were made by means of ultratome LKB V., Sweden. For taking specimens the net Tesla Brno, Czechoslovakia was used. Contrasting was made by plumbic citrate and octane uranyl. The preparations were visualized by means of the transmissive electron microscope Tesla BS 500 (Tesla Brno, Czechoslovakia).

## Results

The survival of *N. gonorrhoeae* is depicted in Fig. 1. Amniotic fluid had an inhibitory effect on *N. gonorrhoeae* in 8 out of 10 samples. In 2 samples taken from the 18th and 19th week of gravidity, *N. gonorrhoeae* multiplied still after 24 h incubation. From Fig. 1 it follows that *N. gonorrhoeae* in concentrations $10^5$–$10^7$ survived in amniotic fluid averagely by 2 h longer than in concentrations $10^2$–$10^4$.

*N. gonorrhoeae* multiplied in liquid culture medium G77L in 7 cases. In 2 cases a considerable decrease in the c.f.u. was observed, but after 24 h it was still $2.1 \times 10^3$ or $1 \times 10^2$ c.f.u./ml of medium (Fig. 2).

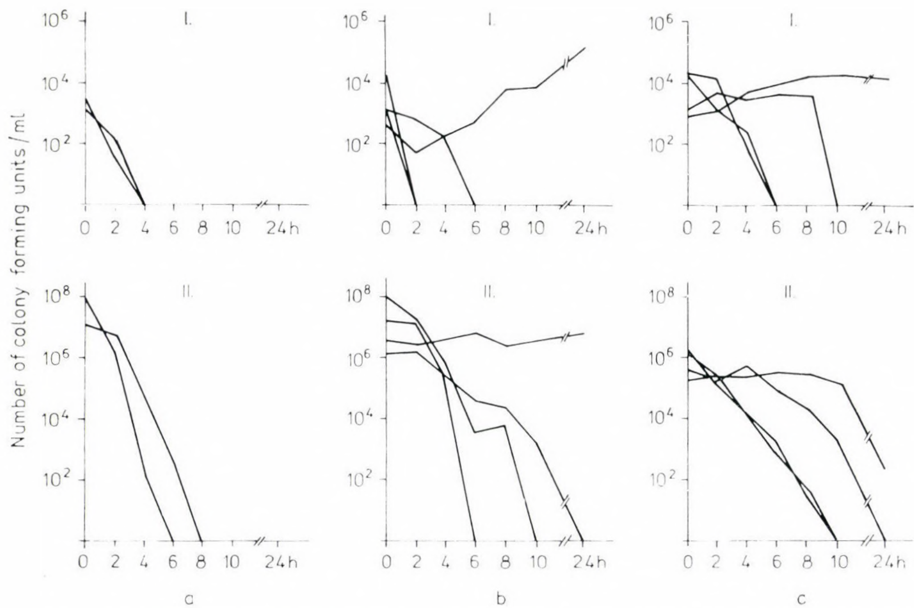


*Fig. 1.* Growth curves of *N. gonorrhoeae* in amniotic fluid; a — 17th, b — 18th, and c — 19th week of pregnancy

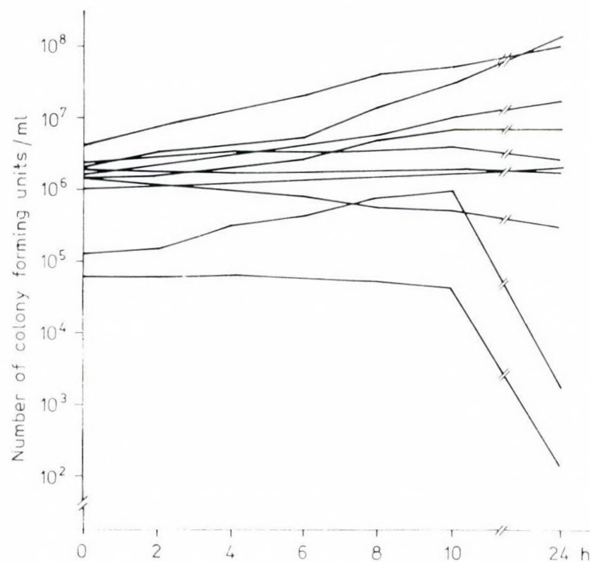


*Fig. 2.* Growth curves of *N. gonorrhoeae* in liquid culture medium G77L

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*Fig. 4.* Electron micrographs of *N. gonorrhoeae* in amniotic fluid, 2 h. A few marked morphologic changes can be observed in sense of vacuolar degeneration of cytoplasm to the destruction of bacterial wall following to initial lysis of *N. gonorrhoeae*. Few gonococci with the formation of vesicles. ×18 000

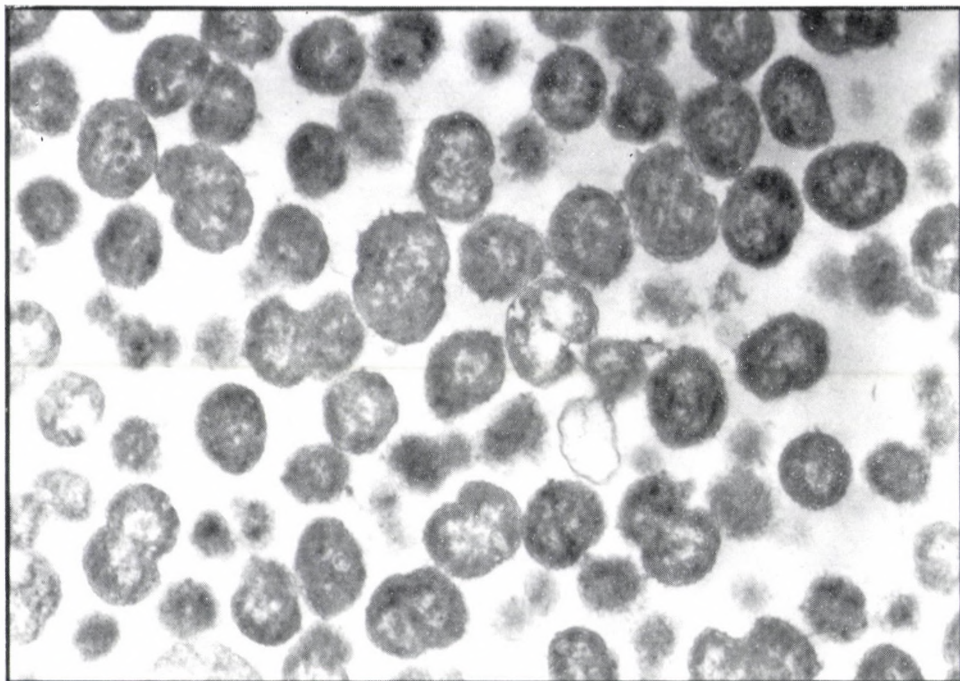
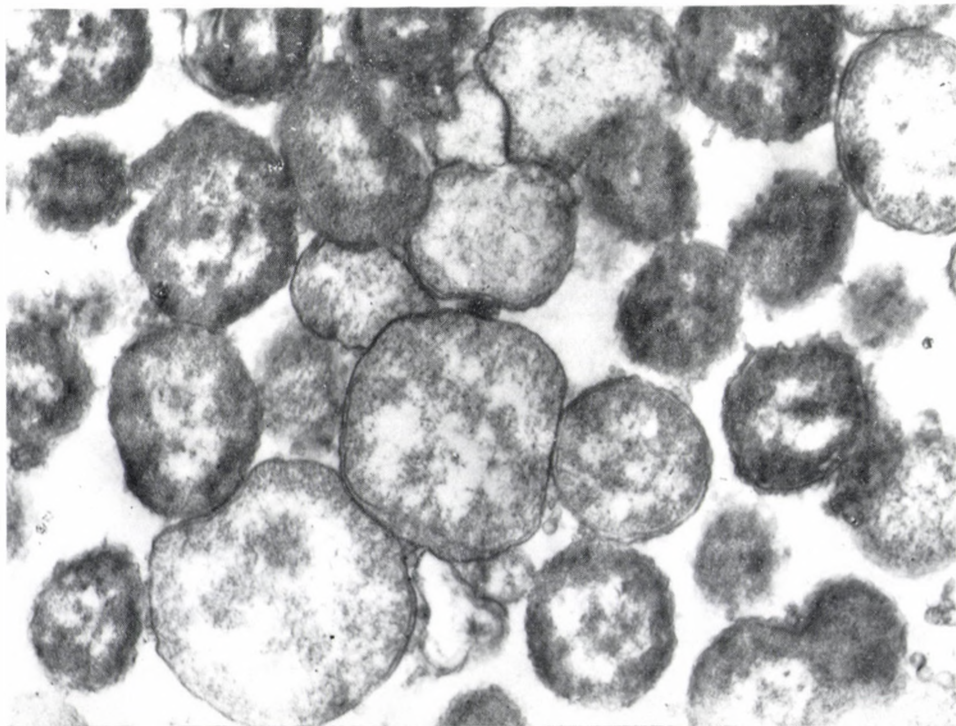


*Fig. 3.* Electron micrographs of *N. gonorrhoeae* in amniotic fluid, 2 h. Most gonococci show typical morphologic picture. A well visible bacterial wall containing two parts — outer membrane and dense lamina — encircles the cytoplasmic membrane. Gonococci adhere mutually in zones of adherence. However, in few places the starting vacuolar degeneration of cytoplasm can be observed, with elucidation of mainly central parts of *N. gonorrhoeae*. ×10 000



All the electron microscopic pictures represent amniotic fluid inoculated with gonococci at higher concentrations, viz. $10^5$–$10^7$/ml, since in these samples we could observe morphologic changes on a larger amount of gonococci. Therefore, these samples were more significant.

Electron microscopic pictures of gonococci were examined after 2 h incubation (Figs 3, 4), after 6 h incubation (Figs 5, 6), and after 24 h incubation (Figs 7, 8).

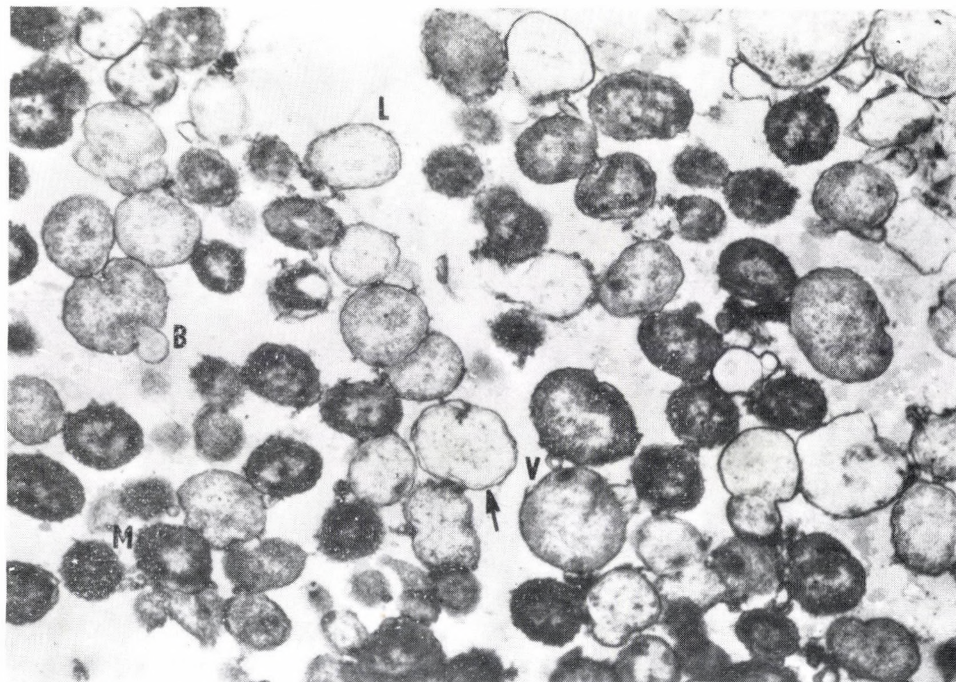



*Fig. 5.* Electron micrographs of *N. gonorrhoeae* in amniotic fluid, 6 h. Complete desintegration of the loci of adherence of gonococci. The coffee beans typical for *N. gonorrhoeae* remain only slightly visible. Marked morphologic changes of bacterial wall — the outer membrane and lamina densa separated from cytoplasmic membrane (arrow). The lamina densa in some places completely disappears as well as the cytoplasmic membrane and only the outer membrane remains preserved. Some of the cells have mesosomes (M). Almost everywhere also the inner structure of *N. gonorrhoeae* is destroyed. Intracellular oedema occurs showing elucidation of the inside of bacterial cell and enlargement of the whole volume. Part of its intracellular structure remains preserved in a narrow margin encircling cytoplasmic membrane; if it remains preserved on the periphery of gonococcus around the outer membrane. The smaller vesicles (V) may have originated from amniotic fluid-induced blebs (B). The above changes in some places result in rupture of bacterial wall and lysis of the cell (L). ×8000

A similar electron microscopic picture as in Figs 7 and 8 were observed also in some samples of amniotic fluid taken at 6 h. However, in some samples of amniotic fluid after 24 h we did not observe any fragments of bacterial wall of gonococci. In such cases no remains of *N. gonorrhoeae* could be observed.

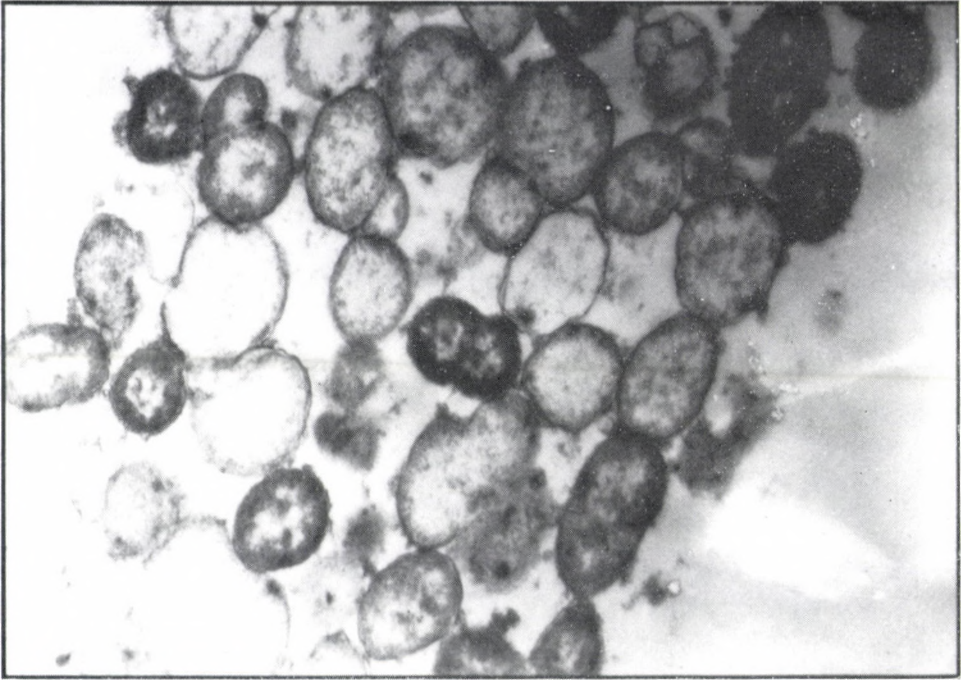


*Fig. 6.* Electron micrographs of *N. gonorrhoeae* in amniotic fluid, 6 h. Marked morphologic changes of intracellular structure and bacterial wall. ×10 000

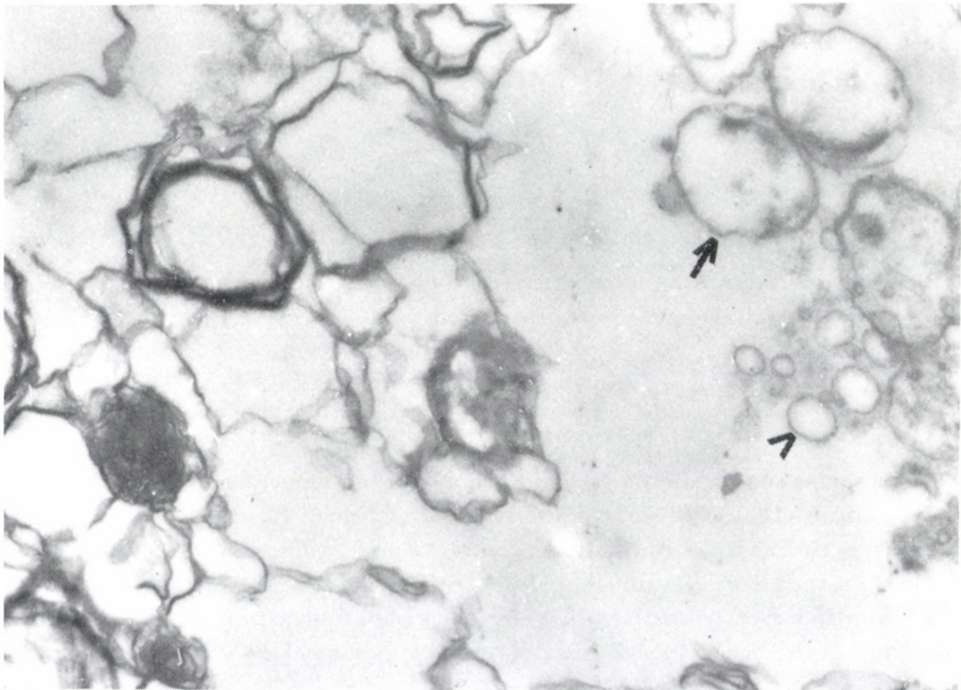


*Fig. 7.* Electron micrographs of *N. gonorrhoeae* in amniotic fluid, 24 h. A focus of preserved gonococci, even if only their contour is visible consisting of the outer membrane complex. The vesicles are induced from amniotic fluid (V). After complete destruction, the fragments of bacterial walls can be seen (arrow). ×10 000

Similar morphologic changes were observed also on gonococci inoculated into the amniotic fluid at $10^2$–$10^4$ *N. gonorrhoeae*/ml. Compared to the higher concentrations the difference was only that more gonococci were affected after 2 h. After 6 h we observed in these cases mostly an expressive destruction of bacterial walls of *N. gonorrhoeae* comparable to that shown in Fig. 8. After

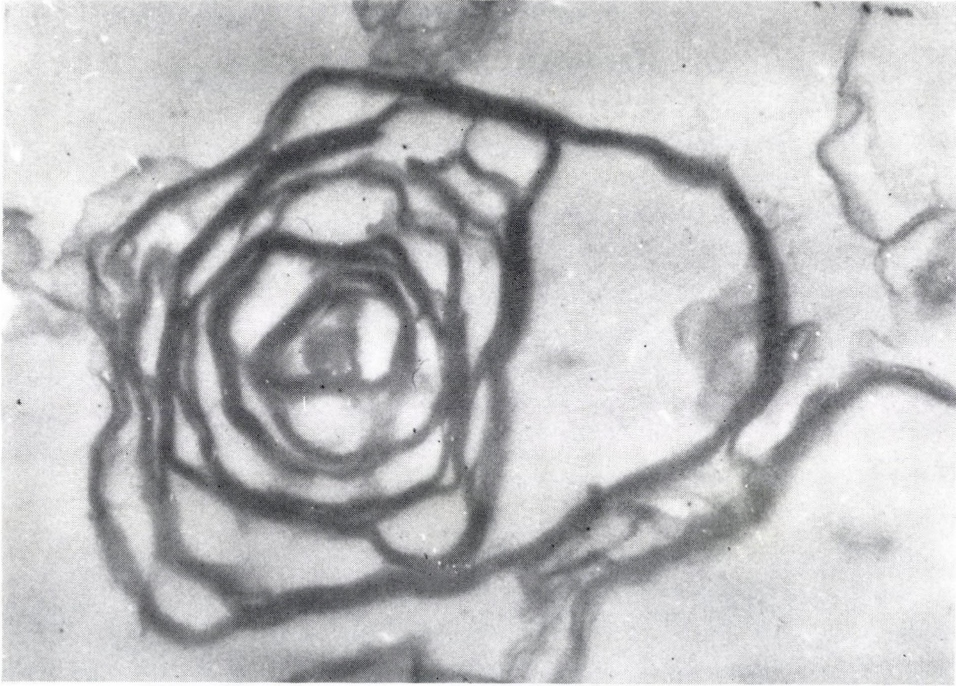


*Fig. 8.* Electron micrographs of *N. gonorrhoeae* in amniotic fluid, 24 h. Spiral structures due to centrifugation of the fragments of bacterial wall. ×30 000

24 h we usually did not observe any fragments of bacterial walls. These observations were confirmed also on growth curves of *N. gonorrhoeae* in amniotic fluid (Fig. 1).

## Discussion

For the study, amniotic fluid from the midtrimester was used, taken always after the first trimester of pregnancy, since transabdominal amniocentesis performed before this term bears a considerable risk of complications [15–18] and it should be considered contraindicated.

Another reason for the follow-up of these problems in midtrimester was the study of the occurrence of gonorrhoea in pregnancy [19], from which follows

that under our conditions the detection of this sexually transmitted disease is considerably higher in midtrimester than in the third trimester.

According to Lowe and Kraus [20] the concentration of gonococci in cervical secretions in non-pregnant women ranges between $4.0 \times 10^2$–$1.8 \times 10^7$ c.f.u. with a geometric mean $1.45 \times 10^5$ c.f.u. (standard deviation, $1.04 \times 10^1$). On the basis of these data we examined the survival of *N. gonorrhoeae* to see whether the defence mechanisms of amniotic fluid of pregnant women are exhaustible or not, and the concentration of gonococci in which they exert in amniotic fluid an inhibitory effect.

On the basis of morphological changes of gonococci observed under electron microscope, the effect of amniotic fluid was in some instances comparable to the effect of benzylpenicillin or ceftizoxime observed upon *N. gonorrhoeae* by Goodel et al. [21] and Korting and Weber [22].

It can be concluded that the amniotic fluid from the midtrimester of pregnancy in majority of our observations showed a considerable antibacterial effect, morphologically comparable to the effect of benzylpenicillin or ceftizoxime. This effect starts already after 2 h incubation of gonococci in amniotic fluid, independently from the amount of the microorganisms. However, it effects more gonococci if they are inoculated into the amniotic fluid in a lower starting quantity ($10^2$–$10^4$ c.f.u./ml).

However, in our study even such cases occurred when the amniotic fluid had minimum effect on *N. gonorrhoee*. In such women, in cases of gonococcal infection gonorrhoeic complications in pregnancy may occur, threatening the mother and the fetus. However, these cases should be endocrinologically and immunologically more thoroughly specified.

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# EPIDEMIOLOGICAL AND MICROBIOLOGICAL DATA ON *SALMONELLA ENTERITIDIS*

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The number of *Salmonella enteritidis* isolations started to rise in humans, eggs and egg products in 4 territories out of the examined 9 territories of Russia in 1986. The spread of *S. enteritidis* infections was connected with the consumption of hen's eggs as it was demonstrated by the analysis of the local outbreaks. Phage type of 1142 *S. enteritidis* strains isolated in Russia was determined using the Hungarian typing scheme. The strains were typable in 95.3% and 12 phage types were found. Phage type 1 was the most frequent (86.7%) among human strains and also among strains originated from hen and egg products. The examined 18226 human *S. enteritidis* strains isolated in Hungary between 1984 and 1989 belonged to 24 phage types and phage type 1 was predominant, the incidence of this type varying between 69.3% and 93.2%. The strains were sensitive to antibiotics, multiresistant strains were found in 1%. Plasmid content was examined of 138 strains; a 38 Md plasmid was carried by all of them and a 96 Md plasmid was harboured by 11 antibiotic-resistant strains. The tested strains produced enterobactin but no aerobactin.

A significant increase has been observed in the incidence of *Salmonella enteritidis* in a number of European countries (Hungary, Germany, Finland, Spain, England) and also in Atlantic coast territories of the USA during the last 5–6 years. The number of *S. enteritidis* isolates reached the 50% of the total number of *Salmonella* serotypes. The investigations cleared up that the consumption of raw or insufficiently heated eggs and poultry was associated with the infection. In several territories of Russia the incidence of *S. enteritidis* increased in the second half of the eighties. Detailed epidemiological analysis of *S. enteritidis* was promoted by the phage typing of this serotype. Several phage typing methods have been described in Poland [1], Israel [2], Hungary [3], England [4] and Spain [5]. This paper gives epidemiological and microbiological information on *S. enteritidis* registered in different territories of Russia between 1983 and 1989.

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## Materials and methods

*Epidemiological data* were collected from 19 territories of Russia and forwarded to the Republic Salmonella Centre. Detailed analysis was made from the data of 9 territories.

*Bacterial strains.* Phage type was determined of a total of 1142 *S. enteritidis strains* (1060 human strains, 82 strains isolated from eggs, poultry products, egg-powder, follicules of hen) isolated from 15 territories where the incidence of *S. enteritidis* was high. Phage type was determined of 18 226 human *S. enteritidis* strains isolated in Hungary between 1984 and 1989. Phage type determination of the latter strains was carried out in the B. Johann National Institute of Hygiene, Budapest.

*Phage typing* was carried out by the method of László et al. [3].

*Antibiotic sensitivity* was tested to chloramphenicol, tetracycline, kanamycin, gentamicin, polymyxin B, streptomycin, ampicillin, carbenicillin.

*Plasmid profile* of 138 strains was determined by the method of Kado and Liu [6].

*Siderophore production* of 36 strains was examined using the method of Rabsch and Reissbrodt [7].

## Results

### *Epidemiological observations*

*Incidence of salmonellae and S. enteritidis.* The informations were collected from 9 territories, 5 were in the European part of the country, 1 in the Uralian region, 1 in Western Siberia, 1 in Eastern Siberia and 1 in the Far Eastern part. The rise in the number of *S. enteritidis* started in 4 territories of the European part of Russia in 1986. This serotype was found for the first time among healthy, perished and emergency slaughtered hens in this year. The frequency of *S. enteritidis* isolations ranged between 100–150 per 10 000 investigated samples and 30–40 per 10 000 samples from eggs and egg-products. This number was not higher among healthy, perished and emergency slaughtered cattle than 15 per 10 000 samples. The frequency of *S. enteritidis* isolates from egg and egg-products increased to 60 per 10 000 samples in 1988. Figure 1 shows the rate of *S. enteritidis* isolated from humans and from eggs and egg-products increased since 1986. During the period 1983–1988 the incidence of *S. enteritidis* in cattle was small.

*Spread of S. enteritidis by egg.* Indirect evidence proved that the tool of transmission of *S. enteritidis* had been hens' egg, in contrast with the other *Salmonella* serotypes, since the end of 1985.

The *Salmonella* serotypes isolated from patients from 72 territories were registered. The frequency of salmonellosis increased only in 16 territories during the period 1984–1987. Out of the 16 territories 15 were supplied with eggs from one poultry farm, the other territories were supplied from other farms (Fig. 2). The data of 19 territories were collected by the Republic Salmonella Centre. It was found that the number of *S. enteritidis* isolations from patients increased in 15 territories from 1985 onwards. Significant change in the incidence of *S. enteritidis* was not observed in the other 4 territories. These 15

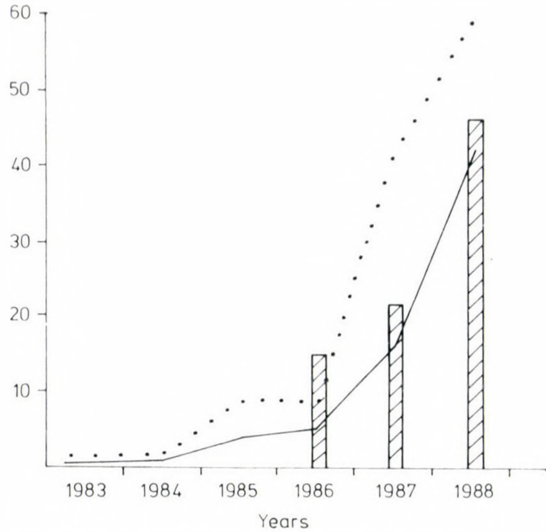


*Fig. 1. S. enteritidis* isolations from humans, eggs and egg products (1983–1988). ——— Incidence of *S. enteritidis* in humans/10 000; shaded columns: in eggs and egg products/10 000; ..... human *S. enteritidis* isolations related to all human salmonella isolations/100

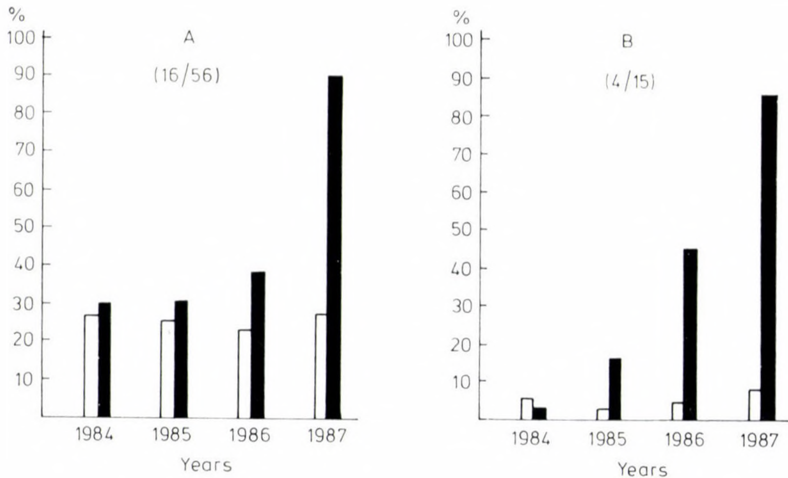


*Fig. 2.* Dynamics of salmonella morbidity in different territories of Russia (1984–1987). A — 72 territories of Russia (all aetiological salmonellae); solid columns: eggs from OPf (numerator); open columns: eggs from other places (denominator); B — 19 territories examined by the Republic Salmonella Centre (only *S. enteritidis*). Columns as above

territories were among the above mentioned ones, which were supplied with eggs from one poultry farm (OPf), the other 4 territories received eggs from other places.

The eggs bred in the poultry farm connected with the increase of *S. enteritidis* were examined bacteriologically. *S. enteritidis* strains were isolated in 3 cases from 60 examined eggs in June, 1986 (5%). Out of 60 pool samples in 3 cases the same microorganism was found. Further examinations gave negative results.

*Analysis of some local outbreaks caused by this serotype.* (i) 22 people got ill who visited a café and consumed buns filled with cream, during the period March 21–25, 1989. The cream contained the white of raw egg and was not treated with heat. *S. enteritidis* was isolated from 16 patients and from the cream, too.

(ii) 21 persons, who visited a canteen of a firm got ill between December 13–14, 1988. The consumed cream was the source of infection. The cream was a mixture of sour cream, milk, cacao, vaniline, jam, sugar and white of raw egg pasteurized in a water bath (70 °C for 30 min). *S. enteritidis* was isolated from 13 patients. The cream was not examined bacteriologically, but it was proved epidemiologically that the white of raw egg was contaminated.

(iii) An outbreak took place after consumption of omlette in a school-canteen between June 11–14, 1988. Twelve persons became ill and *S. enteritidis* was isolated from every patient. The omlette was prepared from raw egg and pasteurized milk and baked. The food was not examined bacteriologically but epidemiological evidence proved that it was responsible for the outbreak.

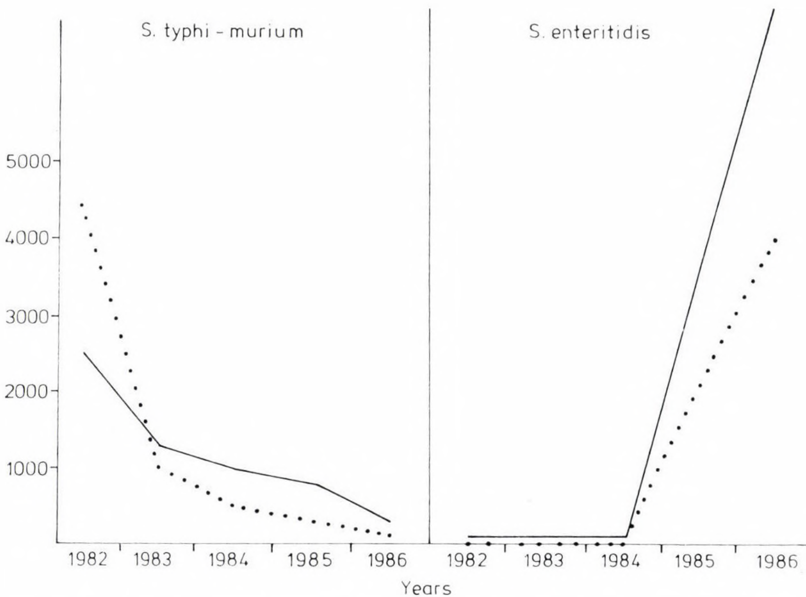


*Fig. 3. S. typhi-murium* and *S. enteritidis* morbidity and deaths among hens. ——— Morbidity; .... infected and died hens

**Table I**

*Phage type distribution of S. enteriditis strains isolated from humans, hens and egg-products*
(Russia 1985–1986)

| Source | Phage types | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 6 | 7 | 9 | 10 | 12 | 13 | 16 | 17 | Nc* | Nt** | Total |
| Human | 86.8 | 0.8 | 0.3 | 0.8 | 0.4 | 0.5 | 0.1 | 2.0 | 0.6 | — | 0.1 | 0.2 | 2.3 | 5.1 | 1060 |
| Meat and organs of hen | 82.4 | — | 5.2 | — | 3.5 | — | — | 1.8 | — | 1.8 | — | 1.8 | 3.5 | — | 57 |
| Egg-products | 92.0 | — | 4.0 | — | — | — | — | 4.0 | — | — | — | — | — | — | 25 |
| Total No. | 990 | 8 | 7 | 9 | 6 | 5 | 1 | 24 | 6 | 1 | 1 | 3 | 27 | 54 | 1142 |
| % | 86.7 | 0.7 | 0.6 | 0.8 | 0.5 | 0.4 | 0.1 | 2.1 | 0.5 | 0.1 | 0.1 | 0.3 | 2.4 | 4.7 | 100.0 |

* Not characteristic
** Not typable

*Spread of S. enteritidis by poultry meat.* The other factor of *S. enteritidis* transmission was the meat of hen. The data, obtained from one territory showed that the incidence of *S. enteritidis* increased among the carcasses of the perished hens, but the increase in the incidence of *Salmonella typhi-murium* was not observed during 1982–1986 (Fig. 3). There was no evidence that the salmonella infection was the cause of death of the birds. It might be assumed that the healthy hens, which were used as food were also infected with salmonellae and this influenced the increase of the number of *S. enteritidis* infections. Epidemiological investigation of a local outbreak served as direct evidence that *S. enteritidis* was transfered by poultry meat. In a children's hospital 38 children and 14 members of the stuff became ill, and 15 children and 4 adults were symptomless excreters between December 26–29, 1989. The source of infection was minced meat, prepared from hen. The meat was boiled for several hours but after it was minced on a table which was not cleaned properly after handling there the raw meat. The hospital got the meat from a farm where *S. enteritidis* was found among the healthy hens by veterinary examinations.

## *Biological properties of S. enteritidis isolated from humans, eggs and poultry-products*

*Distribution of phage types.* Phage types of 1142 strains isolated in Russia were determined. Table I shows the results. The strains were typable in 95.3%. The majority of the strains (86.7%) belonged to phage type 1 and 12 other phage types were found. Phage type 9, 13 and 16 were represented only by one strain. Phage types 2, 10 and 12 were isolated from two territories, phage types 3 and 6 circulated in 5, phage type 4 in 6 territories. Strains of not characteristic

type were found in 5 territories. Phage type 1 was the most frequent in 1985 and also in 1986, but its rate varied from 95.6% to 77.6%. This phage type was predominant in 16 territories, its frequency varied from 60 to 100%. The rate of strains of not characteristic type increased from 0.4% to 9.4% (1985 and 1986). Analysing the phage types according to the source of isolation no difference was found in phage types among strains isolated from children or adults (Fig. 4), except phage type 10, which caused a local outbreak where only adults were concerned. Phage type 1 occurred in 82.4% among strains isolated from meat and organs of hen and 6 other phage types were found. The incidence of phage type 1 was the highest among the strains isolated from egg-products (92%) and 2 other types were represented by 2 strains. All of the strains isolated from eggs and folliculi belonged to phage type 1.

As a comparison, Table II shows the phage type distribution of the 18 226 human *S. enteritidis* strains isolated in Hungary, between 1984 and 1989. The strains were typable in 99.5%. Though 24 phage types were observed, the majority of the strains belonged to phage type 1. According to the previous examinations [3] phage type 7 was predominant in Hungary from 1976 to 1980. There was a change in 1980 and since 1981 phage type 1 became the most frequent type, its incidence reached a peak in 1987 (93.2%) and decreased to 69.3% in 1989.

*Sensitivity to antibiotics.* The majority of the strains was sensitive to antibiotics, only 11 strains were multiresistant, they were resistant to 7 antibiotics, but they were sensitive to gentamicin and polymyxin.

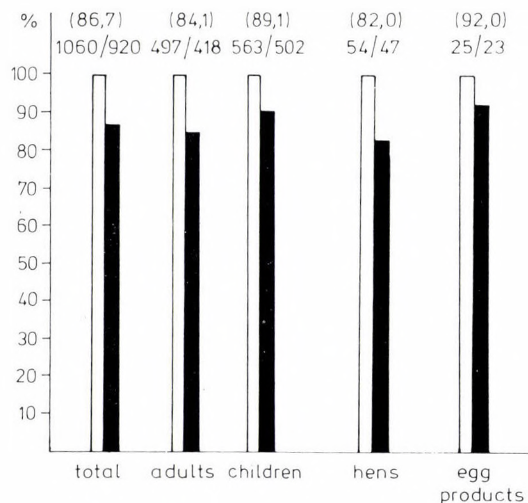


*Fig. 4.* *S. enteritidis* phage types among humans, hens and egg products in 16 territories of Russia (1985–1986). Open columns: all phage types (numerator); solid columns: phage type 1 (denominator). Bracketed figures: percentage of phage type 1

**Table II**

*Phage type distribution of human S. enteritidis strains*
Hungary, 1984–1989

| Phage type | 1984 | 1985 | 1986 | 1987 | 1988 | 1989 | Total | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | No. | % |
| 1 | 72.3 | 74.3 | 86.7 | 93.2 | 85.4 | 69.3 | 14 922 | 81.9 |
| 1a | 1.1 | 0.8 | 0.9 | 0.5 | 1.3 | 1.3 | 180 | 1.0 |
| 3 | 0.1 | 1.0 | 1.2 | 0.4 | 1.1 | 1.3 | 162 | 0.9 |
| 3a | 0.2 | 0.2 | 0.2 | 0.3 | 3.1 | 0.6 | 172 | 0.9 |
| 6 | 0.9 | 1.0 | 0.2 | 0.4 | 0.8 | 1.3 | 138 | 0.8 |
| 7 | 19.9 | 17.5 | 8.0 | 3.0 | 3.9 | 9.4 | 1 564 | 8.6 |
| 17 | 3.5 | 2.2 | 1.4 | 0.8 | 2.7 | 0.8 | 327 | 1.8 |
| 18* | — | — | — | — | — | 13.0 | 400 | 2.2 |
| Others** | 2.0 | 3.0 | 1.4 | 1.4 | 1.7 | 3.0 | 361 | 1.9 |
| Total No. of strains | 1857 | 2091 | 3243 | 3712 | 4239 | 3084 | 18 226 | 100.0 |

* New type (lysis by phages 4 and 8)
** Other types (2, 2a, 4, 4a, 4d, 5, 6d, 8, 10, 10d, 11d, 12, 13, 15, 16, not characteristic, not typable) occurred less than in 0.5%

*Plasmid profile analysis.* Plasmid content was examined of 138 strains of which 127 were sensitive and 11 resistant to antibiotics. A plasmid of molecular weight of 38 Md was carried by all strains. The resistant strains carried a 96 Md plasmid, too.

*Siderophore production.* All of the tested, random selected 36 strains produced enterobactin and did not produce aerobactin.

## Discussion

Among the serotypes not belonging to the typhoid-paratyphoid group, *S. typhi-murium* amd *S. enteritidis* are the most frequent. In the recent years *S. enteritidis* became worldwide a predominant serotype. In some countries, in certain years other serotypes appeared in high number. May be this epidemiological situation is the consequence of the fact that large-scale poultry farms have been developed and the overcrowded circumstances enhanced the risk of salmonella contamination. The birds are usually asymptomatic salmonella carriers and this causes a public health danger. This is not a significant problem for poultry industry and for veterinarians, because the salmonella carriership of the bird do not cause perceptible economic damage. Still, there is no biological ground to explain this striking increase in the incidence of *S. enteritidis*. It is only apparent, that the main factors of transmission of this serotype are the eggs of hens and the meat of broilers. This epidemiologic tendency was

universal in all countries, where the dramatic raise in the incidence of *S. enteritidis* took place. In countries, where these strains were phage-typed it was established that only one or two phage types predominated [3, 8].

It is well known that there are two possibilities how the microorganism penetrates into the egg. One way is the infection of folliculi and the following transovarial transmission of salmonellae. The other way is the contamination of eggs by microorganisms from the feaces of hens. The first way exists in case of "adapted" salmonellae, this type of contamination being rather rare with other *Salmonella* serotypes [9, 10]. The infection of eggs by faeces takes probably place in the oviduct or, unfrequently, during the first minutes after the egg is laid, while the cuticula is moist through the pores or macro or micro cracks of the shell [11–13]. The hypothesis of transovarial transmission of *S. enteritidis* is rather possible [8, 13]. The possibility of the penetration of *S. enteritidis* into the eggs during the first hours after it is laid seems plausible, but it is difficult to understand why has *S. entritidis* an advantage among other salmonellae in penetration. A probable explanation is the thermoresistance of the circulating *S. enteritidis* strains. Data of PHLS [14] for 1984–1988 (17 quarters) testify that in raw eggs *S. typhi-murium* and *S. enteritidis* were found in every quarter and *S. infantis* in 12 quarters. After the pasteurization of eggs *S. typhi-murium* was found in 8 quarters (52.9%), *S. enteritidis* in 7 quarters (47.1%) and *S. infantis* in 1 quarter (8.3%) [14]. On the basis of these data it will be useful to examine the thermoresistance of the circulating *S. enteritidis,* to compare the predominant and other phage types in this respect and the other serotypes, which are isolated from raw eggs.

However, isolations from egg-powder originating from one territory showed that *S. enteritidis* and *S. infantis* were isolated in equal frequency, but the human incidence of *S. enteritidis* was from 11 to 26 times higher than the incidence of *S. infantis*. This might be explained by the different thermoresistance of the two serotypes because egg-powder is used to foods moderately heated before consumption. Recently it was shown that *S. enteritidis* phage-type 4 was more thermoresistant than *S. enteritidis* phage-types 8, 13a and *S. typhimurium* phage-types 110, 141. However, the result may change and depends on the concentration of salmonellae in eggs [15–18].

Concerning the growth of the incidence of *S. enteritidis* it has to be taken into consideration that before the striking rise of the predominant phage type, other phage types circulated [19]. Probably the strains which belong to a definite phage type are connected with some biological properties, which provide them with the ability to adapt to the organisms of humans, birds and to eggs. This problem needs further investigation.

Different phage types are predominant in different parts of the world. In England and Wales, according to Ward et al.'s scheme [4] phage type 4 was the most frequent, and in Russia and Hungary phage type 1 predominated [3].

(Phage type 1 of László et al., as examined by Ward, proved to be identical with the Ward type 1).

The epidemiological situation associated with *S. enteritidis* has economic consequences. There is a need for the united efforts of laboratories of different countries to reduce the number of infections.

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# OXYTOCIN STIMULATES TRANSLOCATION OF PROTEIN KINASE C AND INDUCES ANTIVIRAL STATE IN HUMAN AMNION CELLS

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Association of protein kinase C (PKC) activity to the membrane fraction was observed in oxytocin treated human amnion cells (UAC). In addition, oxytocin was shown to induce an antiviral state and to inhibit multiplication of vesicular stomatitis virus (VSV) in UAC. These observations together with earlier findings indicate that activation of inositol phospholipid breakdown with a consecutive activation of PKC is a common signal transduction pathway in interferon action and hormonal stimulation.

It has been postulated that fetal hormonal signals act upon amnion to trigger labor via prostaglandin production [1]. Oxytocin was identified as a potential hormonal activator of inositol phospholipid breakdown, intracellular free calcium release and $PGE_2$ production in primary cultures of human amnion cells [2]. In addition, it was shown that direct activators of PKC induce considerable increase of $PGE_2$ concentration in human amnion cells. Other studies demonstrated that interferon-receptor interactions stimulate phospholipid turnover [3]. Human alpha, beta and gamma interferon treatment resulted in a two- to three-fold increase in the concentration of diacylglycerol and inositol triphosphate in human fibroblasts. It was also shown that interferon gamma [4] and interferon alpha [5–7] induce translocation of PKC from the cytosol to the cell membrane. Specific inhibition of phosphoinositide hydrolysis [8–10], or inhibition of PKC [10] also indicate that the "inositol phospholipid breakdown-protein kinase C system" plays a significant role in the signal transduction pathway leading to the manifestation of interferon effects. The antiviral effect of phorbol myristate acetate (PMA) [11] and 1-oleoyl-2-acetyl-rac-glycerol (DAG) [8, 9] — direct activators of PKC — support this hypothesis.

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These findings led us to the assumption that there is a common cellular pathway of interferon and hormonal actions. If this assumption is correct, oxytocin should elicit interferon-like effects. Here we examine the effect of oxytocin on the translocation of PKC and on the multiplication and cytopathic activity of VSV in UACs.

## Materials and methods

*For the assay of PKC* suspension cultures of UACs (from V. Sorrentino, University of Rome) were grown in Eagle MEM containing 10% fetal calf serum. Samples containing $2\times10^7$ cells in Dulbecco's phosphate buffered saline (PBS) were treated with oxytocin (Sigma). After intervals indicated, cells were collected at O °C and homogenized by sonication in 0.5 ml homogenization buffer (20 mM Tris HCl, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM phenylmethylsulphonyl fluoride). The homogenate was centrifuged at 48 000 *g* for 30 min at 4 °C. The pellet was resuspended by brief sonication and solubilized (on ice for 1 h) in 0.5 ml homogenization buffer containing 1% TRITON X–100. This suspension was centrifuged at 48 000 *g* for 30 min and the supernatant (membrane fraction) was retained.

*Phospholipid dependent PKC activity* was measured in the detergent solubilized membrane fraction according to Kikkawa et al. [12].

*For virus replication studies* $2\times10^6$ cells were plated into Falcon tissue flasks containing 10 ml culture medium (Parker's 199, supplemented with 10% fetal calf serum). Cultures were incubated overnight at 37 °C (in 5% $CO_2$ atmosphere). The medium was replaced with medium containing 2% fetal calf serum and different concentrations of oxytocin. After incubation periods indicated, the supernatants were removed and the cells were washed with PBS then infected with VSV (Indiana strain) at a multiplicity of 1 plaque forming unit/cell. After 24 h the supernatants were frozen. Virus yields were determined by a cytopathic effect (CPE) assay using 50% tissue culture infections dose ($TCID_{50}$).

## Results and discussion

Figure 1 demonstrates that incubation of suspension cultures of UACs for 10 min with increasing concentrations of oxytocin induce translocation of PKC to the membrane. Figure 2 shows the time course of the effect of oxytocin (50 nM) treatment on the PKC activity of UACs. These results are consistent with the findings of Moore et al. [2] and confirm that oxytocin activates the "inositol phospholipid–PKC system".

Figure 3 shows the effect of oxytocin on VSV yield. Interestingly, the maximal antiviral activity was observed after a relatively short incubation time (3 h). The oxytocin-induced antiviral state was not as strong as that induced by interferon (incubation of UACs for 24 h with 1000 U/ml of human interferon alpha completely inhibited the multiplication of VSV). These findings are consistent with those obtained by the use of noradrenaline treated mouse myocardial cells [13] and support the hypothesis that a common signal transductions mechanism may be involved in interferon and hormonal action. Our data are in agreement with previously published results of Moore et al. [2] and demonstrate that oxytocin receptors are coupled to the PKC containing

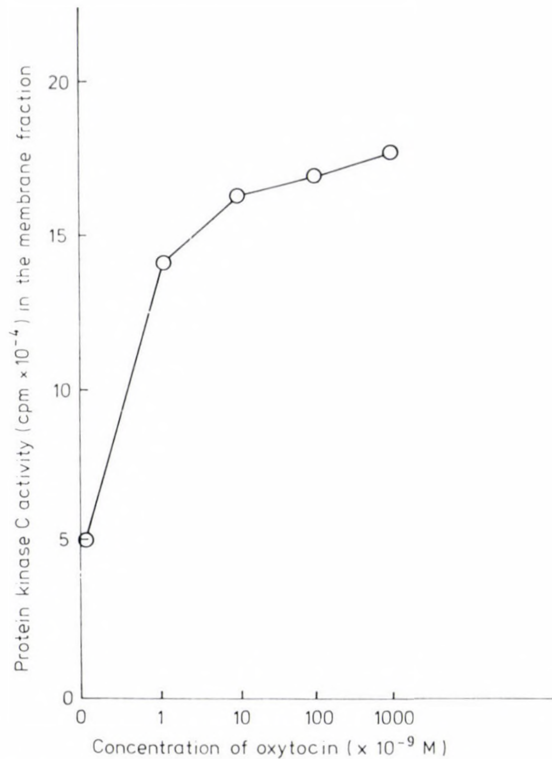


*Fig. 1.* Effect of oxytocin on protein kinase C activity in the membrane fraction. Cells were incubated with oxytocin for 10 min in a suspension culture. PKC activity was defined as that seen in the presence of $CaCl_2$ and lipids minus that seen in the presence of EGTA. A representative example of three similar experiments

signal transduction system, which — when stimulated — results in a decreased multiplication of VSV. The temporal differences between generation of interferon-induced and oxytocin-induced antiviral state reflect, however, deviations in the fine regulation.

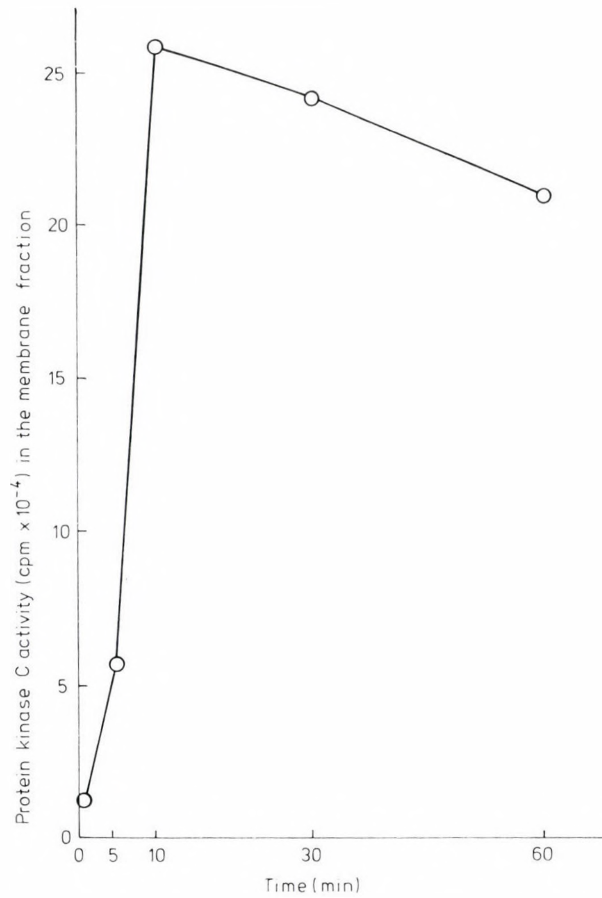


*Fig. 2.* Time course of the effect of oxytocin (50 nM) treatment on the membrane bound protein kinase C activity of UACs. A representative example of three similar experiments

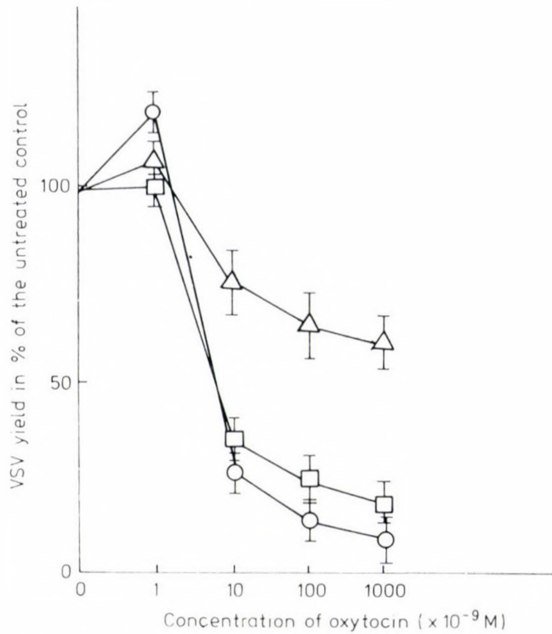


*Fig. 3.* Effect of oxytocin treatment on VSV yield. □, ○, △ symbols mean 1, 3 and 5 h incubation, respectively, using different concentrations of oxytocin before VSV infection. Each point represents the means ± SE from three separate experiments

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# SHIGELLA-TYPE PATHOMECHANISM IN THE "MOUSE MODEL"*

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A strain of *Escherichia coli* K-12 carrying the 140-Megadalton virulence plasmid of the enteroinvasive *E. coli* — J53(pSPl) — showed high virulence in the "mouse model", in chick embryos, but not in the Serény test. It expresses the outer membrane proteins thermoregulatedly, encoded also by the virulence plasmid. In orally infected streptomycin-pretreated mice this strain infects only the large bowel, shows adherence to the epithelial surface, but in its first step preferentially to the mucus excreted by the goblet cells. Epithelial penetration and intracellular multiplication occurs with a characteristic localization of bacteria in the depth of crypts. Consequence of the infection is degeneration of the epithelial surface, its denudation.

Following Freter's method [1] we elaborated a "mouse model" in the sixties to study shigella infections [2]. Essentially, eliminating the aerobic bowel flora of the mouse with high oral doses of streptomycin the animals became susceptible to oral shigella infections. Only virulent strains evoked a long lasting symptomless carriership. This test, called by us "mouse model" was suitable also to study the active or passive protection [3, 4]. On the other hand, streptomycin-pretreated, *E. coli*-free mice are suitable also for the production of an artificial *E. coli* monoflora, by oral administration of any not murine pathogenic *E. coli* strains [5, 6].

The accumulated knowledge of the pathomechanism of shigellae and of the role of chromosomal and plasmid genes make it desirable to reinvestigate the cellular events in this "mouse model". Concentrating on the role of the virulence plasmid, in these experiments we used an *E. coli* K-12 strain (J53) to which the virulence plasmid has been transferred [J53(pSPl)].

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## Materials and methods

*Strains. E. coli* K-12, strain J53, originally obtained from N. Datta (London, United Kingdom) and its shigella-enteroinvasive *E. coli* (EIEC) virulence plasmid carrying derivative, designated as J53(pSPl) prepared in this Institute by transposon mediated conduction [7]. The origin of plasmid was an EIEC strain, *E. coli* O124 No. 34. The virulence tests of J53(pSPl) was compared with a virulent EIEC strain — *E. coli* O143 No. 2 and with its isogenic avirulent mutant No. 2/33.

*Virulence Marker Antigen (VMA).* Rabbit antiserum was produced by the virulent EIEC strain *E. coli* O143 No. 2 absorbed by its avirulent derivative No. 2/33. In ELISA test this absorbed serum distinguishes clearly the virulent and avirulent *Shigella* and EIEC strains. The antigen expressed only in the virulent strains was tentatively designated as Virulence Marker Antigen [8]. Later on this VMA was identified as the plasmid encoded outer membrane protein antigens of IpaB and IpaC [9].

*Mouse model experiments.* Groups of mice kept individually in sterile jars were treated orally with 50 mg streptomycin on two subsequent days. After checking the animals for the elimination of the aerobic bowel flora, they were maintained till the end of experiments under sterile conditions. Oral infection with bacterial strains was carried out by graded doses and the success of the infection determined a week later by controlling the faecal excretion and expressed in $ID_{50}$ (= Infective Dose of 50%) values.

*Chick embryo allantoic test.* Ten days old chick embryos were infected through the allantoic membrane with graded doses of bacteria in a volume of 0.1 ml. The $LD_{50}$ values were calculated after 48 h incubation. *Shigella* strains with preserved penetration capacity kill chick embryos in a dose containing only a few germs [10].

*Serény test* was carried out according to the original method of Serény [11]. Observations concerning the development of keratoconjunctivitis were made on a five day period.

*Histological examinations.* A few days after the oral infection of mice by strain J53(pSPl) the animals were killed and after fixation sections were made from parts of small bowel, coecum and colon. The sections were stained by haematoxylin-eosin and the bacteria were labelled by an immunoperoxidase reaction, namely, by anti-VMA rabbit serum + peroxidase-anti-rabbit IgG.

*Statistical analysis.* $ID_{50}$ and $LD_{50}$ values were calculated by the method of Kärber [12].

## Results

1. *Virulence of the E. coli K-12 derivative — J53(pSPl).* The virulence of the parent *E. coli* K-12 strain J53, its virulence plasmid carrying derivative: J53(pSPl), as well as a virulent (*E. coli* O143 No. 2) and an avirulent (*E. coli* O143 No. 2/33) EIEC strain was determined in different virulence tests. Furthermore, the expression of the plasmid encoded antigen VMA was also tested. The results are summarized in Table I.

From the data presented in this Table it is clear that in the so-called mouse model the strain J53(pSPl) shows an $ID_{50}$ value which is comparable only to the virulent strain of *E. coli* O143 No. 2. In case of the wild-type K-12 (J53), as well as the avirulent EIEC mutant — O143 No. 2/33 — the infectivity values are at least 4 $\log_{10}$ order of magnitude higher. Very similar is the virulence result in chick embryos: J53(pSPl) and O143 No. 2 are highly virulent strains compared to the two other avirulent control bacteria. This $LD_{50}$ value of 10–20 germs means a penetration ability according to our earlier experiences [10]. The only and marked difference between the virulent EIEC strain and J53(pSPl) is that the latter is negative in the Serény test.

On the other hand, the proof for the expression of virulence plasmid encoded genes is the appearance of the VMA marker on the strain J53(pSPl): expressed thermoregulatedly only at 37 °C.

2. *Histological examinations.* The mice were killed shortly and also at different intervals after oral infection by J53(pSPl). The first, preliminary histological studies did not show bacteria in the small bowel or in the coecum — therefore only the sections made from the colon were analyzed further.

**Table I**

*Virulence test of E. coli K-12, its EIEC virulence plasmid carrying derivative as well as, of a virulent (O143,–No. 2) and avirulent (O143, No. 2/33) strains of enteroinvasive E. coli*

| Strains | mouse model test: $ID_{50}$ | chick embryo allantois test: $LD_{50}$ | Serény-test | VMA expression on 30 °C | VMA expression on 37 °C |
|---|---|---|---|---|---|
| *E. coli* K-12, J53 | ~ $10^8$ germs | $2.0 \times 10^4$ germs | — | — | — |
| *E. coli* K-12, J53 (pSPl) | < $10^2$ germs | $1.6 \times 10^1$ germs | — | — | +++ |
| *E. coli* O143, No. 2 | < $10^2$ germs | $1.2 \times 10^1$ germs | +++ | — | +++ |
| *E. coli* O143, No. 2/33 | > $10^6$ germs | $7.0 \times 10^2$ germs | — | — | — |

$ID_{50}$ = Infective Dose of 50%, $LD_{50}$ = Lethal Dose of 50%; observation of guinea pig eyes in the Serény test lasted 5 days; VMA = Virulence Marker Antigen = plasmid coded outer proteins of Ipa B and Ipa C

It seems that the very first step of infection may be an adhesion to the mucus excreted by the goblet cells of the colon. Very well pronounced patches of bacteria can there be seen without heavy adherence to the epithelial surface (Fig. 1).

Perhaps soon after the mucus adherence many pictures show a marked bacterial cover on the epithelial surface in general, but simultaneously intracellularly localized bacteria in groups are also seen. It is already a sign of the intraepithelial multiplication (Fig. 2).

It is also very characteristic — as shown by numerous sections — that the mass of bacteria are preferentially in the depth of crypts — together with intracellular bacterial patches (Fig. 3).

Many sections which were suspected to show a rather late phase of infection show that the epithelial cells are damaged by the infection and in some cases the massive infection leads to the denudation of the epithelial lineage (Fig. 4). It must repeatedly be noted that no clinical symptoms were observed in the animals during the whole course of experiments.

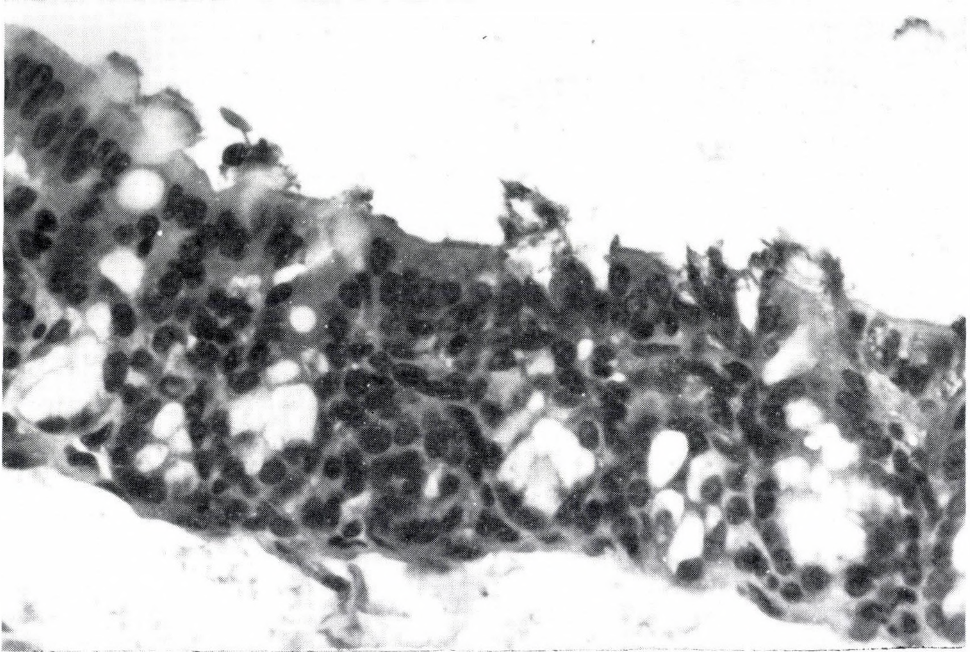

*Fig. 1.* Section of colon from J53(pSPl) infected mouse, stained by haematoxylin–eosin ×400. Bacteria are labelled by an immunoperoxidase reaction. Bacterial adhesion preferentially to the mucus excreted by the goblet cells

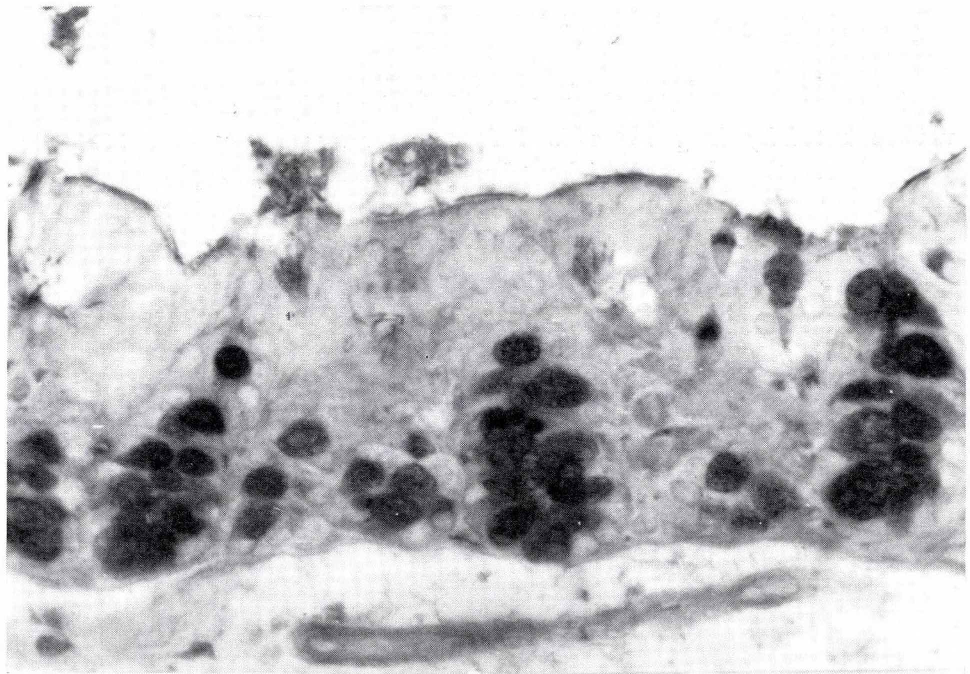

*Fig. 2.* Section of the colon from J53(pSPl) infected mouse, stained by haematoxylin–eosin ×400. Bacteria are labelled by an immunoperoxidase reaction. Mass of adhering bacteria on the epithelial surface and also intracellular bacterial patches

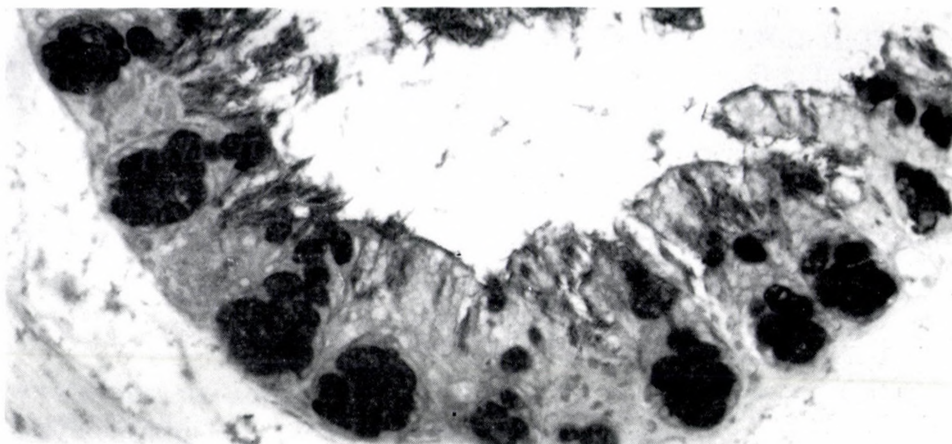

*Fig. 3.* Section of the colon from J53(pSPl) infected mouse, stained by haematoxylin–eosin ×200. Bacteria are labelled by an immunoperoxidase reaction. Characteristic mass of bacteria in the depth of crypts and also intracellularly

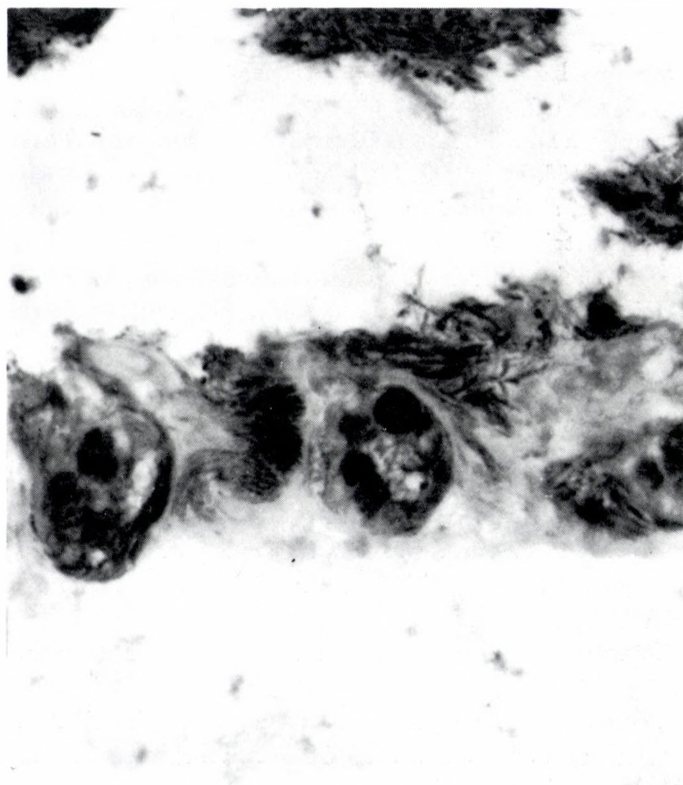

*Fig. 4.* Section of the colon from J53(pSPl) infected mouse, stained by haematoxylin–eosin ×200. Bacteria are labelled by an immunoperoxidase reaction. Denudation of the epithelial surface beside heavy intracellular bacterial growth

## Discussion

To prove the dependence of the *Shigella*-EIEC type pathogens on the 140-Mda virulence plasmid also in the "mouse model", we chose a K-12 strain carrying artificially transferred plasmid. It is true that the chromosomal thermoregulator gene of plasmid, the *vir*R is also carried by *E. coli* K-12 [13], but the *kcp*A gene is not [14]. Therefore taking this latter fact into consideration the negativity of strain J53(pSPl) in the Serény test is not surprising. Besides, this is a bit intriguing because the role of *kcp*A in the induction of the plasmid gene *vir*G and this activity is necessary to the intracellular spreading [14] and against this the intracellular multiplication of the agent in the mouse model seems to be proved. Of course it is an open question that this spreading may be limited and this is responsible for the symptomless character of the infection in the mouse.

Analysing the histopathological data, the first surprise is the clear picture of the adherence, perhaps at first to the mucus and probably later to the brush border of the colonocytes. These data in themselves are not contradicting and not against the numerous other observations. In our recent experiments studying the interactions between enteric bacteria and mucin (as the chief component of mucus) we demonstrated that binding to mucin is a general feature of the enteric bacteria and the mucin binding bacterial ligand is a common outer membrane protein [15]. Pál et al. [16] demonstrated that the epithelial adhesion of shigellae is of a virulence plasmid encoded character and mediated by outer membrane proteins. There are data in the literature both regarding the adhesins responsible for mucus and epithelial adhesion and also the separate adhesins for their receptor. Mouricourt and Julien [17] found that the calf-pathogenic ETEC adhesins K99 and F41 are equal adherence factors for epithelial and mucus receptors. Drumm et al. [18] have a similar finding concerning the rabbit pathogenic *E. coli* strain RDEC-1. On the other hand, Wadolkowski et al. [19] studying a non-murine pathogenic *E. coli* strain demonstrated separate ligands to the mucus and to the epithelial brush border adherence.

The intracellular penetration, multiplication in the epithelial cells the preferable localization of *Shigella* (EIEC) in the depth of crypts is comparable to our knowledge concerning the pathomechanism of this nosological unit [20–22].

For us it was surprising to find a well pronounced epithelial damage despite of the symptomless nature of the mouse model. It is also true that in small laboratory animals mostly death, or very severe clinical symptoms are observed and registered. On the basis of this finding it should be desirable to make more fine examinations in this respect, e.g. to examine microscopical bleedings.

At least, it was mentioned in the Introduction that in streptomycin pretreated, *E. coli*-free mice by the oral introduction of any *E. coli* strain in a higher germ count it is also possible to reach a long lasting carriership, to establish an *E. coli* monoflora. In this case no pathohistological changes, or intracellular bacterial growth can be observed (data not shown), but there is a binding to colonic mucus, growth in it and, as we demonstrated recently, this *E. coli* monoflora composes an intestinal biofilm [23].

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# DETECTION OF *LEGIONELLA PNEUMOPHILA*-SPECIFIC ANTIBODY BY INDIRECT IMMUNOFLUORESCENCE ASSAY

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Antibodies to *Legionella pneumophila* were found by indirect immunofluorescence assay in 525 samples of human serum. The samples were obtained from 451 patients who were suspected of having an acute infectious illness, with mainly respiratory symptoms; 90 patients had antibodies to *L. pneumophila* (19.9%). The results suggest that the prevalence of *L. pneumophila* is greater than had previously been supposed.

The historical origin of research on this new bacteria is found in the 1976 epidemic of legionellosis in Philadelphia, PA, USA. The baecteria were isolated and visualized in 1977 and in 1979 Brenner, Steigerwalt and McDade [1] who classified it in the new family *Legionellaceae* whose only genus was *Legionella*. At the present time 29 different species are known, with a total of 47 serogroups. Of the 14 species which have produced human disease, the most frequent is *L. pneumophila*.

The term legionellosis refers to any of the acute infectious diseases produced by *L. pneumophila* in humans, and can be clinically manifested in three different ways: as Pontiac fever, which is a benign legionellosis; as Legionnaires disease, which is a serious and progressive acute fibrinopurulent pneumopathy usually accompanied by other organic manifestations; and finally, as a legionellosis in patients with depressed immune systems. These last present a more clearly defined clinical profile and are subject to more complications than are the other types.

Serological methods have the advantage that they are the only way of finding out whether or not a patient has had previous contact with a specific microorganism; thus they are excellent techniques for use in epidemiological studies whose purpose is to determine the extent of the presence of a particular micoorganism in a given population. The two most widely used methods are the immunofluorescence assay and the enzyme-linked immunosorbent assay.

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The two goals of this study were to (i) determine the incidence of antibodies to *L. pneumophila* in a population in Cordoba, and (ii) to analyze the influence of certain risk factors on this incidence.

## Materials and methods

*Sera.* From June 1986 to August 1989 a total of 525 sera were taken from 451 patients with predominantly pulmonary symptoms. Of the patients 304 were males and 147 were females, with an age range of from 2 to 88 years; the average age was 43.1 years.

*Assay.* The serum testing was done by indirect immunofluorescence assay using BioDx and SciMedx kits, with the methods developed and described by Wilkinson et al. [2]. For this study we used the reagent that contains a polyvalent mixture of *L. pneumophila* serogroups 1 to 6.

Samples having a titre $\geq 64$ were considered seropositive; this is the limit commonly found in current publications, although some articles have been published which set titre limits of 16 or 32 [3]. Like the majority of authors [2, 4], we have applied the seroconversion criterion when a fourfold or greater increase in the titre was found during convalescence in comparison to the acute phase; this increase had to reach a titre of at least 128.

## Results and discussion

Of the 451 patients included in this study 90 (19.9%) were found to have antibodies to *L. pneumophila*. The prevalence of this bacteria has increased during the years in which the study was done. In the first year 8.2% of the patients (8 out of 97) had antibodies to *L. pneumophila*, in the second year 15.1% (25 out of 166) and in the third year 30.3% (57 out of 188). The progressive increase in the incidence of antibodies suggests that there may be an increase in the environmental presence of *L. pneumophila* in Cordoba, which has led to an increasingly higher level of immunization in the patients studied.

Of titres obtained with seropositive patients 35.3% were 128; 28.6% were 256; 21.0% were 64; 9.2% were $< 64$ and 5.0% were 512. One 1024 titre was obtained in the convalescent stage of the illness.

Figure 1 shows the total of titres in relation to the highest titer value for a seropositive patient, showing a cumulative percentage which refers both to the total number of seropositive patients and to the total number of patients studied. In a study done by Broome et al. [5] there was an 8–26% incidence of antibodies for titre $\geq 128$ and a 2% incidence for titres of $\geq 256$. In another study, in 1985, Borras et al. [3] obtained titres of $\geq 16$ for 26.7% of the subjects; for a group of patients with chronic pulmonary diseases the incidence of antibodies was 11.3% for titres $\geq 64$, 6.5% for titres $\geq 128$, and 3.2% for titres of $\geq 256$.

In the seropositive patients there was a larger number of cases in males than in females throughout the three years of the study, with an overall ratio of 2.1 : 1. However, if one considers the incidence of seropositivity in the cases

studied in comparison to the percentage of male and female patients with antibodies (20.7% and 17.5%, respectively), one finds that the difference is not statistically significant. The average age of the patients studied was 43.1 years which is slightly lower than the average age of those patients with positive serology which was 46.3 years. This indicates that the presence of antibodies is more common in patients older than the average age of the group studied.

As to age distribution of seropositive patients, the greatest number of cases was found in two different age groups, 18.9% in the 20 to 29 years group and 20% in the 40 to 49 years group, followed by 16.7% in the 60 to 69 years group. Of the seropositive patients, 64.4% were of 40 years or more.

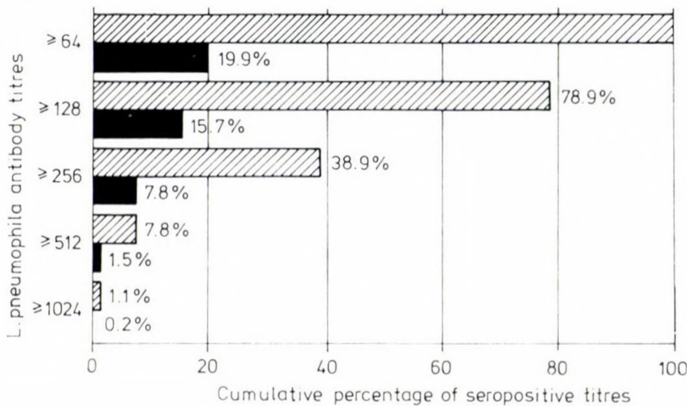


*Fig. 1.* Cumulative percentage of *L. pneumophila* positive titres. Shaded bars, total of seropositive patients; solid bars, total of patients studied

The seropositive patients were in 57.8% from urban areas and in 42.2% from rural areas. This is not a significant difference.

In the literature related to this bacteria diverse factors and illnesses supposed to belie a predisposition to legionellosis are described [5, 6]; these aspects will be discussed with the results. We should point out that 43.3% of the seropositive patients were smokers to some degree, statistically 54.1% of the men and 20.7% of the women. Alcoholism has been noted as a related factor in some of the literature and although there is no solid basis for a discussion of this here, it should be noted that this affects 17.8% of the seropositive patients studied. Finally, we found that in the case of two particular patients both of whom, after taking a trip together, showed symptoms clinically compatible with an acute *L. pneumophila* infection.

A significant percentage (34.4%) of the seropositive patients were found to have some history of respiratory illness. Out of these patients 11.1% were

suffering, or had previously suffered from pulmonary tuberculosis, (3.3% and 7.8%, respectively). Another 11.1% had previously had an infectious pulmonary disease, 7.8% a chronic pulmonary disease and 4.4% had had some other type of pulmonary affection. In terms of the other predisposing illnesses, the existence of immunodepression in 20.0% of the seropositive cases should be noted; this was due to the fact that 6.7% of the patients were undergoing renal transplantation, 3.3% were HIV positive, 4.4% suffered from some immunodepressive illness (leukaemia, etc.) and 5.5% were under immunosuppressing treatment. Of the other illnesses considered, renal insuffiency (12.2%), cardiopathy (7.8%) and hepatic diseases (6.7%) might have been predisposing factors.

Of the total number of seropositive patients (whose etiological causes were later verified by their clinical records) 7.8% had had a non-infectious disease, 8.9% non-pulmonary infectious disease, 5.5% non-infectious pulmonary problems; 3.3% had suffered from pulmonary tuberculosis, 20.0% from pneumonia, 33.3% had been victims of an atypical pneumonia resulting from microorganisms other than *Legionella*, and finally, 21.1% were found to have illnesses compatible with an acute *L. pneumophila* infection.

As to the latter, whether or not seroconversion was demonstrated, three conditions had to be met. First, a clinical description of a pulmonary disease with symptoms and X-ray compatible with legionellosis; second, that in spite of blood cultures, sputum cultures, different serological tests and other microbiological procedures, no other significant finding was encountered except for a high titre or seroconversion for *L. pneumophila;* and last, that treatment with erythromycin led to the resolution of the disease, with the exception of those cases that resulted in the death of the patient.

Of 21.1% of the cases with findings compatible with an acute *L. pneumophila* infection, 14.4% produced only titres of $\geq 128$ with no seroconversion, while in 6.7% of the cases seroconversion was demonstrated. One of the patients with seroconversion had a fatal septic shock, subcutaneous bilateral emphysema and Guillain-Barré syndrome, rarely described in the neurological complications of legionellosis [7].

During this study there have been four cases of suspected cross-reactions with *Mycoplasma pneumoniae* and two with *Chlamydia psittaci.*

Although Bouza and Rodriguez-Creixems [8] and Aliaga et al. [9] believe that direct diagnosis will be increasing, serology, because of its simplicity and the fact that it is a non-invasive method, will continue to be used to confirm the diagnoses in those patients with atypical pneumonia. Serology will continue to be useful in the ex juvantibus diagnoses of patients undergoing treatment with erythromycin and josamycin.

We conclude this article by referring to the 1984 prediction of Pujadas [10]; that Legionnaires' disease is more frequent in Spain than had originally

been thought, and that diagnosis of this illness will rise as awareness of it increases and as microbiological studies of *L. pneumophila* are done and their results disseminated. This study corroborates that prediction.

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# MIXED CULTIVATION OF *TRICHODERMA REESEI* AND *ASPERGILLUS OCHRACEUS* FOR IMPROVED CELLULASE PRODUCTION

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*Aspergillus ochraceus* IMI 317911 was screened to be a high cellobiase producing strain (2.40 IU/ml). The mixed batch cultivation of *Trichoderma reesei* QM 9414/Rut C–30 and *A. ochraceus* IMI 317911 resulted in a balanced enzyme as compared to singly grown cultures. The milled rice straw (6%, w/v) as carbon source was found suitable for production of cellulases resulting in a Filter Paper Activity (FPA) of 1.83 IU/ml and a cellobiase production of 1.63 IU/ml after 7 days of stirred cultivation. An overall cellulase productivity of 10.89 IU/l/h and an enzyme ratio to cellobiase of 1.12 was achieved.

Cellulose is most abundant renewable resource, utilizable for production of fuels, chemicals and food [1]. The cellulose can be enzymatically degraded into monomeric glucose units by synergistic action of exo-glucanase ($C_1$), endoglucanase (cx) and beta-glucosidase (cellobiase), the constituents of cellulase enzyme complex [2]. Therefore, cellulase enzyme filtrate containing these constituents in balanced ratio is necessary for efficient hydrolysis [3]. Since cellulase production involves 40% of process cost in the bioconversion of cellulose into ethanol [4], the production of a balanced enzyme complex with high specific activity, using abundant carbon source such as a lignocellulosics is of prime importance for process economics [1].

Various approaches so as to achieve balanced enzyme complex for efficient hydrolysis are (a) supplementation of cellulase complex with a beta-glucosidase from another source (often an *Aspergillus* species [5]); (b) optimization of physiological conditions for production of balanced enzyme complex [6, 7]; and (c) selection of *Trichoderma reesei* mutants with high cellobiase activity [8]. However, the concept of mixed cultivation of hypercellobiase producing strain along with *T. reesei* in a single step seems to be suitable and economical approach [3]. Therefore, in the present studies mixed cultivation of *T. reesei* strains alongwith *Aspergillus ochraceus* have been employed for cellulase production using rice straw as carbon source.

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## Materials and methods

*Cultures.* The fungal culture of *T. reesei* Rut C–30, was obtained from National Research Council, Ottawa, Canada. Other cultures of *T. reesei* QM 9414, *A. ochraceus* IMI 317911, *Aspergillus terreus* IMI 317912, *Aspergillus niger* IMI 317915, *Aspergillus japonicus* IMI 317916, *Aspergillus nidulans* IMI 317917, *Aspergillus flavus* IMI 317918 and *Chaetomium virescens* IMI 317939 used in the present study were procured from the Department of Microbiology, PAU, Ludhiana, India. The cultures were grown in GYE broth (glucose, 1%; yeast extract, 0.25%; peptone, 0.25%) and maintained at 4 °C on Potato Dextrose Agar Medium (peeled potato extract, 250 g/l; glucose, 1%) and sub-cultured fortnightly.

*Cellulase production.* Erlenmeyer flasks of 250 ml capacity contained 100 ml Mandel's Basal Medium [9]: glucose, 10 g; peptone, 1.0 g; $(NH_4)_2SO_4$, 1.4 g; $KH_2PO_4$, 2.0 g; $MgSO_4$, 0.3 g; urea, 0.3 g; proteose peptone, 1.0 g; Tween 80, 2.0 g per litre. The following trace elements were added (mg/l): $FeSO_4$, 5.0; $MnSO_4$, 1.96; $ZnSO_4$, 3.52; $CoCl_2$, 2.5. Aliquots of the medium were inoculated with 250 mg of freshly grown mycelium of each culture. For the mixed cultivation of *T. reesei* strains with *A. ochraceus*, equal amounts of both cultures (175 mg) were aseptically inoculated simultaneously. The flasks were incubated for 7–14 days at 30 °C under shaking conditions (150 r.p.m.).

*Substrate type and concentration versus cellulase production.* The Erlenmeyer flasks (250 ml) containing 100 ml basal medium alongwith (a) rice straw (2 mm), (b) cellulos (BDH, chemicals) as carbon source was used to evaluate cellulase production. The experiments were carried out at 2, 4 and 6% (w/v) substrate concentrations. The basal mineral medium ingredients were increased by same magnitude, so as to maintain a constant C : N ratio [10]. All experiments were performed in triplicates.

*Enzyme assay.* The crude enzyme filtrate was assayed against filter paper (Whatman No. 1), carboxymethyl cellulose [11] and cellobiose [12] as substrates. The enzyme activity was expressed in terms of International Units (IU/ml) = $\mu$ moles of reducing sugars liberated/min/ml culture filtrate.

## Results

*Screening for hypercellobiase producing fungal strains.* Seven cellulytic fungi were cultivated and screened for their cellobiase, FPA and carboxymethyl cellulase (CMCase) activities. The fungal strain of *A. ochraceus* IMI 317911 was found to produce maximum cellobiase, resulting in 1.35 and 2.40 IU/ml activity after 7 and 14 days of incubation, respectively. The CMCase activity was maximum in *A. terreus* IMI 317912 (0.29 IU/ml) after 7 days of incubation, whereas *A. ochraceus* IMI 317911 exhibit maximum activity against FPA, of all the screened fungal strains (Table I).

*Submerged batch fermentation for cellulase production.* The results in Table II indicate that maximum FPA (0.69 IU/ml) was recorded in *T. reesei* QM 9414 as well as mixed culture of *T. reesei* Rut C-30 and *A. ochraceus* IMI 317911. The cellobiase activity in mixed culture cultivations was more than their corresponding singly grown culture.

*Effect of substrate type and concentration on cellulase production.* (i) *Cellulose as carbon source for fungal cultivation.* The submerged batch cellulase production at varied cellulose concentrations of 20, 40 and 60 g/l were studied. The results in Table III show that mixed culture cultivation of *T. reesei* QM 9414/Rut C-30 with *A. ochraceus* IMI 317911 resulted in higher FPA, as compared to singly grown cultures of *T. reesei*, after 7 and 14 days of incubation,

**Table I**

*Screening of fungi for cellobiase production*

| Cultures | Enzyme activity (IU/ml) | | | | | |
|---|---|---|---|---|---|---|
| | CMCase | | FPA | | Cellobiase | |
| | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days |
| *Chaetomium virescens* IMI 317939 | 0.18 ±0.03 | 0.10 ±0.02 | 0.02 ±0.003 | — | 0.16 ±0.02 | 0.18 ±0.01 |
| *Aspergillus ochraceus* IMI 317911 | 0.17 ±0.01 | 0.11 ±0.02 | 0.04 ±0.002 | 0.04 ±0.004 | 1.35 ±0.12 | 2.40 ±0.20 |
| *A. terreus* IMI 317012 | 0.24 ±0.03 | 0.17 ±0.02 | 0.03 ±0.002 | 0.02 ±0.001 | 0.38 ±0.07 | 1.16 ±0.13 |
| *A. niger* IMI 317915 | 0.11 ±0.04 | 0.16 ±0.02 | 0.01 ±0.003 | 0.01 ±0.004 | 0.77 ±0.09 | 0.96 ±0.13 |
| *A. japonicus* IMI 317916 | 0.17 ±0.06 | 0.15 ±0.03 | 0.03 ±0.003 | 0.02 ±0.003 | 0.96 ±0.15 | 0.96 ±0.09 |
| *A. nidulans* IMI 317917 | 0.07 ±0.01 | 0.03 ±0.008 | 0.02 ±0.001 | — | 0.67 ±0.03 | 0.64 ±0.06 |
| *A. flavus* IMI 317918 | 0.08 ±0.01 | 0.02 ±0.01 | — | — | 0.96 ±0.11 | 0.48 ±0.00 |

Production conditions: cellulose concentration, 1% (w/v); pH 5.0; temperature, 30 °C, agitation, 150 rpm

— Not detected. S.E. at 5%

**Table II**

*Batch production of cellulase with single and mixed fungal cultures*

| Culture | Enzyme activity (IU/ml) | | | | | |
|---|---|---|---|---|---|---|
| | FPA | | CMCase | | Cellobiase | |
| | 7 days | 14 days | 7 days | 14 days | 7 days | 14 days |
| *T. reesei* QM 9414 | 0.43±0.13 | 0.69±0.08 | 2.46±0.23 | 2.32±0.14 | 0.27±0.06 | 0.29±0.02 |
| *T. reesei* Rut C-30 | 0.39±0.09 | 0.65±0.06 | 1.85±0.16 | 1.52±0.17 | 0.25±0.03 | 0.34±0.11 |
| *T. reesei* QM 9414 and *A. ochraceus* IMI 317911 | 0.39±0.12 | 0.41±0.10 | 1.27±0.19 | 0.93±0.13 | 0.37±0.02 | 0.50±0.05 |
| *T. reesei* Rut C-30 and *A. ochraceus* IMI 317911 | 0.43±0.11 | 0.69±0.12 | 1.89±0.16 | 0.92±0.07 | 0.41±0.07 | 0.55±0.13 |

Fermentation conditions: cellulose concentration, 1% (w/v); temperature, 30 °C; pH 5.0; agitation, 150 rpm, S.E. at 5%

**Table III**

*Batch fermentation for cellulase production using varied concentrations of cellulose by single and mixed cultures*

| Substrate (%w/v) | Culture* | Enzyme activity (IU/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | FPA | | | | Cellobiase | | | |
| | | 7 days | | 14 days | | 7 days | | 14 days | |
| | | single | mixed | single | mixed | single | mixed | single | mixed |
| 2 | a | 0.97 ± 0.13 | 0.92 ± 0.17 | 0.82 ± 0.03 | 1.03 ± 0.11 | 0.24 ± 0.03 | 0.27 ± 0.08 | 0.36 ± 0.07 | 0.42 ± 0.04 |
| 4 | a | 0.48 ± 0.09 | 0.76 ± 0.12 | 0.76 ± 0.07 | 1.02 ± 0.08 | 0.25 ± 0.01 | 0.33 ± 0.06 | 0.38 ± 0.02 | 0.43 ± 0.06 |
| 6 | a | 0.47 ± 0.03 | 0.54 ± 0.07 | 0.69 ± 0.09 | 0.89 ± 0.04 | 0.40 ± 0.02 | 0.43 ± 0.05 | 0.43 ± 0.03 | 0.58 ± 0.07 |
| 2 | b | 0.92 ± 0.13 | 0.87 ± 0.04 | 1.02 ± 0.11 | 1.10 ± 0.09 | 0.23 ± 0.03 | 0.43 ± 0.05 | 0.49 ± 0.05 | 0.53 ± 0.02 |
| 4 | b | 1.24 ± 0.14 | 1.34 ± 0.15 | 1.28 ± 0.11 | 1.63 ± 0.18 | 0.28 ± 0.06 | 0.64 ± 0.11 | 0.45 ± 0.07 | 0.71 ± 0.01 |
| 6 | b | 1.03 ± 0.03 | 1.29 ± 0.08 | 0.95 ± 0.06 | 1.52 ± 0.07 | 0.39 ± 0.04 | 0.66 ± 0.07 | 0.27 ± 0.03 | 0.80 ± 0.06 |

Production conditions working volume, 100 ml; temperature, 30 °C; pHe 5.0; agitation, 150 rpm; S.E. at 5%
* a, single: *T. reesei* QM 9414; a, mixed: *T. reesei* QM 9414 and *A. ochraceus* IMI 317911
b, single: *T. reesei* Rut C-30; b, mixed: *T. reesei* Rut C-30 and *A. ochraceus* IMI 317911

**Table IV**

*Batch fermentation for cellulase production using varied concentrations of rice straw by single and mixed fungal cultures*

| Effective cellulose (g/l) | Substrate (%, w/v) | Culture* | Enzyme activity (IU/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | FPA | | | | Cellobiase | | | |
| | | | 7 days | | 14 days | | 7 days | | 14 days | |
| | | | single | mixed | single | mixed | single | mixed | single | mixed |
| 7.85 | 2 | a | 0.49 ± 0.08 | 0.27 ± 0.03 | 0.45 ± 0.04 | 0.40 ± 0.07 | 0.30 ± 0.02 | 0.37 ± 0.06 | 0.56 ± 0.03 | 0.88 ± 0.09 |
| 15.70 | 4 | a | 0.42 ± 0.02 | 0.43 ± 0.02 | 0.40 ± 0.003 | 0.90 ± 0.07 | 0.40 ± 0.04 | 1.20 ± 0.06 | 0.84 ± 0.05 | 1.36 ± 0.11 |
| 23.55 | 6 | a | 0.38 ± 0.03 | 0.90 ± 0.09 | 0.45 ± 0.06 | 0.62 ± 0.03 | 0.81 ± 0.06 | 0.91 ± 0.09 | 0.78 ± 0.03 | 0.98 ± 0.15 |
| 7.85 | 2 | b | 0.52 ± 0.03 | 0.67 ± 0.07 | 0.53 ± 0.07 | 0.89 ± 0.10 | 0.41 ± 0.03 | 0.83 ± 0.11 | 0.52 ± 0.06 | 1.32 ± 0.13 |
| 15.70 | 4 | b | 0.64 ± 0.06 | 1.60 ± 0.11 | 0.73 ± 0.05 | 1.79 ± 0.17 | 0.87 ± 0.12 | 1.34 ± 0.18 | 0.67 ± 0.07 | 1.55 ± 0.09 |
| 23.55 | 6 | b | 0.76 ± 0.09 | 1.83 ± 0.13 | 0.92 ± 0.10 | 1.33 ± 0.12 | 1.07 ± 0.16 | 1.63 ± 0.13 | 0.93 ± 0.11 | 1.59 ± 0.14 |

Fermentation conditions: substrate, milled rice straw; other conditions as in Table III
* Cultures as in Table III

except at 2% (w/v) cellulose concentration after 7 days. The cellulase activity started declining at higher substrate concentration, involving *T. reesei* QM 9414 as well as its mixed culture; whereas mixed cultivation of *T. reesei* Rut C-30 and *A. ochraceus* IMI 317911 produced maximum FPA (1.63 IU/ml) and cellobiase (0.80 IU/ml) at 4 and 6% (w/v) substrate concentrations, respectively.

(ii) *Rice straw as carbon source for fungal cultivation.* Cellulase production using milled rice straw at 2, 4 and 6% (w/v) equivalent to effective cellulose concentrations of 7.85, 15.70 and 23.55 g/l, respectively, was evaluated. The cellulase production was found to be improved as compared to cellulose as carbon source (Table IV). The mixed culture of *T. reesei* Rut C-30 and *A. ochraceus* IMI 317911 produced maximum cellulase as FPA (1.83 IU/ml) and cellobiase (1.63 IU/ml) at 6% (w/v) substrate level, after 7 days of cultivation. The resultant filtrate was having comparatively balanced enzyme complex with FPA: cellobiase of 1.12 and overall cellulase (FPA) productivity of 10.89 IU/l/h.

## Discussion

*T. reesei* mutant strains QM 9414 and Rut C-30 are known to be amongst best producers of cellulases but, although these fungal strains excrete high levels of extracellular cellulose solubilizing activity, but the beta-glucosidase/cellobiase yields are generally low. The low beta-glucosidase content in the enzyme mixture results in imbalanced enzyme complex, which affects the efficient hydrolysis of cellulosics [2]. The deficiency of beta-glucosidase in enzyme mixture is marked with accumulation of cellobiase in the hydrolyzing syrup, that in turn inhibits cellulase action and ultimate glucose yield. Workers have suggested that a balanced enzyme ratio FPA: cellobiase of nearly 1.0 is suitable for achieving efficient saccharification [7]. The mixed cultivation of *A. ochraceus* and *T. reesei* strains of QM 9414/Rut C-30 have been experimented for overcoming this artifact in the present studies.

The growth studies involving cultivation of mixed and single cultures on basal medium containing glucose as carbon source have shown higher biomass yields and almost equal extracellular protein when compared to singly grown culture of *T. reesei* [13]. The compatibility of these cultures to grow in presence of each other is further reflected by relatively more balanced enzyme upon mixed cultivation on cellulose (1%, w/v).

The effect of substrate type and concentration on ultimate enzyme yield and its constituents complex have also been shown by these studies. The cellulose powder was found to be less suitable than rice straw at higher substrate concentrations. Moreover, the *T. reesei* QM 9414 was found to be more prone to catabolite repression as compared to Rut C-30 as also advocated by Allen

and Roche [14]. But the cellobiase production was not affected by high substrate concentrations. Rice straw has been found to be better inducer for cellulase as well as cellobiase production, since the mixed cultivation of *T. reesei* Rut C-30 and *A. ochraceus* resulted in higher enzyme yield alongwith balanced enzyme ratio.

The cellulase production seems to be influenced by many factors such as strain used, cultural conditions and substrate type and concentration. The relationship between these variables have pronounced affect on ultimate enzyme yield and complex [15]. The use of lignocellulosics such as rice straw as carbon source for enzyme production can cut down the process cost considerably [1] and moreover the mixed cultivation of the organisms for suitable enzyme yield and ratio with higher hydrolytic potential [13] can make the enzyme based bioconversion process economically feasible.

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# COMPARISON OF HAEMAGGLUTINATION INHIBITION AND INDIRECT FLUORESCENT ANTIBODY TESTS TO DETECT CERTAIN FLAVIVIRUS ANTIBODIES IN EQUINES

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Formalinized goose erythrocytes were used in haemagglutination inhibition (HI) and indirect fluorescent-antibody (IFA) tests to detect antibodies to Japanese encephalitis (JE) and West Nile (WN) viruses in equines. Paired serum samples from 31 cases having clinical symptoms of flaviviral infections (JE and WN viruses) and 45 controls were examined. For HI test, formalinized goose erythrocytes were used as such, whereas in IFA test, formalinized goose erythrocytes were first coated with respective viral antigens separately and later used to detect antibodies. By employing HI and IFA tests, paired samples having a titre same or less than two fold rise over the control sera were considered normal for both the viruses. IFA test was found to be a method of choice, due to its sensitivity over HI test.

Arboviral infections are most common in India. Earlier reports have revealed serological evidence of arboviral infections in horses and mules from Himalayan border areas [1, 2]. A large number of assays like complement fixation (CF) test, haemagglutination inhibition (HI), immunofluorescence (IF) test and enzyme linked immunosorbent assay (ELISA) have been tried for serodiagnosis of arboviral infections by several investigators [3–6].

Döller et al [7] have described the limitations of CF test due to anticomplementary activity of serum substances. For haemagglutination and haemagglutination inhibition tests, erythrocytes can not be stored for longer periods. To eliminate this problem, Porterfield [3] and Clarke and Casals [8] described the use of Dextrose-Gelatin-Veronal (DGV) for erythrocyte storage for longer periods. Later, Nagarkatti et al. [9] reported that these cells, if stored for more than a week in DGV, cannot be used. In these tests, results are affected by the nonspecific serum components [10–13]. Among others, indirect fluorescent-antibody (IFA) test is comparable with respect to its sensitivity but is more cumbersome with monolayer cell cultures [14].

In the present investigation formalinized goose erythrocytes were employed to detect antibodies to Japanese encephalitis (JE) and West Nile (WN) viruses in serum samples of horses/mules suspected of equine encephalitis by HI and IFA.

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## Materials and methods

*Serum samples.* Thirty one paired serum samples were collected from horses and mules with clinically suspected arboviral infections during the acute and convalescent phases of illness. Serum samples from 45 normal equines were included as control.

*Antigen preparation.* Live JE and WN viruses were obtained from National Institute of Virology, Pune, India and propagated in brains of 1–3 days old suckling mice for antigen preparation. The antigens were purified according to the method of Aizawa et al. [15] and used to prepare immune ascitic fluids [8]. Both JE and WN antigens had a HA titre of 1:640 [16].

*Preparation of formalinized goose erythrocytes (FGRBCs).* Goose erythrocytes were collected and formalinized as described by Csizmás [17]. The formalinized GRBCs were stored as 10% suspension in virus adjusting diluent pH 6.8 at 4 °C [9].

*Haemagglutination inhibition test.* HI titres of ascitic fluids and serum samples were determined according to the method of Ghose and Rao [16] using purified antigens of JE and WN viruses. Positive and negative controls were also included in the test.

*Indirect fluorescent antibody test.* Antigens were coated onto FGRBCs as described by Döller et al. [7]. In brief, a 0.1 ml suspension of 2.5% (v/v) formalinized GRBCs in virus adjusting diluent (VAD, 45 ml of 0.2 M $Na_2HPO_4$ and 55 ml of 0.2 M $NaH_2PO_4$ prepared in 0.15 M saline, pH 6.8) was incubated with 0.1 ml of purified JE and WN antigens (HA units — 16 to 32, each) separately at 4 °C overnight. Antigen coated FGRBCs were pelleted out at 480 g for 5 min at 4 °C and resuspended in 1 ml methanol for 10 min at room temperature. After centrifugation at low speed, the subsequent pellets were washed twice with phosphate-buffered saline pH 7.0 (PBS) and resuspended in 0.25 ml of distilled water (FGRBCs concentration 1%).

Two μl of antigen coated FGRBCs were loaded per spot separately on two multi-spotted immunofluorescence slides (Flow Labs, UK). These slides were dried and kept at —20 °C until used. Before staining, slides were dried at room temperature for 30 min to remove moisture. Later, 20 μl of twofold serially diluted ascitic fluids prepared against JE and WN antigens and serum samples from horses/mules were loaded per spot separately. The slides were then incubated for 60 min in a moist chamber at 37 °C. Subsequently the slides were washed twice with PBS, rinsed with distilled water, air dried and stained with anti-mouse-IgG-FITC conjugate and anti-equine-IgG-FITC conjugate (Sera Lab, UK) for ascitic fluids and serum samples, respectively. Later, the slides were examined under fluorescence microscope as described earlier [18]. Positive and negative controls were also included in the test.

## Results

The titres of immune ascitic fluid raised against JE and WN wiruses were determined by HI and IFA, separately. They were found to be 1 : 1280 by HI and 1 : 2560 by IFA for both viruses.

Similarly paired serum samples from 31 equines were tested by HI and IFA in order to detect the presence of antibodies against JE and WN viruses. The antibody levels in 45 serum samples of apparently healthy animals (control) were found to be $\leq$ 1 : 10 and $\leq$ 1 : 20 by HI and IFA, respectively for both viruses.

Table I presents comparison of results obtained with acute and convalescent phase sera in clinically suspected cases of flaviviral (JE and WN) infections in equines using HI and IFA tests. Seroconversion from negative to positive and fourfold or more rise in the titre between the paired sera was considered as an evidence of group B arboviral infections. By HI 22 sera were positive for JE and 18 for WN viruses, while the corresponding positive samples by IFA test were 22 and 22, respectively.

**Table I**

*Comparison of acute and convalescent phase sera in 31 clinically suspected cases*

| | JE | | WN | |
|---|---|---|---|---|
| | HI | IFA | HI | IFA |
| 1. Seroconversion from negative to positive | 8 | 2 | 7 | 3 |
| 2. Four fold or more rise in titre | 14 | 20 | 11 | 17 |
| Total number of positive sera | 22 | 22 | 18 | 20 |
| 3. Same or twofold fall/rise in titre | 9 | 9 | 13 | 11 |

JE = Japanese encephalitis virus
WN = West Nile virus

**Table II**

*Comparison of the titres obtained for JE and WN viruses by IFA and HI tests in acute and convalescent phase sera*

| Reciprocal titres | JE | | | WN | | |
|---|---|---|---|---|---|---|
| | 0–20 | 40–160 | 320–2560 | 0–20 | 40–160 | 320–2560 |
| Acute Sera | | | | | | |
| HI | 19 | 12 | 0 | 11 | 16 | 4 |
| IFA | 1 | 23 | 7 | 3 | 20 | 8 |
| Convalescent Sera | | | | | | |
| HI | 2 | 24 | 5 | 1 | 26 | 4 |
| IFA | 0 | 8 | 23 | 0 | 8 | 23 |

The data presented in Table II shows the number of serum samples in a given titre range for both acute and convalescent cases using HI and IFA tests. Acute phase sera show a low titre of JE and WN antibodies by both HI and IFA tests, whereas most of the samples from convalescent phase group exhibited high antibody titre. It appears that increase in titre of convalescent sera over the acute phase sera may be due either to the presence of cross-reacting antibodies or to the simultaneous presence of antibodies to both viruses.

## Discussion

The present study describes the use of antigen coated FGRBCs in indirect fluorescent antibody test to detect antibodies to JE and WN viruses in serum samples of animals clinically suspected of equine encephalitis. This was

first attempted with immune ascitic fluids raised against these viruses under laboratory conditions and compared with HI to check the reproducibility and sensitivity of this modification. Samples from clinically suspected animals were tested employing HI and IFA tests, where the latter was found to show high sensitivity.

In this investigation formalinized goose erythrocytes have been used as antigen carrier by indirect adsorption of antigen onto it. Various treatments have been described to prevent the loss of antigen from goose erythrocytes. Döller et al. [7] used methanol to prevent the antigen loss. Prior to antigen adsorption, Nagarkatti el al. [19] advocated the formalinization of cells.

Porterfield [3] and Clarke and Casals [8] described the use of chick or goose erythrocytes stored in DGV. However, cells stored in DGV due to increased fragility can not be used for more than one week. Our results with FGRBCs have been found reproducible and further supported the use of formalinized cells, the storage of which for longer periods has not affected the IFA results.

The difficulties in HI test due to nonspecific serum components have been discussed by several previous workers [10–13]. This property of serum presents problems of low sensitivity to the test. Therefore, IFA was performed to evaluate and compare with HI results by using antigen coated FGRBCs. Difficulties in HI due to nonspecific serum components were not encountered with IFA as described in this investigation.

The present investigation is therefore useful where formalinization of goose erythrocytes provides longer storage stability. Use of formalinized antigen coated GRBCs for indirect fluorescent antibody test makes it a valuable tool for routine diagnosis of JE and WN infections in equines.

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# DIFFERENCES AND SIMILARITIES IN THE SENSITIVITY OF LYMPHOCYTIC AND MACROPHAGE PLASMA MEMBRANE TO DEOXYCHOLATE

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Human tonsillar lymphocytes separated on nylon wool and rat macrophages showed different sensitivity to deoxycholate (DOC) treatment at a low (0.24 mM, 0.01%) concentration for 3 h. The T cell-enriched fraction was stimulated more readily by PHA whereas the B-cell enriched fraction lost its adherence and a decrease of chromium binding capacity was observed after the detergent treatment. Rat peritoneal macrophages under the same conditions lost their chromium label and lysozyme content, whereas their adherence and phagocytic capacity decreased dramatically without affecting their binding capacity. Higher sensitivity to the detergent was observed in peritoneal macrophages compared to tonsillar lymphocytes when various DOC concentrations were used. These findings proved that this low concentration DOC treatment, at least in macrophages, touched mainly the adhesive proteins and the dynamics of the membrane and not its receptor-associated properties.

Several factors which influence the cell surface may lead to a number of changes in the intracellular biochemical processes. Some of these modifications (e.g. binding of hormones or antigens to their receptors) play a physiologically important role. Several agents (drugs, lectins) have been used for studying the mechanisms of these membrane changes [1–3]. Detergents are also suitable for these investigations because they can disturb the hydrophobic lipid-protein interactions [4]. However, the majority of detergents used for protein solubilization have been applied at concentrations above the critical micellar concentration that leads to the complete destruction of the membrane structure [5, 6], only a few data are available on the application of relatively low detergent concentration which save the normal state of the cells [7–9].

The integration of surface proteins into the membrane structure is not uniform. Peripheral proteins are attached to the surface by weak bonds; moreover, several other membraneous glycoproteins are loosely or closely integrated [10] into the lipid bilayer. These integrated proteins might have various functions in the transmission of hormone signals, in the stimulation of macromolecular biosynthesis, in cell-cell contact or in the binding of several compounds that have different functions in immunological processes [11–15].

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We have published earlier the effects of a mild treatment with deoxycholate (DOC) on tonsillar lymphocytes that made possible to solubilize a lymphokine-like membraneous glycoprotein [8]. In another publication we described that hydrophobic drugs inhibiting the protein synthesis and exerting lysosomotropic effects can inhibit the protein synthesis and bacterial phagocytosis of rat macrophages without affecting the binding capacity [16]. Another hydrophobic lysosomotropic compound, L-leucine-O-methylester was also found to be a mild agent to macrophage plasma membrane but causing severe damages inside the cells [17, 18]. In this paper we describe the effect of the low concentration DOC treatment on several membrane-bound functions of lymphocytes and macrophages: mitogenic stimulation, chromium labelling, adherence, phagocytosis and binding of bacteria and enzyme secretion.

## Materials and methods

*Cells.* Lymphocytes were prepared from human individual tonsil pairs freshly removed from 3–6 years old children [8]. Macrophages were prepared from Wistar rats (LATI, Gödöllő, Hungary) after injecting casein or thioglycollate intraperitoneally for 3 days [16, 17]. The peritoneal cells were left to be adhered for 30 min and the adhered cells were considered as macrophages.

*Cell separation.* Tonsillar lymphocytes were separated on nylon wool columns. The adherent cells were eluted in Eagle medium containing 2% human AB serum and the cell subfractions were checked on the basis of their 5'-nucleotidase activity and rosette forming capacity [19–22].

*Isotonic and deoxycholate treatment.* Lymphocytes were incubated in Eagle medium whereas macrophages in Hanks medium for 180 min at 37 °C. In the case of DOC treatment 0.24 mM (0.01%) or 0.024–2.4 mM (0.001–0.1%) DOC applied at final concentrations in the media. In a number of experiments the DOC-treated samples were divided into two groups: after removing the detergent by washing with the media, the first group was resuspended in the media without DOC and the other one in DOC-contraining media. The first group was designated as "pre-treated cells" the other as "DOC-treated cells".

*Mitogenic stimulation.* In 1.0 ml medium $2\times10^6$ lymphocytes were incubated in the presence of 5 $\mu$g PHA (Phaseolus vulgaris lectin) [23] for 48 and 72 h at 37 °C. Then a 60 min pulse labelling was applied with 55 kBq $^3$H-thymidine (sp. radioact. 370 GBq/mmol, UVVVR, Prague). Cells were precipitated and washed twice with 0.5 N PCA (perchloric acid), the precipitate was hydrolyzed in 0.5 N PCA at 90 °C for 15 min: the radioactivity was counted in a liquid scintillation spectrometer (Beckman LS 300) and DNA was determined according to Burton [24].

*Chromium uptake and release.* Lymphocytes were labelled by a method described earlier [25]. Macrophages were adhered ($3\times10^6$ cells in Petri dishes) and 185 kBq $Na_2CrO_4$ (sp. radioact. 1.85 TBq/mmol, IZINTA, Budapest) was added for 60 min at 37 °C. Cells were extensively washed in PBS (phosphate buffered saline) and radioactivity within the cells was measured in a gamma-scintillation spectrometer (Gamma NK 350, Gamma Works, Budapest). Release of radiochromate was also measured according to Szabó et al. [25] during 60 min.

*Determination of lysozyme content.* The lysozyme activity was measured on the basis of its lysing effect on *Micrococcus luteus* followed at 570 nm [26].

*Preparation of FITC (fluorescein isothiocyanate)-labelled bacteria and proteins. M. luteus* bacteria were labelled with FITC according to Vray et al. [27]. Labelled *M. luteus* cells were opsonized by autolog serum for 15 min at 37 °C. IgG was prepared from rat serum and aggregated by a repeated treatment at 63 °C for $3\times20$ min, precipitate was removed by centrifugation at 20 000 *g* for 30 min. The aggregated IgG was purified on a Sepharose column and was labelled with FITC [28]. Protein content was determined according to Lowry [29].

*Determination of phagocytosis and binding.* The ingestion of *M. luteus* was determined according to Vray et al. [27]. The number of bound bacteria was measured similarly but an

excessive washing with PBS was applied instead of a lysozyme treatment. The binding of the FITC-labelled aggregated IgG was determined as follows: the macrophages were incubated at 4 °C for 60 min with the IgG, then washed five times and lysed and the fluorescence was measured in a Hitachi fluorimeter using 492 nm and 525 nm as excitation and emission wavelengths, respectively.

*Statistical evaluation.* S. E. M. values were calculated in each experiment from 3–4 simultaneous samples. Data represents results referred to the samples derived from individual tonsils because the variability of these tonsils is very high. Results for macrophages are referred to a pooled cell population derived from 3 animals.

## Results

*DOC increases the PHA-stimulation of non-adherent cells.* Figure 1 shows that the stimulation index of DOC-treated non-adherent cells is significantly higher than in non-treated cells. This increase was observed only in non-adher-

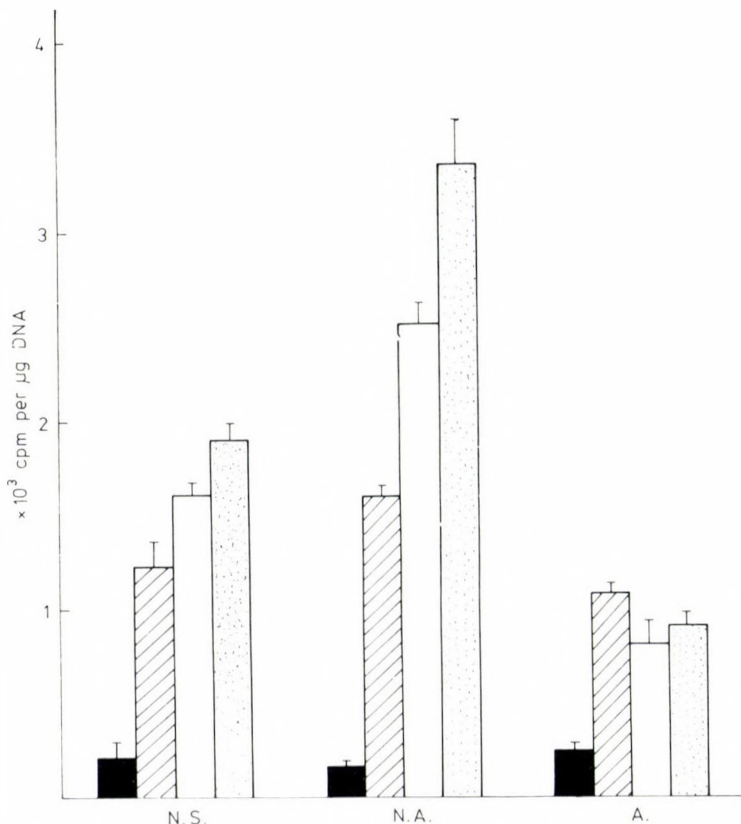


*Fig. 1.* The effect of DOC-treatment on the PHA-stimulation in human tonsillar lymphocytes. N. S., non separated lymphocytic fraction; N. A., non adherent (mainly T lymphocyte) population; A., adherent (mainly B lymphocyte) population. Solid columns, no PHA; shaded columns, PHA only; open columns, PHA + 0.24 mM DOC, detergent removed after 3 h and medium changed for the stimulation in DOC-free Eagle medium-10% serum; stippled columns, PHA + 0.24 mM DOC during the stimulation. Vertical bars indicate S. E. M. (n = 3)

ent cells which were identified mainly as T lymphocytes. The enhancing effect of DOC was more pronounced in the presence of the detergent but it was also observed in pre-treated cells. The effect of detergent was not observed either in 72 h stimulated samples or in Con A (Concanavalin A, jack been lectin)-stimulated lymphocytes (data not presented).

**Table I**

*The alterations of chromium labelling after isotonic incubation and 0.12 mM DOC-treatment in separated lymphocytes*

| Cell population | | Freshly prepared lymphocytes | | Isotonic incubation | | 180 min DOC | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | removed[a] | | present[b] | |
| Non-separated | A | 88.12+/−0.93 | | 76.62+/−5.94 | | 76.15+/−1.25 | | 59.22+/−10.50 | |
| | B | 98.33 | (100) | 73.39 | (74.6) | 75.54 | (76.8) | 62.14 | (63.2) |
| Non-adherent | A | 117.67+/−9.93 | | 110.85+/−1.35 | | 109.15+/−8.00 | | 83.25+/− 1.45 | |
| | B | 120.70 | (100) | 98.99 | (82.0) | 101.50 | (84.0) | 88.93 | (73.6) |
| Adherent | A | 68.95+/−0.55 | | 61.52+/−2.42 | | 54.85+/−5.97 | | 38.85+/−1.66 | |
| | B | 84.60 | (100) | 58.22 | (68.8) | 61.71 | (72.9) | 50.32 | (59.4) |

Data were calculated from 3 samples of a single tonsil (A, mean+/−S.E.M.). The average of 4 experiments from different individual tonsils are shown in "B", without S.E.M. and the percentage of chromium uptake is referred to the control (in brackets). The labelling is given in cpm $^{51}$Cr per $\mu$g cellular DNA

[a] DOC (0.24 mM) was present for 180 min but it was removed before adding radiochromate
[b] DOC (0.24 mM) was present for 180 min and during radiochromate labelling, too

Freshly prepared cells were labelled immediately after isolation, isotonic incubation was performed in Eagle medium for 180 min and cells were then labelled with radiochromate

*Radiochromate labelling decreases mainly from adherent cells.* When lymphocytes were incubated for 180 min at 37 °C in Eagle medium or were treated in DOC-containing medium, their $^{51}$Cr-labelling decreased. The chromium uptake was significantly lower when the detergent was present during the labelling. Diminution was more pronounced in adherent fraction (enriched in B lymphocytes) compared to non-adherent cells (Table I). The distribution of radiochromate was also influenced by these treatments. Both isotonic incubation and DOC-treatment resulted in the enhancement of labelling in the intracellular insoluble fractions (data not shown).

*The effect of the isotonic and DOC-treated incubations on the nylon wool separation itself.* The isotonic incubation in Eagle medium and the DOC-treatment did not affect cell recovery by nylon wool separation method. However, the proportion of the adherent population (B cells) decreased in the consequence of any incubation. When DOC was present during the separation this decrease was more definite on the basis of the cellular DNA content and cell number. The diminution of 5'-nucleotidase activity of adherent population was also observed (Table II).

*DOC-treatment of macrophages diminishes the phagocytosis without affecting the binding of bacteria.* Table III shows that DOC-treatment over 12 mM final concentration decreased the FcR (Ig-Fc receptor) and C3b (complement 3b) mediated ingestion of *M. luteus.* On the other side, the effect could not be due to the damage of surface receptors because neither the attachment

**Table II**

*The effect of isotonic incubation and 0.12 mM DOC-treatment on the separation of tonsillar lymphocytes on nylon wool*

| | | Freshly prepared lymphocytes | Isotonic incubation | 180 min DOC | |
|---|---|---|---|---|---|
| | | | | removed[a] | present[b] |
| Cell recovery % | A[a] | 53.1 +/−11.9 | 54.1 +/−13.1 | 53.1 +/−13.3 | 47.9 +/−9.5 |
| | B[b] | 54.2 | 50.6 | 46.9 | 52.0 |
| Proportion of cells adh/non-adh. | A[a] | 2.31+/− 0.39 | 1.21+/− 0.57 | 1.38+/− 0.72 | 1.30+/− 0.71 |
| | B[c] | 2.03+/− 0.11 | 1.78+/− 0.36 | 1.63+/− 0.45 | 1.34+/− 0.13 |
| Proportion of 5'-nucleotidase adh./non-adh.[c,d] | | 3.09+/− 0.77 | 0.89+/− 0.23 | 0.86+/− 0.11 | 0.48+/− 0.25 |

A: data based on cell counting in light microscope; B: data based on cellular DNA content determined according to Burton [24]

[a] Calculated from 6 experiments

[b] Calculated from 2 experiments (without S.E.M.) with cells derived from different individual tonsils

[c] Calculated from 3 experiments

[d] Proportion of specific activities of the 5'-nucleotidase

of the bacteria to the cell surface nor the binding of FITC-labelled aggregated IgG was impaired. The effect of the detergent was more pronounced when it was present during the whole process but it was also observed in pre-treated samples especially at higher (over 24 mM) concentrations.

*Radiochromate and lysozyme release is observed even at lower DOC-concentrations.* The radiochromate was found to be released at 12 mM DOC indicating a high sensitivity of the macrophage membrane to this detergent. The control release was increased threefold by 24 mM DOC during 60 min. The chromium release is expressed in cpm radioactivity per $10^6$ adherent cells and shown in Fig. 2. The lysozyme and protein content of the macrophages was also released into the medium during 180 min. Lysozyme content of the medium was measured and is shown in Table IV. It can be seen that 12 mM DOC caused a significant release and this is enhanced by increasing the concentration of the detergent.

*The adherence of macrophages is impaired by DOC-treatment.* In Hanks medium 24 mM DOC decreased the adherence of macrophages during 30 min at 37 °C. It was proved both by cell counting and on the basis of radiochromate

**Table III**

*The effect of DOC-treatment on various macrophage functions*

| DOC conc. | | *M. luteus*-C3b phagocytosis | *M. luteus*-C3b binding | *M. luteus*-IgG (FcR) phagocytosis | *M. luteus*-IgG (FcR) binding | IgG aggregate binding |
|---|---|---|---|---|---|---|
| Control | | 5.68+/−1.70 | 13.1+/−2.5 | 9.40+/−1.74 | 12.0+/−2.1 | 19.4+/−5.4 |
| 0.024 mM | A | 4.50+/−0.49 | 19.9+/−3.5 | 7.80+/−0.30 | 17.2+/−0.7 | 17.0+/−3.3 |
| | B | 5.35+/−0.72 | 16.1+/−4.7 | 8.00+/−1.02 | 11.9+−0.7 | 23.1+/−6.0 |
| 0.12 mM | A | 1.55+/−0.17 | 16.6+/−1.8 | 6.10+/−0.99 | 15.2+/−2.3 | 24.4+/−5.0 |
| | B | 3.82+/−0.35 | 18.0+/−4.1 | 8.10+/−0.24 | 20.2+/−2.9 | 22.2+/−2.3 |
| 0.24 mM | A | 0.19+/−0.14 | 18.6+/−2.6 | 4.10+/−0.20 | 18.9+/−2.9 | 24.7+/−3.6 |
| | B | 0.74+/−0.20 | 17.3+/−1.8 | 5.60+/−1.21 | 20.0+/−3.8 | 17.1+/−1.7 |
| 0.48 mM | A | 0.16+/−0.11 | 15.6+/−4.7 | 1.40+/−0.28 | 16.6+/−1.5 | 17.0+/−3.2 |
| | B | 0.27+/−0.1 | 17.2+/−3.0 | 2.70+/−0.88 | 15.9+/−2.9 | 13.1+/−0.1 |

Phagocytosis: phagocytic index is expressed in ingested bacteria per macrophage per hour; Binding: bound *M.luteus* is given in bound bacteria per cell, bound aggregate in pg aggr. IgG per cell.

A: DOC was present during the whole process

B: DOC was present for 3 h and then removed, phagocytosis and binding was performed as usual in DOC-free medium. For details see in Materials and methods.

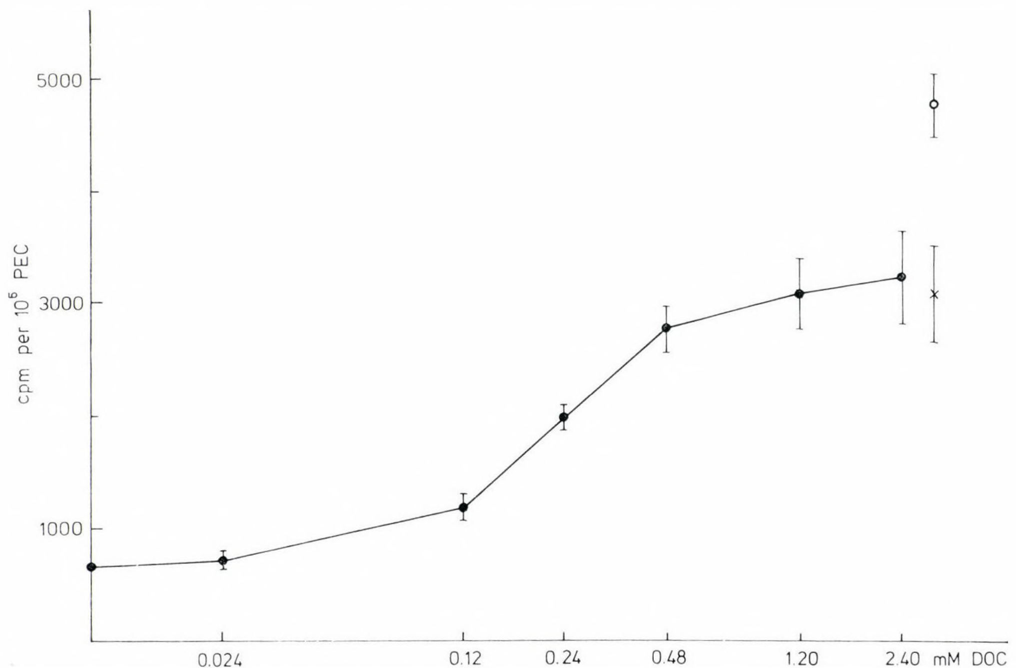


*Fig. 2.* The effect of DOC on the radiochromate release from rat peritoneal macrophages (PEC). ●—●, release in the presence of the indicated DOC concentration; x, release after lysing in water for 60 min at 37 °C; ○, total labelling after washing the cells exhaustively. Vertical bars indicate S.E.M. (n = 6)

**Table IV**

*The effect of DOC-treatment on the adherence and the lysozyme and protein release of rat peritoneal cells*

| DOC conc. | Chromium uptake[a] | Adhered cell number[b] | Lysozyme release[c] | Protein release[d] |
|---|---|---|---|---|
| Control | 1656+/−400 | 280+/−20 | 0.84+/−0.22 | 43+/−9 |
| 0.12 mM | 1101+/−113 | 200+/−21 | 1.12+/−0.18 | 53+/−3 |
| 0.24 mM | 358+/− 42 | 43+/− 7 | 2.28+/−0.33 | 76+/−9 |

[a] cpm radiochromate per $10^6$ macrophages
[b] Cells counted in a light microscope per 0.1 $mm^2$
[c] $\mu g$ lysozyme released per $3 \times 10^6$ PEC
[d] $\mu g$ protein released from $3 \times 10^6$ PEC

labelling as shown in Table IV. In vivo thioglycollate elicited cells were more adherent than resident cells but the DOC-treatment caused the same effect on both populations.

## Discussion

The adherent subfraction of nylon wool separated tonsillar lymphocytes contains mainly B cells whereas the non-adherent population consists of T lymphocytes [19–22]. An increasing effect of a mild deoxycholate treatment on PHA stimulation was observed only in the T-cell enriched population (Fig. 1) without influencing its lectin binding capacity [30]. Two possible explanations were found to this observation: (i) the DOC-treatment may induce the chedding of membraneous proteins making new receptors available; (ii) the separation of the lymphocytes diminished the cell-to-cell interactions and blocked a moderating effect of B cells on T lymphocyte stimulation. These effects are probably combined and the resulted effect can be responsible for the exclusive stimulation of T lymphocytes for three reasons: (i) DOC-treatment may influence the surface of adherent cells above all (Tables I and II) and the enhancement of the stimulation is characteristic of T cells; (ii) DOC has no any significant effect in mixed populations; (iii) the stimulation of separated, non-treated T lymphocytes was much lower compared to treated T cells. These results confirm that the effect of DOC may be a consequence of the lost B–T cell interactions after the separation. Usinger et al. [31] made similar observations on bovine T cells depleted of monocytes and B lymphocytes. A further explanation is possible on the basis of the interactions between DOC and membrane lipids. Rode et al. [32, 33] showed than Con A stimulation was mediated by membraneous phospholipids. Nevertheless, the stimulation of the two different mitogens should not be identical.

The impairment of phagocytic process without changing the binding was observed in macrophages (Table III). The binding of the ingested particles is the condition of any phagocytosis and it is facilited by opsonization by IgG or C3b, depending on the receptor involved in the ingestion. Fc receptors mediate the phagocytosis of IgG-opsonized bacteria and they can bind aggregated IgG, too. Bacteria opsonized by complement are bound and ingested via C3b-receptors. Our results suggest that these receptors are not impaired by this mild treatment; the inhibition of the phagocytosis may be due to the damage of the plasma membrane or to the damage of the cytoskeleton. Similar results were obtained by hydrophobic drugs [8] and the involvement of intracellular processes is more likely in the case of the increase of the PHA-stimulation, too. The effect of DOC could not be distinguished from the isotonic incubation in short term lymphocyte cultures; the DOC treatment was reversible. $^{51}$Cr-uptake equally decreased in the lymphocytes cultured in isotonic medium with or without the detergent before the labelling if compared to the uptake of freshly prepared cells (Table I). The detergent diminished the chromium uptake when it was present during the labelling. These observations suggested the following explanation: the shedding of surface proteins might cause the decrease of the chromium label. The proteins touched by the isotonic or DOC-incubation should be responsible for the binding and eventually for the uptake of the radiochromate. When the DOC is present during the labelling process the negative charge of the detergent may also cause a repulsion of the negatively charged chromate anion, too. The higher sensitivity of B cell membrane can be explained by the fact that this cell is more adherent and its membrane contains a high number of adhesive proteins that are also impaired by the DOC-treatment. The adhesion proteins called integrins were studied recently [34] and several proteins responsible to the adhesion were described. An "adhesive sequence" consisting of Arg-Gly-Asp (RGD peptide) was also identified [35, 36].

An alternative explanation for the decreasing effect of the isotonic incubation is possible on the basis of the observation of Staub et al. [37]: the metabolic activity of the tonsillar lymphocytes was lower compared to freshly prepared cells and the tonsillar B cells should be considered as activated cells [38]. According to these findings a transition of tonsillar lymphocytes to the resting state is suggested. This change may be reflected in the decrease of the chromate uptake, too

In macrophages, the plasma membrane showed a higher sensitivity for the detergent treatment compared to the lymphocytes. DOC at 0.05% concentration caused a significant release of chromium (Fig.2) and of the lysozyme and protein content of the cells into the medium (Table IV), but did not affect the intact state of the lymphocytic plasma membrane [8]. This increased sensitivity of the membrane may be related to its involvement in the important

physiological functions in the macrophage function e.g. adherence, spreading, etc. It should be emphasized that the adherent B lymphocyte membrane was also more sensitive to the DOC-treatment than the T cell membrane as shown in Table I.

The questions discussed above made us study the direct effect of DOC on the adherence of macrophages. In Hanks medium containing 0.01% DOC used for the adherence there was a drastic decrease in the number of the adhered cells proved by both the chromium uptake of adhered cells and the increased number of non-adhered cells after 30 min at 37 °C (Table IV). This observation supported that DOC promoted the shedding of the adhesion proteins from the surface. A similar phenomenon was observed in lymphocytes: a part of the more adherent B cells lost its surface-bound adhesive proteins (Table II). The B lymphocyte marker 5'-nucleotidase activity [22] was also lower in the lymphocytes incubated in isotonic or DOC-containing media (Table II). This observation suggested that non-freshly prepared B lymphocytes dispose of a different surface compared to freshly prepared cells; the loss of the shed surface proteins resulted in the decrease of the proportion of adherent cells and this may lead to the elution of a number of B lymphocytes on nylon wool together with non-adherent T cells. For this reason, the nylon wool separation can be performed correctly only with freshly prepared lymphocytes.

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# PLASMIDS ENCODING FOR ERYTHROMYCIN RIBOSOMAL METHYLASE OF *STAPHYLOCOCCUS EPIDERMIDIS* AND *STAPHYLOCOCCUS SIMULANS*

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Two 1.7 Md plasmids of *Staphylococcus epidermidis* and three ones of *Staphylococcus simulans* determining inducible macrolide-lincosamide resistance are identical as judged by restriction endonuclease fingerprinting. These plasmids designated pEI2101, pEI9105, pEI107, pEI1108 and pEI6104, respectively, belong to the incompatibility group 12. Dot-blot hybridization by photobiotin-labelled gene probe developed from *S. aureus* erythromycin ribosomal methylase gene showed cross hybridization between methylase-coding reference plasmids and the tested ones. The examined plasmids proved to be no transmissible in mating experiments into *S. aureus* recipients.

Resistance to macrolide, lincosamide and streptogramin B (MLS) antibiotics is exhibited by three different systems: modification of the target in the cell; extracellular inactivation of the drug molecule and decreased cell wall penetration [1–4]. Mechanisms of the resistance based generally on post-transcriptional methylation of adenine of 23 SRNA [2, 3]. These strains are usually resistant to all or more MLS antibiotics simultaneously. Detoxification of the antibiotics depends on substrate-specific enzyme production usually without cross-resistance between non-related MLS antibiotics [1, 4]. Both types occur also in Gram-positive organisms and in *Enterobacteriaceae*. Further alternative way of parcial MLS resistance is an alteration of membrane permeability due to the product of the plasmid-borne *erp*A [5] or *msr*A [6] genes in *Staphylococcus epidermidis*.

The usual resistance phenotypes to MLS antibiotics in the genus *Staphylococcus* were grouped by Kono et al. [7] and Hashimoto et al. [8]. In Hungary, the cumulated occurrence of plasmid-borne group B (erythromycin and oleandomycin induced macrolide-streptogramin resistance) and group C2 (erythromycin and oleandomycin induced macrolide-lincosamide resistance) phenotypes were reported among *S. aureus* strains isolated from three nurseries in Pest county in the late seventies [9–12]. The resistance determinants of C2 phenotype strains were located on 1.7 or 1.8 Md plasmids [11, 12]. Incidence of the same phenotype in coagulase-negative staphylococcal (CNS) strains of

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clinical origin happened in 17.7% [13]. Five isolates of them harboured 1.7 Md plasmids that seemed to be identical with the above-mentioned *S. aureus* plasmids by size and the determined phenotype [14]. Further experiments to examine the phenotypical and genetical nature of these plasmids and identification of the gene product by hybridization probe developed from *S. aureus* erythromycin ribosomal methylase *(erm)* gene are presented in this paper.

## Materials and methods

*Bacterial strains.* All bacterial strains used in plasmid determination and genetical experiments are listed in Table I.

*Plasmids.* The strains harbouring plasmids for incompatibility *(Inc)* group testing and conjugation are shown in Table II and Table III, respectively.

*Protoplast fusion* was performed according to Götz et al. [22] on DM3 plates [23] by use of wild-type *S. epidermidis* and *S. simulans* donors and *S. aureus* BI 7801 [15], a streptomycin and rifampicin resistant mutant of SA 113 [24], as recipient. For selection of recombinant clones erythromycin, 6.8 mg/l; chloramphenicol, 45 mg/l; tetracycline, 15 mg/l; and rifampicin, 45 mg/l were used in 1/4 volume of soft agar covered 3 h incubation after plating. The recombinants were identified by antibiogram, clumping factor production and sensitivity to *S. aureus* phage 80L2 [25].

Transduction was carried out by phage 80 $\mu\alpha$ [26] or 80L2 by method of Iordănescu [27].

*Incompatibility group* of plasmids were determined by 80 $\mu$ $\alpha$-dependent transduction [17, 18, 28].

*Mating experiments* were done in mixed culture of donor and recipient strains [29]. *S. aureus* BI 7801 [15] was used as recipient. To control the mating system SA 403 *S. aureus* donor strain [17, 19] offered by Witte [30] was used.

*Pure plasmid DNA* was prepared by CsCl gradient density method [31].

*Restriction endonuclease digestion* was performed according to New England Biolabs catalog [32]. The following enzymes (Reanal) were used: *Acc*I, *Cla*I, *Eco*RI, *Hinc*II, *Hind*III, *Hpa*I, *Kpn*I, *Mbo*I, *Sac*I, *Sph*I, *Xho*I.

*DNA electrophoresis* was carried out on 0.8% horizontal agarose gels (Type I, Sigma) in tris/borate buffer [33]. *Hind*III digested phage lambda DNA was used as molecular size control.

*Restriction fragments for molecular cloning* were separated from agarose gels by the method of Langridge et al. [34].

*Construction of pE103-BM chimera plasmid* for cloning of methylase gene was done by V. L. Motin and M. S. Pokrowskaya (Gamaleya Institute, Moscow). Approximately 1.1 kb *Hind*III/*Cla*I fragment of 1.7 Md *Inc*12 pE103 plasmid [12] was cloned by *Hind*III/*Acc*I digested pUC19 vector [35] in *Escherichia coli* HB101 host [36]. For hybridization *Hind*III/*Eco*RI fragment of pE103-BM plasmid was labelled with photobiotin (bio-5-dUTP) [36–38].

*Dot-blot hybridization* was performed on nitrocellulose membrane (Amersham) in 6×SSC at 42 °C [37, 38]. Hybridization was detected by alkaline phosphatase labelled streptavidine conjugate (Amersham). One microgram quantities of samples were used. Phage lambda DNA and pE103-BM plasmid DNA were sampled as negative and positive control, respectively.

## Results

Using five C2 phenotype strains and a rifampicin/-streptomycin resistant derivative of a restriction and modification deficient *S. aureus* the fusion of protoplasts resulted recombinant clones to selected markers in frequency $6\times \times 10^{-8}-8\times 10^{-5}$ (Table IV). The recombinants resistant to MLS antibiotics by inducible mode harboured the 1.7 Md plasmids of the CNS parent strains.

**Table I**

*Staphylococcal strains*

| Strain | Species | Source* | Resistance phenotype** | Reference |
|---|---|---|---|---|
| LK 2101 | *S. epidermidis* | throat | Pc Tc Cm C2 | 14, 15 |
| LK 9105 | *S. epidermidis* | sinus punctate | Pc Cm C2 | 14, 15 |
| LK 1107 | *S. simulans* | nose | Pc Tc C2 | 14, 15 |
| LK 1108 | *S. simulans* | nose | Pc Tc C2 | 14, 15 |
| LK 6104 | *S. simulans* | blood culture | Pc Tc Cm C2 | 14, 15 |

* All strains were isolated by the Microbiology Laboratory of László Central Hospital for Infectious Diseases, Budapest, Hungary [14]

** Pc penicillin G, Tc tetracycline, Cm chloramphenicol, C2 erythromycin and oleandomycin induced MLS resistance

**Table II**

*Staphylococcal plasmids for insompatibility testing*

| Plasmid | Phenotype | Incompatibility group | Host* | Reference |
|---|---|---|---|---|
| pT181 | Tc | *Inc*3 | SA 389 | 16, 17 |
| pSA4502 | Tc | *Inc*3 *Inc*11 | SA 757 | 18 |
| pSA6502 | Tc | *Inc*3 *Inc*12 | SA 1094 | 18 |

* All strains were kindly supplied by S. Iordănescu (Institute Cantacuzino, Bucharest, Roumania)

**Table III**

*Staphylococcal plasmids used in mating experiments[1] and in dot-blot hybridization[2]*

| Plasmid | Host | Phenotype* | Reference |
|---|---|---|---|
| pT127[1] | SA 403** | Tc | 17, 19 |
| pCRG1600[1] | CRG 1600** | Pc Gm | 20 |
| pE1764[2] | SA 1104*** | C2 | 18 |
| pE194[2] | SA 484*** | C2 | 17, 21 |

* Abbreviations: Pc penicillin G, Tc tetracycline, Gm gentamicin, C2 MLS resistance inducible by erythromycin and oleandomycin

** Strains originated from W. Witte (Institut für Experimentelle Epidemiologie, Wernigerode, Germany)

*** Strains were provided by S. Iordănescu (Institute Cantacuzino, Bucharest, Roumania)

Determination of *Inc* group of the tested plasmids exhibited an unilateral incompatibility with *Inc*12 plasmids (data not shown).

In mixed culture of the strains the transmission of pT127 plasmid occurred in $5 \times 10^{-7}$ frequency. Using CNS donor and *S. aureus* recipient strains

**Table IV**

*Recombination frequencies in protoplast fusion experiments between S. epidermidis or simulans donor and S. aureus recipient*

| Donor | Selection | Recombination frequency* | Resistance pattern of derivatives | Frequency* |
|---|---|---|---|---|
| LK2101 | Em Rp | $2.1\times10^{-6}$ | C2 Rp Sm | $2.0\times10^{-6}$ |
| | | | C2 Tc Rp Sm | $1.0\times10^{-7}$ |
| | Tc Rp | $2.4\times10^{-6}$ | Tc Rp Sm | $1.9\times10^{-6}$ |
| | | | C2 Tc Rp Sm | $5.0\times10^{-7}$ |
| LK 6104 | Em Rp | $5.0\times10^{-5}$ | C2 Rp Sm | $4.6\times10^{-5}$ |
| | | | C2 Cm Rp Sm | $4.0\times10^{-6}$ |
| | Cm Rp | $6.7\times10^{-5}$ | Cm Rp Sm | $4.2\times10^{-5}$ |
| | | | Tc Cm Rp Sm | $5.0\times10^{-6}$ |
| | | | C2 Cm Rp Sm | $2.0\times10^{-5}$ |
| | Tc Rp | $1.2\times10^{-4}$ | Tc Rp Sm | $6.0\times10^{-5}$ |
| | | | Tc Cm Rp Sm | $3.0\times10^{-5}$ |
| | | | C2 Tc Rp Sm | $1.0\times10^{-5}$ |
| | | | C2 Tc Cm Rp Sm | $2.0\times10^{-5}$ |
| LK 1107 | Em Rp<br>Tc Rp | 0<br>0 | | |
| LK 1108 | Em Rp | $8.2\times10^{-5}$ | C2 Rp Sm | $8.2\times10^{-5}$ |
| LK 9105 | Em Rp | $4.5\times10^{-7}$ | C2 Rp Sm | $3.9\times10^{-7}$ |
| | | | C2 Cm Rp Sm | $6.0\times10^{-8}$ |
| | Cm Rp | $3.6\times10^{-7}$ | Cm Rp Sm | $2.3\times10^{-7}$ |
| | | | C2 Cm Rp Sm | $1.3\times10^{-7}$ |

* Calculated on recipient's colony forming units
Abbreviations: Em erythromycin, C2 MLS resistance inducible by erythromycin and oleandomycin, Rp rifampicin, Tc tetracycline, Cm chloramphenicol, Sm streptomycin

no recombinants resistant to erythromycin and the recipient's marker were observed. Neither were the plasmids encoding for inducible MLS resistance mobilized by pCRG1600 conjugative gentamicin resistance plasmid which was

transmissed from *S. aureus* CRG 1600 host into the C2 type plasmid harbouring recombinants with frequencies of $2\times10^{-6}$–$6.5\times10^{-4}$.

Before the restriction endonuclease analysis the plasmids were transduced into *S. aureus* NCTC 8325 host by phage 80L2. The digestion of the CNS plasmids with restriction endonucleases resulted fragments identical in size in case of every plasmid (Table V).

**Table V**

*Restriction enzyme digestion of pEI plasmids of coagulase negative staphylococcal strains*

| Enzyme (s) | Size of fragments (bp) |
|---|---|
| None | 2426 |
| *Hinc*II | 1800 660 |
| *Hind*III | 2150 200 80 |
| *Hpa*I | 1650 780 |
| *Kpn*I | no site |
| *Mbo*I | 1460 580 380 |
| *Sac*I | linearized |
| *Sph*I | no site |
| *Xho*I | no site |
| *Hind*III + *Mbo*I | 1850 300 200 80 |
| *Hind*III + *Sac*I | 1825 320 200 80 |

In dot-blot hybridization we demonstrated homology between the pEI1108, pEI2101, pEI6104, pEI9105, pE1764 plasmid DNAs and the *erm*C harbouring pE194 plasmid DNA by the gene probe sourced from pE103 *S. aureus* plasmid (Fig. 1).

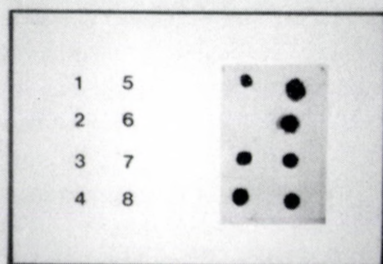


*Fig. 1.* Dot-blot hybridization by photobiotin-labelled methylase-specific gene probe. Samples: 1 pE103-BM, 2 phage lambda, 3 pEI2101, 4 pE1764, 5 pEI1108, 6 pE194, 7 pEI6104, 8 pEI9105. Samples 3, 5, 7 and 8 originated from coagulase-negative staphylococcal hosts

## Discussion

Harbouring of different resistance genes by CNSi means an important therapeutical problem because they may serve as resistance gene reservoirs for the clinically more significant *S. aureus* [30–40]. Although the different mechanisms of MLS resistance of *S. aureus* have been studied in detail, only few data are available regarding their occurrence and molecular epidemiology in the related CNS species. The MLS resistance was found in 36.7% of CNS strains isolated in a general hospital in Spain [41], it was particularly frequent in *S. epidermidis* (57%) in contrast to *Staphylococcus saprophyticus* (12%). The high-level resistance to all MLS drugs predominated. The incidence of inducible MLS resistance among randomly selected CNS population proved 19% in England [42] and 17.7% in Hungary [13].

The genetical background of resistance was studied only in few cases. Plasmid determined constitutive MLS resistance in *S. epidermidis* was reported by Parisi et al. [43]. The plasmid pNE131 harbours *erm*M methylase gene homologous with *erm*C gene of pE194 in more than 95% [44]. The plasmid pNE131 is indistinguishable from pE103 *S. aureus* plasmid by restriction endonuclease fingerprinting [12] which was used to develop gene probe [36].

Ross et al. [6, 45] isolated and characterized a 31.5 kb plasmid in *S. epidermidis* coding inducible resistance to macrolides but not to lincosamides and streptogramins. This novel resistance phenotype was first observed in *S. aureus* isolates in our laboratory, and the responsible determinant was located on *Inc*l plasmids, also coding for beta-lactamase production and resistance to some heavy metal ions [9, 10, 46].

Plasmids determining non-MLS resistance were identified in connection with altered membrane permeability in *S. epidermidis* [5], and in CNS strains producing lincomycin inactivating enzymes [1, 47].

Our present study demonstrates that the inducible MLS resistance of the five tested CNS isolates was coded by 1.7 Md plasmids as suggested by preliminary curing experiments [14]. Four of these plasmids exist permanently in *S. aureus* host followed protoplast fusion. Only the pEI1107 plasmid proved to be intransmissible to *S.aureus* recipient by the same way.

The examined pEI1108, pEI2101, pEI6104, and pEI9105 plasmids belong to the *Inc*12 incompatibility group, similarly to the pE103 [12] and pE1764 [18], *S.-aureus* plasmids isolated in Hungary [9, 11, 12, 48], and differing from *Inc*11 plasmid pE194 isolated in Roumania [17, 21].

Comparison of pEI1108, pEI2101, pEI6104, and pEI9105 plasmids by restriction endonuclease digestion resulted identical patterns [15]. To compare restriction endonuclease fingerprints to *S. aureus* plasmids physical maps suggests a close relationship of CNS pEI plasmids to plasmids determining a similar character in *S. aureus* of Hungarian origin [12, 15]. Also the comparison

of gene products presented in this report proved genetical connexion within the genus *Staphylococcus:* the results of hybridization performed in strong-stringency conditions showed high homology between the tested plasmids and the *erm*C methylase coding pE194 *S. aureus* plasmid, although the genes are located on plasmids of different incompatibility.

All these observations supported the interspecific transmission of plasmid vehicles and/or resistance genes as it was demonstrated on gentamicin, tetracycline, chloramphenicol, MLS plasmids [39, 40, 49, 50] or on erythromycin-resistance determinants of streptococci and staphylococci [2, 51, 52].

In our hands, these plasmids proved to be non self-transmissible in a well-controlled mating system and were not mobilized by the standard pCRG1600 [20] and neither by two conjugative gentamicin resistance plasmids of clinical origin [15]. This experience could be interpreted by lack of stable covalent linkage between large "sex factor activity" plasmid and smaller MLS plasmid resulted absence of their cotransfer. Similar observations were reported by McDonnel et al. [40].

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# PLASMIDS DETERMINING ENZYMATIC INACTIVATION OF LINCOMYCIN IN *STAPHYLOCOCCUS EPIDERMIDIS* A PRELIMINARY REPORT*

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Coagulase-negative staphylococcal strains isolated from immunocompromised patients harboured in 41% non-MLS type lincomycin resistance determinant. Two kinds of resistance plasmids were detected in *Staphylococcus epidermidis* isolates of this origin in connection with lincomycin resistance. One of them represented by pBI1 and pBI84 (1.4 Md in size) determines no other resistance marker. The pBI109PGL plasmid determines also penicillinase production and aminoglycoside resistance, its molecular mass is 31 Md. Hybridization using *lin*A, *lin*A' and *lin*A-like specific gene probes suggested occurrence of genes of lincosamid-inactivating enzyme belonging to the *lin* gene family but differring from the previously characterized determinants.

In the last decade there has been an increasing number of reports describing the incidence of coagulase-negative staphylococcal (CNS) species in clinical infections [1–5]. The CNS species have been increasingly recognized as nosocomial pathogens in patients whose defences were compromised by an implanted foreign body or by administration of immunosuppressive drugs [1, 3]. The incidence of strains resistant to a variety of antimicrobials increased the value to identify them in more detail [1, 4, 6–9]. Several authors have demonstrated that CNSi (mostly *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*) exhibit resistance to a wider spectrum of antibiotics than *Staphylococcus aureus* [1, 6, 9].

A total of 152 CNS isolates were collected from clinical samples of 14 patients hospitalized after bone-marrow transplantation at the National Institute of Haematology and Blood Transfusion, Budapest, during an 18-month period in 1987–1989 [10]. The strains were characterized by phage typing [11], slime production [12], plasmid pattern, antibiogram and biotyping based on carbohydrate utilization and deoxyribonuclease production [13]. Different body sites of all patients without clinical manifestation of staphylococcal infection were continuously colonized by CNS isolates belonging to one to five

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species, in spite of the permanent antibiotic treatment and usage of disinfectants. The species distribution showed the predominance of *S. epidermidis* and *S. haemolyticus* (45–45%). The total typability was found 68% using the phages in routine test dilution, 16 lytic patterns occurred. All but one strain was multiply resistant. The evaluation of results obtained by the mentioned methods refuted the horizontal spread of strains: several patients' isolates with identitical phage pattern and slime character, belonging to the same species, usually differed in their antibiogram and plasmid pattern; there was no correlation between slime production, phage pattern and any resistance marker.

Surprisingly, a new marker, single resistance to lincomycin but not to clindamycin was observed in 41% of isolates, belonging to the species *Staphylococcus capitis, S. epidermidis, S. haemolyticus* and *Staphylococcus hominis*, respectively. This phenotype was detectable in 0.2–8% of different staphylococcal species in France [14]. Accumulation of this rare non-MLS type resistance (resistance not extended to all members of macrolide-lincosamide-streplogramin antibiotics) in the given epidemic population suggested "gene epidemy" between CNS strains colonized immunocompromised hosts. Preliminary characterization of the resistance determinants is summarized in this communication.

## Materials and methods

*Bacterial strains.* Three clinical isolates of *S. epidermidis* sourced from the National Institute of Haematology and Blood Transfusion, Budapest, were selected (Table I). *S. haemolyticus* BM 4610, a pIP855 harbouring wild strain, its plasmid-free derivative BM 4610-1 [14, 15], *S. aureus* BM 4611, the original host of pIP856 [16] and *lin*A-like control strains *Staphylococcus cohnii* "Despres" and "Retaut" originated from R. Leclerq (Hospital Henri Mondor, Université Paris XII, Créteil, France).

*Plasmids.* Recombinant plasmids pAT151 (pAT22 $\Delta$ *Acc*I fragment) [17, 18] harbouring *lin*A gene and pAB3 (pUC8 $\Omega$/pIP856/*Hind*III) [18] harbouring *lin*A' gene sourced from A. Brisson-Noël (Diagnostics Pasteur, Marnes la Coquette, France).

*Minimal inhibitory concentration of the antimicrobials* was determined by agar dilution method [19]. The following drugs were used: lincomycin (Lincocin, Upjohn), clindamycin (Dalacin C, Upjohn), amikacin (Likacin, Lisapharma), gentamicin (Chinoin), tobramycin (Brulamycin, Biogal), kanamycin (V/O "Medexport"), neomycin (Russel), netilmicin (Netromycin, Schering), paromomycin (GYKI), streptomycin (EGYT), spectinomycin (Sigma).

*Beta-lactamase production* was tested by iodine-starch reaction [20].

*Detection of lincomycin-inactivating enzyme* was according to Gots [21]. *Micrococcus luteus* ATCC 9341 (HNCMB 117001) test organism was received from the Hungarian National Collection of Medical Bacteria, Budapest. BM 4610 and BM 4611, the *lin*A and *lin*A' harbouring strains were used as positive controls and BM 4610-1 as a negative control, respectively.

*Curing of resistance markers* was performed according to Bouanchaud et al. [22] on MIC of ethidium bromide.

*Transduction* was performed by phage 80L2 [23] according to Iordănescu [24] into *S. aureus* NCTC 8325 recipient.

*Protoplast fusion* was carried out by method of Götz et al. [25], using wild-type *S. epidermidis* donors. *S. aureus* SA 35, a novobiocin-resistant mutant of NCTC 8325 was used as recipient sourced from W. Witte (Institut für Experimentelle Epidemiologie, Wernigerode, Germany). For selection of recombinant clones 16 mg/l of lincomycin (Lincocin, Upjohn) and 2 mg/l of novobiocin (Inamycin, Hoechst) were used in 1/4 volume of soft agar covered 3 h

incubation after plating. The recombinants were identified by antibiogram, clumping factor production and phage sensitivity.

*Mating experiments* were made in biphasic cultures of donor and recipient strains [26] in nutrient broth supplemented with 0.01 M $CaCl_2$, 0.7% yeast extract and 0.02% agarose [27]. *S. aureus* SA 35 was used as recipient. The mobilizing factor pCRG1600 [28] harbouring *S. aureus* CRG 1600 strain was received from W. Witte, Germany. Selective concentrations were 0.5 mg/l of novobiocin and 4 mg/l of lincomycin or 10 mg/l of gentamicin.

*Total staphylococcal DNA samples* were prepared by 50 mg/l lysostaphin (Sigma) followed by phenol/chloroform extraction [14].

*Pure plasmid DNA* was obtained by CsCl gradient density method [29].

*Restriction endonuclease digestion* was performed according to the manufacturers' instructions. The following enzymes were used: *Acc*I (New England Biolabs), *Bgl*II (Nippongene), *Bsp*XI (Appligene), *Eco*RI (Pharmacia), *Hind*III and *Pst*I (Amersham, both).

*DNA electrophoresis* was carried out on 0.8% horizontal agarose gels (Litex) in tris/borate buffer [30]. Plasmid size was calculated from their restriction endonuclease fragments. *Hind*III or *Hind*III and *Eco*RI digested phage lambda DNAs (Nippongene) were used as molecular size controls.

*Gene probe DNAs*. Specific gene probes for *lin*A, *lin*A' and *lin*A-like sequences [14] were received from A. Brisson-Noël. The probe fragments were multiplicated in M13mp18 system [31]. Gene amplification was performed by polymerase chain reaction (PCR) technic [32] using *Taq* polymerase (Amersham), dNTPs (Pharmacia), and the following oligonucleotides: as "—20" 5'TGACCGGCAGCAAAATG3' and as "reverse" 5'CAGGAAACAGCTATGAC3'. The samples were denaturated at 90 °C for 1 min and were subjected to 40 cycles of amplification as follows. Samples were cooled to 50 °C for 1 min, heated for extension to 72 °C for 30 s and denatured again at 94 °C for 30 s. Finally the specimens were kept at 72 °C for 5 min and at 30 °C for 5 s.

*Dot-blot hybridization* [33] was performed on Hybond N membrane (Amersham). Two micrograms of samples were transferred. Fragments of DNA to be used as probes were cloned in M13mp18 replicative-form DNA [31] and transfected into *Escherichia coli* JM 101 [34], and single-stranged recombinant DNAs were purified and labelled [35] with [alpha-$^{32}$P]dCTP (Amersham) using 17mer "—40" universal primer (United States Biochemical Corporation) and Pharmacia produced Klenow fragment and dNTPs.

*Southern blotting* [33] of electrophorized samples was carried out onto nylon filter (Schleicher and Schuell). $^{32}$P-labelled gene probes were prepared by nick translation [33] using Nick Translation Kit (Pharmacia) and PB 10205 [alpha-$^{32}$P]dCTP (Amersham) as radioactive component. Labelled DNA was collected chromatographically on Sephadex G50 or NICK-Column (Pharmacia).

*Hybridization*. All $^{32}$P-labelled probes were hybridized to DNA immobilized on nitrocellulose or nylon filters in 6×SSC at 37 °C overnight [33]. Autoradiography was followed overnight and for 48 h simultaneously at —70 °C by Fuji X-Ray films.

## Results

Three multiply resistant lysostaphin sensitive *S. epidermidis* strains were selected as resistance donors in fusion experiments. The isolation frequencies of fusants are presented in Table II. All the progenies of strain numbered OKI 109/1989 were resistant to penicillin, gentamicin and chloramphenicol, too. The lincomycin resistance determinants were transduced into the standard *S. aureus* NCTC 8325 recipient and the antibiograms and plasmid profiles were tested. The derivatives obtained from OKI 1/1989 and OKI 84/1989 hosts proved to be resistant only to lincomycin, they harbour an 1.4 Md plasmid (Fig. 1).

On the other hand, transduction by phage 80L2 propagated on OKI 109/1989 derivative resulted two kinds of transductants: one of them was resistant to penicillins, aminoglycosides and lincomycin, likewise the other one

**Table I**

*Staphylococcus epidermidis strains used to determine genetic localization of lincomycin resistance*

| Strain* | Source | Resistance pattern** | | Slime | Phage pattern |
|---|---|---|---|---|---|
| OKI 1/1989 | skin | Lm Pc Gm Tm Km | Tc SXT | + | Ph13/+ |
| OKI 84/1989 | foreskin | Lm Pc Gm Tm Km | Tc SXT | + | Ph13 |
| OKI 109/1989 | skin | Lm Pc Gm Tm Km (Pm) Cm | Tc SXT | — | U4/U14/U15/U16/ /U20/U33/U46 |

* All strains were isolated at the Bacteriology Department of National Institute of Hygiene, Budapest, Hungary

** Abbreviations of tested resistance markers: Lm lincomycin, Pc penicillin G, Gm gentamicin, Tm tobramycin, Km kanamycin, Pm paromomyicn, Cm chloramphenicol, Tc tetracycline, SXT co-trimoxazole, determined by disc diffusion method. Brackets symbol moderate susceptibility

lack for aminoglycoside resistance. Both types possess a large plasmid designated pBI109PGL and pBI109PL, their size being 31 Md and 20.3 Md, respectively. Curing of plasmids by ethidium bromide was observed in frequencies of $1.7\times \times 10^{-2}$ and $6.7\times 10^{-2}$, respectively.

The MIC values of lincomycin resistant *S. aureus* transductants are documented by Table III. All the derivatives produced lincomycin inactivating enzyme as was detected by Gots' test.

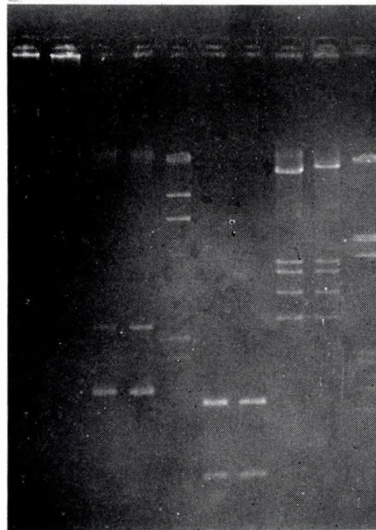



*Fig. 1.* Plasmids of *S. epidermidis* encoding lincomycin inactivating enzymes. Samples: a 8325 (pBI109PL), b 8325 (pBI109PGL), c 8325 (pBI1), d 8325 (pBI84), e *Hind*III digested phage lambda, f *Acc*I digested pBI1, g *Acc*I digested pBI84, h *Bgl*II digested pBI109PGL, i *Bgl*II digested pBI109PL, j *Hind*III and *Eco*RI double digested phage lambda

**Table II**

*Isolation frequencies of recombinant colonies obtained by protoplast fusion between clinical isolates of epidermidis and novobiocin resistant S. aureus*

| Donor | Selection | Recombination frequency* | Resistance pattern of derivatives | Frequency* |
|---|---|---|---|---|
| OKI 1/1989 | Lm Nb | $2.3 \times 10^{-7}$ | Lm Pc Nb | $2.310 \times ^{-7}$ |
| OKI 84/1989 | Lm Nb | $1.3 \times 10^{-4}$ | Lm Pc Tc Nb | $3.2 \times 10^{-5}$ |
| | | | Lm Pc Nb | $7.8 \times 10^{-5}$ |
| | | | Lm Nb | $2.0 \times 10^{-5}$ |
| OKI 109/1989 | Lm Nb | $8.6 \times 10^{-6}$ | Lm Pc Tc Cm Gm Nb | $7.5 \times 10^{-6}$ |
| | | | Lm Pc Cm Gm Nb | $1.1 \times 10^{-6}$ |

* Calculated on recipient's colony forming units
For abbreviations see Table I; Nb = novobiocin

**Table III**

*M.I.C. values of S. aureus strains harbouring lincomycin-resistance determinants of S. epidermidis*

| Strain | MIC (mg/l)* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lm | Cl | An | Gm | Tm | Km | Nm | Nn | Pm | Sm | Sn |
| 8325 (pBI1) | 32 | 0.06 | 4 | $<1$ | $>1$ | 4 | $<1$ | $<1$ | 256 | 8 | 128 |
| 8325 (pBI84) | 64 | 0.25 | $<1$ | $<1$ | $<1$ | $<1$ | 1 | $<1$ | 32 | 8 | 64 |
| 8325 (pBI109PGL) | 32 | 0.25 | 8 | 1024 | 512 | $>1024$ | $<32$ | 64 | 64 | 16 | 64 |
| 8325 (pBI109PL) | 32 | 0.5 | $<1$ | $<1$ | $<1$ | $<1$ | $<1$ | $<1$ | 16 | 4 | 64 |

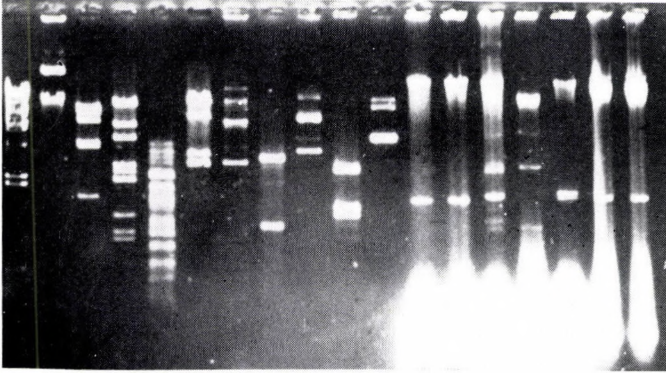
* Abbreviations: Lm lincomycin, Cl clindamycin, An amikacin, Gm gentamicin, Tm tobramycin, Km kanamycin, Nm neomycin, Nn netilmicin, Pm paromomycin, Sm streptomycin, Sn spectinomycin

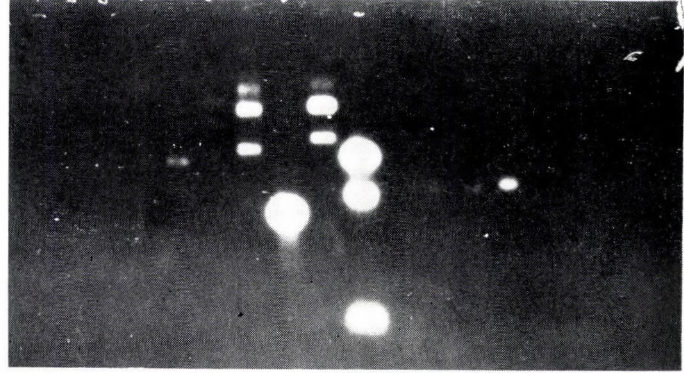
Using $^{32}P$-labelled *lin*A-like probe in dot-blot hybridization we found strong homology with the total DNA samples of the lincomycin-resistance plasmid harbouring derivatives and the positive control plasmid DNAs (data not shown). Further on southern blot hybridizations were performed by using of *lin*A, *lin*A' and *lin*A-like probes to identify exactly the genes and to determine their localization on the novel type pBI109PGL plasmid. The results show the *lin* gene to be situated on 13 kb *Xba*I, 14.8 kb *Hpa*I, 2.3 and 1.35 kb *Eco*RI/*Hind*-III, and 16.5 kb *Eco*RI/*Sal*I fragments of pBI109PGL plasmid. There was no observed interpretable difference between the three trials, the examined samples hybridized with all the three probes in different degrees (Fig. 2).

In mating experiments we found pBI1, pBI84, pBI109PGL and also the pBI109PL plasmids to be non-conjugative in *S. aureus* and they were not mobilized by pCRG1600 sex-factor activity plasmid.

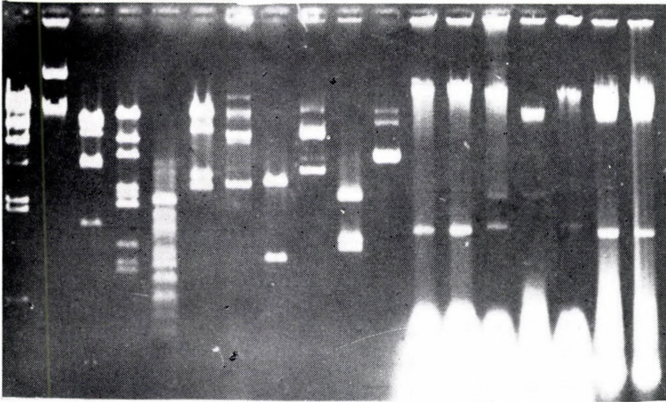
a b c d e f g h i j k l m n o p q r a b c d e f g h i j k l m n o p q r

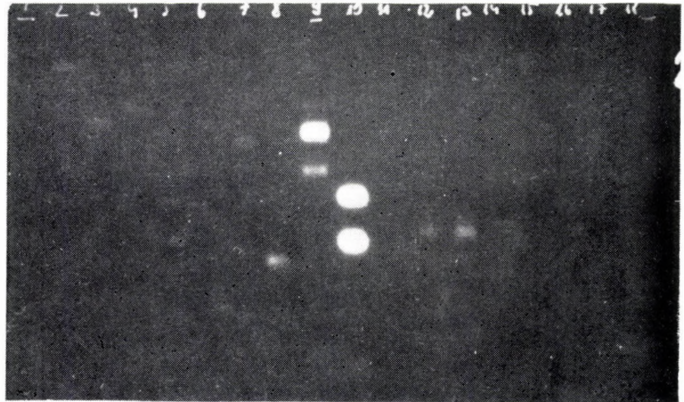
A





B





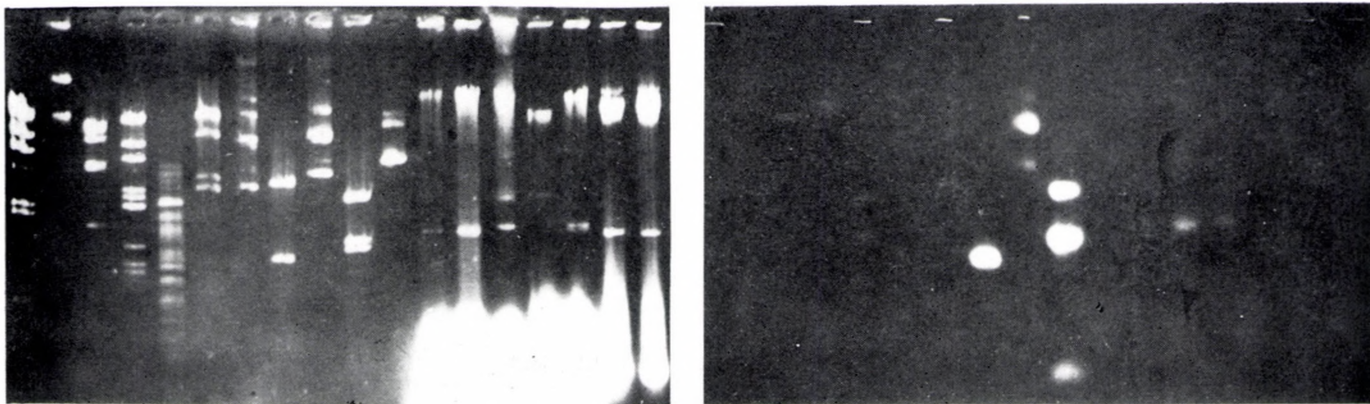



*Fig. 2.* Southern blotting of DNA samples determining enzymic lincomycin inactivation. Samples: a phage lambda/*Hind*III, b pBI109PGL, c pBI109PGL/*Xba*I, d pBI109PGL/*Hpa*I, e pBI109PGL/*Eco*RI + *Hind*III, f pBI109PGL/*Eco*RI + *Sal*I, g pAT151, h pAT151/*Eco*RI + *Acc*I, i pAB3, j pAB3/*Hind*III + *Bsp*XI, kp BI50 (a constitutive macrolide-lincosamide resistance determinant), l 8325 (pBI1), m 8325 (pBI84), n BM 4610, o BM 4610-1, p BM 4611, q "Despres", r "Retaut". b–k purified plasmid DNAs, l–r total DNA preparates were used. A: *Lin*A specific, B: *lin*A' specific, C: *lin*A-like specific probes. Left sides show electrophoretograms, the right side the conferring autoradiograms

## Discussion

The non-MLS type resistance to lincosamides was detected first in *S. haemolyticus* of clinical origin isolated in Paris, France [15]. This mode of resistance is due to adenylation of the drug molecule [14, 16, 35, 36]. Two *lin* genes (lincosamide inactivation nucleotidylation) were identified and sequenced in the genus *Staphylococcus*. Occurence of *lin*A was observed in *S. aureus, S. epidermidis, S. haemolyticus* and *S. hominis*, the closely related *lin*A' gene was harboured by *S. aureus, S. cohnii* and *S. epidermidis* strains [35]. The genes located on small non-conjugative plasmids. The reference plasmid of *lin*A designated pIP855 originated from *S. haemolyticus*, its size is 2.5 kb (1.6 Md) [15]. Gene *lin*A' was isolated from the 2.6 kb (1.7 Md) pIP856 *S. aureus* plasmid [16]. MIC values for strains harbouring these plasmids [14] were identical with those for our isolates. The size and the lack of non-conjugative character of plasmids pBI1, and pBI84 suggest a possible relationship between the plasmids of French and Hungarian origin, however a large plasmid, as the pBI109PGL, encoding also beta-lactamase and aminoglycoside-modifying enzymes, was not published yet.

The *lin* genes were cloned and the gene-specific DNA probes were developed in *E. coli* [14, 16, 17]. In our experiments we used pAT151 recombinant plasmid as *lin*A control constructed from pBR329 vector and a 1.1 kb *Eco*RI/*Acc*I *lin*A harbouring fragment of pIP855 [17, 18], and pAB3 as *lin*A' control was obtained by cloning a 2.6 kb *Hind*III pIP856 fragment conferring lincomycin resistance into pUC8 vector, respectively [18]. That fact explains the aspecific hybridization observed on 2.7 kb fragment of pAB3 using M13mp18-dependent gene probes.

All strains harbouring neither *lin*A nor *lin*A' gene possess a related 178 bp fragment, the so-called *lin*A-like sequence [14]. Thus *lin*A-like probe is usable for screening of presence of any gene belonging to the *lin*-family.

In this preliminary study we describe two small 1.7 Md non-conjugative plasmids of *S. epidermidis* conferring enzymatic inactivation of lincosamides and a larger one exhibits a new class of *lin* plasmids harbouring also beta-lactamase and aminoglycoside-modifying enzyme production. Both types of plasmids harbour a gene belonging to the *lin*A relation group but differring from both known genes. Further experiments are involved to clear up phylogenetical connexions of *lin*-related genes. Experiments for clarifying the exact localization of genes on the novel pBI109PGL plasmid and for determining the mechanism of resistance are in progress.

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# BOOK RECEIVED

## J. P. Armitage and J. M. Lackie (eds): Biology of the Chemotactic Response, Soc. Gen. Microbiol., Symp. 46. Lectures held at the University of York, 1990, December. Cambridge University Press, Cambridge–New York–Port Chester–Melbourne–Sydney, 1991. Hardback, 404 pages, 103 figures and tables. Price Ł 55 or USD 110.

Research workers tend to accept symposial editions with suspicion, because these are frequently just collections of short papers dealing with partial and minor problems of a topic. This "Biology of the Chemotactic Response" does not belong to such kind of symposium editions. Even the reader who is interested only superficially in this field can obtain an adequate information about the present status of our knowledge on chemotaxis.

The first three papers are concerned with the general theoretical problems: G. A. Dunn considers the basic principles of taxis, kinesis and stimulus. Schnitzer et al. provide a mathematical model for the "strategies" in chemotaxis and R. T. Tranquillo is similarly modelling the questions of chemoattractant gradient and the chemotactic movement response.

On the other hand, R. M. Macnab gives a summary on the genetics, structure and the morphogenetic pathway of bacterial flagellum based on recent data of *Escherichia* and *Salmonella* research. Hazelbauer et al. describe the modern views of the chemotactic signal transfer, while Matsumura et al. are discussing the signal complex.

Dingwall et al. compare the above facts with the flagellar biogenesis, chemotaxis, etc. of the monotrichous *Caulobacter*. J. P. Armitage et al. make an overview on the methylation-independent taxis in different bacteria. The paper of D. R. Zusman et al. concerns with the chemotactic motility of a gliding species of *Myxococcus xanthus*, while D. Desterhelt and W. Marwin are discussing these topics in the case of *Halobacterium halobium* including a representative of the "third branch of the living world" — an archaebacterium.

The presentations of J. E. Segall, P. C. Newell, and J. van Houten comprise protozoonic chemotaxis and signalling systems in *Dictyostelium discoideum* and *Paramecium*.

The field of the last three lecture are the eukaryotic cells, namely leukocyte chemotaxis in general (P. C. Wilkinson), about their signal transduction between receptor and motor apparatus (P. H. Naccache et al.) and about the inflammatory leukocyte chemoattractans (K. B. Bacon et al.).

The lectures are accompanied by well-constructed figures, closed by a list of up-to-date literature and the volume has a subject list.

In opinion of the Reviewer, some of papers are very useful for all microbiologists, immunologists, and for all members of the medical, veterinary, etc. communities who are interested e.g. in the general problem of phagocytosis.

*Iván Kétyi*

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# INSTRUCTIONS TO AUTHORS

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**Abstract.** This should not exceed 200 words and should outline briefly the purpose of the study and detail important findings and the authors' principal conclusions. Redundant phrases, generally known information and repetition should be avoided.

**Introduction.** This part should state briefly the nature and purpose of the work and cite recent important work by others.

**Materials and methods.** Describe microorganisms, methods, apparatus, procedure and statistical methods in sufficient detail to allow other authors to reproduce the results. This part may have subheadings like "Bacterial strains" or "Culture media".

**Results.** The experimental data should be presented clearly and concisely. Avoid repeating information presented in tables and figures.

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*Acta Microbiologica Hungarica, 39 (2), pp. 105–132 (1992)*

# DATA FOR THE PROPERTIES OF *LISTERIA* STRAINS

(A REVIEW)

B. RALOVICH

*Hungarian Meat Research Institute, Budapest*

(Received October 22, 1991)

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It seems that taxonomy of listeriae is in a fluid stage. Between beta-haemolytic property and virulence of these bacteria a strong relationship has been demonstrated. These characters are coded by *hly*A gene which seems to belong to a monocistronic unit. Virulent *Listeria* strains possess specific

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surface antigens and can enter as well as multiply in host cells. Their exact human virulence is not known. Listeriae can grow from around 0 to 42 °C and can survive either the effect of frost or of temperature of 48 °C. They may form biofilm on different surfaces. Their growth kinetics, persistance and heat resistance can be influenced by many factors (type of growth environment, pH, acidulants, salts, chemicals, antibiotics, plant substances, enzymes, humidity, atmosphere, temperature, prior temperature-effects, microbial competition and the length of influences). Radiation sensitivity of *Listeria* is usual. Plasmid mediated antibiotic resistant mutant of *Listeria monocytogenes* is verified.

## Introduction

The WHO and FAO Expert Committee rediscussed microbiological aspects of food hygiene in 1976. *Listeria monocytogenes* was not listed among bacteria which could cause food-borne disease. Different aspects of this concept were disputed in 1983 at Budapest [1]. During the past 10 years very important changes have happened. The number of bacteriologically confirmed cases of listeriosis has generally increased. Smaller or more serious epidemics were observed over the world. The attention was focused on the transmitter role of foodstuffs. Because of the epidemic outbreaks, the high rate of mortality and the food-borne character of these infections WHO organized two discussions [2, 3] and supported another one [4]. Besides these different meetings have also be arranged [5–8].

Irritant opinions which were in contrast with both the microbiological and epidemiological facts and medical mentality could also be read [9].

Now it is quite clear that good animal husbandry, food technology and food hygiene play important role in prevention of human listeria infections. For the purpose of the effective prevention the newest informations on characters of *Listeria* strains are summarized in this review. *L. monocytogenes* was first described by Murray et al. [10]. From ill patients of mononucleosis it was isolated by Nyfeldt in 1929 [11].

## Taxonomy

*Taxonomic position* of *Listeria* strains has changed time to time. At presents, the genus *Listeria* consists of seven species *(L. monocytogenes, L. innocua, L. welshimeri, L. seeligeri, L. ivanovii, L. grayi* and *L. murrayi)* which are biochemically very similar to each other but are somewhat different in DNA homology [12–14]. We think perhaps more adequate to subdivide the genus to three species only [15]. The first one may be *L. monocytogenes* which has four subspecies or biotypes *L. monocytogenes* subsp. *monocytogenes, innocua, welshimeri* and *seeligeri*, the second is *L. ivanovii* and the third *L. grayi* which consists of two subspecies or biotypes: *grayi* and *murrayi*. King et al. [16] published that "... among

the *Listeria* there is a very tight cluster of species highly related to *L. monocytogenes* including *L. innocua*, *L. welshimeri*, *L. seeligeri*, *L. ivanovii*. Although some species level sequence variation is observed, for the most part it is no greater or more consistent than that which is observed at the strain level among *L. monocytogenes* isolates, for example. This holds true even for the most variable regions of 16S and 23S rRNA. In contrast *L. grayi* and *L. monocytogenes*, which clearly highly related to the other *Listeria* show a more consistent pattern of nucleotide sequence differences with them."

*The members of this genus* are Gram-positive, short to long rods which contain flagellae. Their most important differential diagnostic properties are listed in Table I.

**Table I**

*Characteristic properties of Listeria species*

| Species | Beta-haemolysis | Pathogenicity | Acid from Rh | Acid from Xy | Acid from Ma | $NO_2$ from $NO_3$ | $H_2O_2$ activity | Arginine hydrolysis |
|---|---|---|---|---|---|---|---|---|
| *L. monocytogenes* | + | + | + | – | – | – | + | – |
| *L. ivanovii* | + | + | – | + | – | – | + | – |
| *L. seeligeri* | d[a] | d | – | + | – | – | + | – |
| *L. innocua* | – | – | d | – | – | – | + | – |
| *L. welshimeri* | – | – | d | + | – | – | + | – |
| *L. grayi* | – | – | – | – | + | – | + | – |
| *L. murrayi* | – | – | d | – | + | + | + | – |

[a] Different. Rh, rhamnose; Xy, xylose; Ma, mannitol

## Pathogenicity

*The pathogenic property* of *Listeria* has been studied since 1924 [10]. We have emphasized several times that a strong relationship exists between beta-haemolytic ability and virulence of these strains, however, exceptions can also be observed. Beta-haemolysis on sheep or horse blood agar is one of the best and most simple marker of virulence [17–19]. Haemolytic *L. monocytogenes* possesses the ability to enter and multiply in host cells. This process was observed during ocular infections of human and animals [20, 21], and later in experimental listeria cystitis and enteritis [22, 23]. Ralovich et al. [24] published that their non-haemolytic *L. monocytogenes* strain (serotype 4ab, No. 10) could gain entrance into the ocular epithelial cells but it was not able to multiply in them and therefore, did not cause keratoconjunctivitis. Other researchers verified that non-haemolytic mutants of *L. monocytogenes* did not escape from the phagosomes and did not appear free in the cytoplasm [25]. Recent genetic

studies verified the pathogenetic importance of haemolysin (listeriolysin O) [25–27]. Haemolysin production and virulence is coded by *hly*A gene. The gene was completely sequenced [28]. The listeriolysin O gene seems to belong to a monocistronic unit [29]. Haas et al. [30] established a library of chromosome DNA of *L. ivanovii* in the pTZ19R plasmid system using *Escherichia coli* DH5 alfa as the host. These genes directed the production of exoproteins which were involved in cytolytic process. Kreft et al. [31] purified and characterized cytolysines from *L. monocytogenes* serovar 4b and *L. ivanovii* strains. They produced several phenotypic mutant of *L. ivanovii*.

The virulence of the *Listeria* strains is a multifactorial phenomenon that can spontaneously disappear. The spontaneous lost of haemolytic activity is known [32]. Changes in the original antigenicity and/or biochemical properties of the strains can be expected. The change in the different characteristics does not necessarily take place at the same time, therefore, different variants may be formed [33]. We found spontaneous alteration in phage type, and also change of phage type in consequence of induced mutation [34]. Some observations have supported the hypothesis that virulent *Listeria* possesses specific surface antigens. Presence of these antigens may inhibit the agglutination of the virulent *L. monocytogenes* strains serotype 1/2 in the antiserum prepared by *Listeria* No.1830 [33]. Kuhn and Goebel [35] isolated a mutant of *L. monocytogenes* which was impaired in the synthesis of a major extracellular protein (p60). The p60 mutant lost the capability of invading non-professional phagocytic 3T6 mouse fibroblast cells. Kathariou and Pine [36] verified the existence of extracellular proteins, too. They also published that the production of listeriolysin is usually sensitive to several environmental factors including components of the culture media and temperature of growth of the bacterium.

Virulent *Listeria* strains are pathogenic for both humans and animals. Their virulence is determined genetically, however, its phenotypical expression or degree is strongly influenced not only by the condition of the strain but also by biological circumstances of the host which may play an important role in manifestation of an outer *Listeria* infection – see the effect of underlaying illnesses [18, 37–40], or in the transformation of a symptom-free carrier state into a disease – see cofactors [18–41]. The exact human virulence of *Listeria* strains is not known. It is supposed that the consumption of a food containing virulent listeriae in a germ count higher than $10^3$/g may be dangerous for a healthy person. In a pregnant woman or an immunocompromised host less listeriae may also cause disease. Factors influencing the virulence of *Listeria* were summarized [18]. Iron is a critical determinant of the host-parasite interaction. Despite the abundance of iron in a host, its availability to microbes is restricted by the iron-binding and transport systems of the host. The acquisition of iron is thus essential, although not sufficient, for virulence [42]. Cowart and Foster [43] found that virulent *L. monocytogenes* strains exhibited faster rates of

growth as a function of iron than did the avirulent strains. They also noted that serum was microbiostatic, but this microbiostasis was overcome either by saturating the serum transferrin with iron or by increasing the number of organisms initially inoculated into the serum. They observed that listeriae removed iron from a complex by a reductive pathway.

Golnazarian et al. [44] studied infectious dose of *L. monocytogenes* and those factors which could influence its value. They observed that hydrocortisone treatment decreased the $ID_{50}$ value significantly, pregnancy also decreased it but the difference was not significant. Susceptibility of mutant and cimetidine treated mice was not different from that of normal controls. Schlech III [45–46] wrote that oral feeding of virulent *L. monocytogenes* to pregnant Sprague-Dawley rats resulted in fetal loss, including stillbirth. Severity of the infection was dose dependent. Conner et al. [47] studied food-borne, environmental and clinical isolates of *L. monocytogenes* (218 strains) and other *Listeria* species (32 strains) for pathogenicity in immunocompromised mice. All non-monocytogenes species were non-pathogenic. Fifteen out of the 218 *L. monocytogenes* strains were also non-pathogenic. Pathogenic isolates were haemolytic for sheep blood. In contrast, many non-pathogenic isolates were weakly haemolytic. The initial source and serotype of the isolate appeared not to be related to pathogenicity in immunocompromised mice. Some other data indicate an association between human virulence and serological type of *L. monocytogenes* [48]. Ortel supposed a link between phage-pattern and meningitis causing ability of *L. monocytogenes* serotype 4b strains [40].

## Other characteristics

*Listeria* strains have some very interesting properties. Data connected with those will be presented in the following parts of this review.

### *Growth temperature*

*Listeria* strains can multiply from around 0 °C through between 45 and 50 °C. Walker et al. [49] observed that minimum growth temperatures ranged from −0.1 to −0.4 °C for three strains. Lag times of 1–3 d and 3 to >34 d were at 5 and 0 °C, respectively, and the corresponding generation times ranged from 13–24 h and 62–131 h in chicken broth and UHT milk. Junttila et al. [50] published that values of minimum growth temperature varied between 0.5 and 3.0 °C for 100 *Listeria* strains on Trypticase Soy Agar – TSA –. Their results showed that *L. monocytogenes* strains grew better than non-haemolytic listeriae under cold conditions. Schaack and Marth [51, 52] observed that *L. monocytogenes* could grow in autoclaved (121 °C, 15 min) sterile skim milk at 21, 30, 37 and 42 °C. Papageorgiou and Marth [53]

inoculated *L. monocytogenes* in autoclaved samples of skim milk and deproteinated whey strengthened with 6 or 12% NaCl and incubated at 4 and 22 °C. The pH values of the 6% salted whey, 6% salted skim milk, 12% salted whey, and 12% skim milk were 5.65, 6.20, 5.50, and 6.00, respectively. Generation times of *L. monocytogenes* strains in 6% salted whey at 22 °C (3.67 and 3.56 h) were significantly shorter than those in 6% salted skim milk at 22 °C (4.31 and 4.42 h). At 4 °C they ranged between 37.49 and 49.43 h in 6% salted products. Maximum populations reached at 22 and 4 °C were significantly higher in 6% salted whey than in 6% salted skim milk. *L. monocytogenes* gradually decreased in numbers in 12% salted products incubated at 22 °C. One strain was inactivated within 85 d in 12% salted skim milk or within 110 d in 12% salted whey, the other survived for more than 130 d under the same conditions. Loss of viability by both strains was similar in 12% salted whey and skim milk after 130 d of storage at 4 °C. Ferguson and Shelef [54] inoculated pasteurized or sterilized commercial soymilk and stored at 22 or 5 °C. Doubling times were 1.33 h at 22 °C and 1.57 d at 5 °C. *Listeria* population persisted in soymilk with little change for more than 36 d at 22 °C for 2 months at 5 °C. Foegeding and Leasor [55] studied the growth of *L. monocytogenes* strains in liquid whole egg. Each strain grew in it at temperatures between 4 and 30 °C except one which did not grow at 4 or 10 °C. Generation times ranged from 24 to 51 h at 4 °C and from 7.8 to 31 h at 10 °C. Viability of *L. monocytogenes* was studied in raw and heat-treated (121 °C, 15 min) whole eggs, albumen, or yolks during storage at 5 and 20 °C [56]. Studies with raw eggs showed that the organism grew only in egg yolks (generation times of 1.7 d and 2.4 h at 5 and 20 °C, respectively). Cell numbers in whole eggs initially declined and then levelled off. A sharp decline in cell numbers was observed in the raw albumen. In contrast, *Listeria* grew in all heat-treated egg samples. Generation times for cooked whole eggs, yolks, and albumen were 1.9, 2.3, and 2.4 d at 5 °C, and 2.6, 2.6, and 3.5 h at 20 °C, respectively. Notermans et al. [57] controlled the survival of *Listeria* species in liquid egg products (albumen, yolk, whole egg) and whole egg containing 25% sucrose. *Listeria* strains were inactivated in egg albumen at 22 °C. At 4 °C no inactivation was found in any of the all products. If sucrose was added to the whole egg product a pronounced and temperature-dependent decrease in numbers of *Listeria* took place. After prolonged incubation *Listeria* displayed a resistance to the defence mechanism. Sizmur and Walker [58] examined 60 samples of salads. The listeria-positive samples were left at 4 °C for four days. The numbers of listeriae increased indicating that the organism could survive and multiply during storage of the products. Steinbruegge et al. [59] studied the ability of *L. monocytogenes* to survive and grow on head lettuce. Contaminated samples of lettuces were placed into plastic bags and stored at 5, 12 and 25 °C. Lettuce juice was also inoculated and stored at 5 °C. Behaviour of *L. monocytogenes* was variable. In most trials, numbers increased by several log cycles during 14 d of storage, but in several cases growth

never occurred or did not persist for 14 d. Lettuce juice held at 5 °C was able to support growth of *L. monocytogenes*. Berrang et al. [60] inoculated vegetables with *L. monocytogenes* and stored at 4 and 15 °C under controlled atmosphere storage and air. *L. monocytogenes* was monitored over 21 d at 4 °C and 10 d at 15 °C. It was observed that the populations increased during storage and controlled atmosphere storage did not influence the rate of growth.

*Biofilm-formation*

*Listeria* strains have a very interesting character. They can attach to stainless steel, glass, polypropylene and rubber surfaces after short contact times (20 min or 1 h) at ambient (20 °C) and cold (4 °C) temperatures. They produce a fibrous-like material similar in appearance to acidic polysaccharide fibrils which were observed by Herald and Zottola [61] in case of *Yersinia enterocolitica* [62, 63]. Real importance of this "biofilm-formation" is not known yet. Frank and Koffi [64] published that surface adherent microcolonies of *L. monocytogenes* were more resistant to benzalkonium chloride, anionic acid sanitizer and heat than the planktonic cells. Shin Ho Lee and Frank [65] inactivated *L. monocytogenes* cells adherent to stainless steel with hypochlorite. They observed that allowing *L. monocytogenes* to grow on stainless steel surfaces for a matter of days would significantly increase its resistance to hypochlorite sanitizer.

*Heat resistance*

Golden et al. [66] studied the effect of heating on 4 *L. monocytogenes* strains in TPB. Only slight decreases in the viable populations of the strains were detected after 75 min at 50 °C. D-values of the strains were at 52 °C between 38.3 – 64.1 min; at 54 °C between 14.4 – 31.6 min and at 56 °C between 5.7 – 16.0 min. Populations of three strains were reduced to non-detectable levels after 10 min at 60 °C but viable cells of one strain were recovered for 30 min. All strains heated at 52, 54 and 56 °C were injured and their sensitivity to NaCl on TPA increased.

Several publications reported the possibility that *L. monocytogenes* might survive the minimum heat treatment required to pasteurize milk. Doyle [67] infected cows with *L. monocytogenes*, verified that the organism was within the milk polymorphonuclear leukocytes, heated the milk in a HTST pasteurizer and controlled the milk for *Listeria*. Surviving cells were recovered from milk of six of the nine trials done at 71.4 – 73.9 °C for 16.4 sec, but were not detected in milk heated at 76.4 – 77.8 °C for 15.4 sec. Farber et al. [68], Farber [69] observed that no viable *Listeria* were detected in milk after the treatment of 16.2 s at temperatures of 69 °C and above, however, their survival at 60 – 67.5 °C was verified. Fernandez [70] wrote:

"we must conclude that a normal or correct pasteurization of milk is usually sufficient to destroy *Listeria*." Lemaire et al. [71] had the same opinion. Lovett et al. [72] stated that HTST (71.7 °C for 15 s) pasteurization inactivates *L. monocytogenes*. Donelly's conclusion was: "... no studies to date have suggested that currently employed pasteurization procedures have presented problems of survival of *L. monocytogenes* ..." [73]. The thermal resistance of one strain of each of *L. ivanovii, L. seeligeri* and *L. welshimeri* and three *L. monocytogenes* strains was determined in raw and sterile milk [74]. The samples were heated at temperature ranging from 52.2 to 71.1 °C for various contact times. D-values in raw milk at 57.8 °C were between 128.7 – 151.3 s for *L. ivanovii, L. seeligeri* and *L. welshimeri* and 330.0 – 528.6 s for *L. monocytogenes* strains; at 63.3 °C 18.8 – 32.0 s and 31.0 – 46.1 s; and at 68.9 °C 2.1 – 2.9 s and 2.8 – 4.0 s, respectively. In sterile milk at 52.2 °C D-values were between 1247.9 – 1779.1 s and 1704.8 – 2848.3 s; at 57.8 °C 179.1 – 228.7 s and 290.2 – 440.5 s; at 63.3 °C 20.1 – 25.5 s and 49.6 – 68.0 s; and finally at 68.9 °C 3.4 – 5.2 s and 6.2 – 9.1 s, respectively. That is, the heat resistance of *L. monocytogenes* appeared somewhat greater than that of the other *Listeria* spp. in both kinds of milk, but the difference was not statistically significant. They stated that HTST processing is adequate for pasteurization of raw milk.

Farber et al. [75] found that D-values of 10 strains of *L. monocytogenes* in ground meat and ground meat with cure (salt, white pepper, nitrite, dextrose, lactose, corn syrup) ranged from 1.01 min at 62 °C to 13.18 min at 56 °C and from 7.06 min at 62 °C to 50.0 min at 58 °C, respectively. Mackey et al. [76] determined D-values of 27 *L. monocytogenes* and 2 *L. ivanovii* strains in Tryptone Soya Broth – TBS –. They observed a fourfold differences in $D_{57}$-values (from 6.5 to 26 min) between the least and most heat resistant strains, but no strain was unusually heat resistant. $D_{70}$-values were between 0.1 – 0.2 min in meat. They stated that a treatment equivalent to heating at 70 °C for 2 min was sufficient to inactivate *L. monocytogenes* during cooking of raw meat and that curing salt (3.5% NaCl, 200 ppm nitrite and 300 ppm nitrate) enhanced heat resistance of *L. monocytogenes* in the mixture of beef and beef fat.

In the case of hot dogs a minimum internal temperature of 71.1 °C may be borderline in term of ensuring a listeria-free product [77]. Zaika et al. [78] published that the process for cooking frankfurters (70 min for the frankfurter to reach an internal temperature of 71.4 °C) should kill *L. monocytogenes* ($10^3$ or less/g) encountered in raw meats, but they also mentioned that the margin of safety for the process was relatively small, particularly, if higher levels of *L. monocytogenes* were present. Heating beaker sausage to an internal temperature of 62.8 °C for 1 h inactivated listeriae to undetectable levels; in contrast, heating pepperoni at 51.7 °C for 4 h inactivated *L. monocytogenes* only after the drying cycle but not before it [79]. Bhaduri et al. [80] determined thermal destruction of *L. monocytogenes* strains in a

liver sausage slurry (1:1, liver sausage batter and water) using a submerged ampoule technique. D-values for *L. monocytogenes* Scott A grown at 37 °C were 8.9 min at 57.2 °C, 2.4 min at 60.0 °C, and 1.1 min at 62.8 °C (Z = 6.2 °C) based on analysis of the linear portion of the survivor curves. D-values of 6.6, 1.6 and 0.4 min (Z = 4.65 °C) were obtained when the data were analyzed using non-linear techniques. $D_{60}$-values was 1.0 min for another strain and 1.6 min for the third one. When Scott A was grown at 19 °C, there was a decrease in thermal resistance ($D_{60}$ = 0.8 min). Their date indicated that *L. monocytogenes* had a thermal resistance in liver sausage comparable to that observed in other food system.

The ability of *L. monocytogenes* to survive and proliferate on chicken processed using moist heating method was investigated by Harrison and Carpenter [81]. Chicken breast samples were inoculated with $10^6 - 10^7$ microorganisms/g, cooked to one of five different cooking temperatures (65.6, 71.1, 73.9, 76.7, 82.2 °C), then either vacuum packaged or wrapped in an oxygen permeable film and stored at 4 °C for up to 4 weeks or at 10 °C for up to 10 days. By the fourth week of storage at 4 °C, the *L. monocytogenes* population in all of the samples, except those cooked to 82.2 °C increased significantly. Storage at 10 °C allowed microbial populations in 6 of the 10 treatments to significantly increasing within 10 days.

Lund et al. [82] found that in a domestic 650 W oven cooking poultry to an internal temperature of about 70 °C resulted in a $10^6$-fold lethality to *L. monocytogenes*. In contrast, the surviving listeriae were observed on chicken breasts cooked to one of five different internal temperatures (65.6, 71.1, 73.9, 76.7, 82.2 °C) in a microwave oven then vacuum-packaged or wrapped in an oxygen permeable film and stored for up to 4 weeks at 4 °C or 10 d at 10 °C [83]. The presence of listeriae on cook-chill food after microwave cooking to an internal temperature at 70 °C was reported by Sheeran et al. [84], too. Gilbert et al. [85] found that the retail precooked, ready-to-eat poultry and chilled meals (mainly poultry) showed high frequency of listeria contamination (12% and 18%, respectively), which fact could be explained by inadequate cooking and/or recontamination after cooking. Similar findings had been published by Kerr et al. [86].

There are some data relating to eggs, too. Foegeding et al. [55, 87] wrote that D-values of each *L. monocytogenes* strain in raw liquid whole egg were similar to D-values reported in milk. They ranged from 22.6 min at 51 °C to 0.20 min at 66 °C. Minimal pasteurization parameters (60 °C, 3.5 min) for liquid whole egg resulted in 99–99.9% inactivation (populations reduced 2 to 3 log cycles) of the *L. monocytogenes* strain tested. Thermal death times (F-values) for *L. monocytogenes* were also determined between 62 and 73 °C a submerged capillary tube procedure [87]. At 62 °C, F = 16 min and at 69 °C F = 1.6 min. Minimal pasteurization of egg would not result in product free from *L. monocytogenes* if initial populations were large.

*Effect of frost*

*Listeria* strains could survive the frost [66, 88, 89]. However, Golden et al. [66] observed that a storage at −18 °C in Tryptose Phosphate Broth − TPB − resulted in injury of *L. monocytogenes* cells. The injured cells were recovered on Tryptose Phosphate Agar − TPA − but not on TPA supplemented with 8% NaCl [66]. Palumbo and Williams [90] studied the ability of *L. monocytogenes* to survive freezing and frozen storage at −18 °C in ground beef, ground turkey, frankfurters, canned corn, ice-cream mix, and tomato soup. Injury of *L. monocytogenes* as a result of freezing and frozen storage was also investigated. *L. monocytogenes* survived freezing and frozen storage well in five of the examined foods, except tomato soup and the organism was not injured when the pH of the foods was 5.8 or above, and was quantitatively recovered on listeria-selective media. In contrast, the organism showed a decline in viable count after extended frozen storage in tomato soup, was injured, and could not be quantitatively recovered in listeria-selective media.

*Prior temperature-effects*

Prior temperature-effects on *Listeria* were also studied. Buchanan and Klawitter [91] examined the effect of pre-inoculation temperature on the subsequent growth of *L. monocytogenes* at 5 °C in TPB, UHT milk, canned dog food and raw ground beef (untreated and irradiation-sterilized). In TPB, the duration of the lag phase was decreased when aerobic and anaerobic cultures were initially grown at ≤28 and ≤13 °C, respectively. Differences in lag phase durations were also observed when *L. monocytogenes* initially cultured at 19 and 37 °C were grown at 5 °C in UHT milk and some of the dog food varieties. Subsequent exponential growth rates and maximum population densities of the 5 °C cultures were not affected by temperature history. Growth of *L. monocytogenes* was not observed in either untreated or irradiation-sterilized raw ground beef. While temperature history could affect the growth kinetics of *L. monocytogenes* at 5 °C, it did not account for the lack of growth in raw meat, suggesting that there is an inhibitory condition or component in ground beef that is lost upon cooking. Smith et al. [92] studied the effect of growth temperature on *L. monocytogenes*. It had a profound effect on injury and death of washed cells that were suspended in phosphate buffer and exposed to 52 °C for 1 h. The temperature had low lethality for cells grown at 37 or 42 °C, but there was a $10^3-10^4$-fold increase in killing for cells grown at 28, 19, 10 or 5 °C. There was little injury with exposure to 52 °C of cells grown at 5, 10 or 19 °C, but injury increased as the temperature of growth increased. When cells were grown anaerobically, lethality induced at 52 °C increased as the growth temperature decreased, but there was more injury under anaerobic conditions than for aerobically grown cells. The results

indicated that *L. monocytogenes* cells growing at low temperatures were more susceptible to heat induced death.

Prior heat-shocking of *L. monocytogenes* in medium or cured meat (at 48 °C for 2 h) resulted in an increase in survival on the strain during the final heat-treatment (64 °C). When the heat-shocked bacteria were held at 4 °C their thermotolerance could be detected 24 h after the prior heat shocking yet [93, 94]. Linton et al. [95] heat-shocked cells of *L. monocytogenes* Scott A in TSB + 0.6% yeast extract – TSB+YE – at 40, 44 and 48 °C for 3, 10 and 20 min, followed by heating at 55 °C for 50 min in order to determine an optimum heat shock response. Most heat shocking temperatures significantly increased thermal resistance. Increasing heat shock temperature and time allowed the organism to survive much longer than non-heat-shocked cells at 50 to 65 °C. The optimal heat shock condition was 48 °C for 20 min where D-values at 55 °C increased 2.3-fold in non-selective agar and 1.6-fold in selective agar. Heat shocking conditions may be created in pasteurization or minimal thermal processing of food allowing increased heat resistance of pathogenic and spoilage microorganisms.

*Tolerance to pH*

Several studies indicate that *Listeria* strains could tolerate wide range of pH (3.8–9.2) in bacteriological media. The tolerated pH was very dependent on the incubation temperature, oxygen tension and salt concentration. Sorrelles et al. [96] verified that *L. monocytogenes* could grow at pH 4.4 and could persist as well as tolerate a low pH at low (10 °C) temperature. Parish and Higgins [97] studied survival of *L. monocytogenes* at pH levels of 3.6–4.8 in pH adjusted orange juice where growth did not occur at 4 °C. Survival times were less for lower pHs but death occurred more slowly at refrigeration temperatures than at 30 °C. It was also observed by Cole et al. [98] that *Listeria* survival at low pH and high salt concentration was strongly temperature dependent. When the pH was low (<4.4) and salt concentration high (>10%) *Listeria* strains could survive for weeks only at low temperature (5 °C). When the incubation temperature was 30 °C then growth could be observed at pHs higher than 4.6 in the presence of salt (0–12%). When the temperature was 10 °C then listeriae grew at pHs >4.8 and 0–10% of salt concentration. But at 5 °C multiplication was only observed at pH 7.0 and 0–8% of salt concentration. Buchanan and Klawitter [99] described that when the pH was 4.5 *Listeria* cells in TPB only survived at 5–10 °C, multiplied at 19–28 °C and they died off relatively rapidly at 37 °C under aerobic condition. Their opinion was that *L. monocytogenes* better tolerated adverse conditions when the incubation temperature was somewhat suboptimal and/or oxygen concentration was lowered.

The fate of *L. monocytogenes* during refrigerated storage was determined on several processed meat products including ham, bologna, wieners, sliced chicken, sliced turkey, fermented semidried sausage, bratwurst and cooked roast beef. The meats were surface inoculated, vacuum packaged and stored at 4.4 °C. *L. monocytogenes* survived but did not grow on summer sausage, grew only slightly on cooked roast beef, grew well on some wiener products, ham, bologna and bratwurst and grew exceptionally well on sliced chicken and turkey. The rate of growth depended largely on the type and the pH of the product [100]. Karches and Teufel [101] stated that the fate of listeriae in fresh Zwiebelmettwurst depended mainly upon the pH of the sausage. The pH could be lowered by cure and/or starter culture and the multiplication of *L. monocytogenes* was inhibited for some days.

Papageorgiou and Marth [102] observed that *L. monocytogenes* could grow in Feta cheese during the initial stage of ripening and survived for more than 90 days in it at 4 °C even the pH was low (4.3). Results of their other work [103] indicated that *L. monocytogenes* could also grow during the initial stage of manufacture of Blue cheese until its pH was reduced to 5.0 or below. The rate of death of the organism during the first 50 days of ripening was substantial, but later, as the pH of cheese increased listeriae survived more successfully. Growth of *L. monocytogenes* was registered in orange serum prior to the reduction in viable cell numbers at pH 4.8 and 5.0 during different temperatures of storage [97].

*Acid-injury*

Although *Listeria* strains can tolerate acid conditions, "acid-injury" of these germs is known [104]. Injuring effect of different acidulants was not the same. Hydrochloric acid was less inhibitory than the organic acids, and there was some difference among the effect of the organic acids, too. Acetic acid was most detrimental to *L. monocytogenes* followed in order by lactic and citric acids. It was observed that *L. monocytogenes* did not survive the effect of 0.12% acetic acid and 0.25% lactic and citric acids in Nutrient Broth – NB – at 37 °C [105, 106]. Temperature could also affect the effect of the acids [96, 106]. In case of cold-pack cheese foods the acidification with lactic acid, lactic plus acetic acid or acetic acid decreased the length of survival of *L. monocytogenes* [107].

Conner et al. [108] also observed that inhibitory pH of different acids is different: pH 5.0 for propionic acid, 4.5 for acetic and lactic acids and 4.0 for citric and hydrochloric acids. Initial populations of viable cells were reduced to $<10$ c.f.u./ml within 1-3 weeks at 30 °C, whereas at 10 °C *L. monocytogenes* survived for 11-12 weeks in acetic, citric or propionic acid-adjusted media and for 6 weeks in media adjusted with HCl or lactic acid. Ita and Hutkins [109] studied the effect of citric, acetic, and hydrochloric acids on growth, survival and intracellular pH ($pH_{in}$)

of *L. monocytogenes*. Under the experimental conditions, *L. monocytogenes* grown in TSB-YE survived even when the pH was reduced to 3.5. Although citric and lactic acids were more effective in lowering the $pH_{in}$, acetic acid had the greatest effect on cell survival. Their results suggested that inhibition of *L. monocytogenes* by acids was caused not by a decrease in the $pH_{in}$ "per se" but rather by specific effects of undissociated acid species on metabolic or other physiological activities. Zeitoun and Debevere [110] used buffered lactic acid systems and unbuffered lactic acid solutions in their experiments. They studied the effect of treatment on microbial decontamination and on shelf life of poultry. The treatment of poultry carcasses with 10% (w/v) lactic acid/sodium lactate buffer (pH 3.0) immediately after slaughter exerted an antimicrobial effect, maintained a low pH in the chicken skin during storage, and prolonged the shelf life without apparent adverse effects on sensory quality. Dickson [111] observed when acetic acid (0.5 – 2.0%) was included in water spray chilling cycles then their effectiveness is reducing levels of *L. monocytogenes* on beef lean and fat tissue was enhanced. A reduction of up to 3 log cycles (99.9%) was achieved. The reduction was less on lean tissue than on fat tissue.

When viability of *L. monocytogenes* was studied in raw and heat-treated whole eggs, albumen, or yolks during storage it was observed that the pH influenced the length of survival of this microorganism [56]. Laird et al. [112] determined the viability of *L. monocytogenes* strains in artificial egg washwater at different temperatures and pH values. After a 4 h incubation, less than a 1-log decrease in viability of the strains was found at 33 °C with alkaline detergent (pH 8.0-10.5); however, up to a 3-log decrease was found in neutral pH controls lacking detergent. At 42 °C, survival was generally poorer; complete loss of viability was found within 2 h at neutral pH. Viability of all strains was markedly lower in synthetic washwater at the lower pH values (7.0-9.0) containing whole egg than in washwater from which whole egg was omitted.

### *Effect of modified atmosphere*

*Listeria* strains are aerobic and facultatively anaerobic microorganisms. Some anaerobic media – thioglycolate broth, cooked meat broth (Holman) medium [113, 17] – have been proposed to use for their primary cultivation since 1951. Buchanan et al. [114] as well as Buchanan and Phillips [115] verified that oxygen content of the medium must be considered when assessing the growth of *L. monocytogenes* and its sensitivity to the bacteriostatic activities. At 5 °C anaerobic incubation tended to favour growth of listeriae. In the same time anaerobic incubation clearly enhanced the bacteriostatic and bactericidal activity of sodium nitrite. In a medium with pH 4.5 *L. monocytogenes* died off when the incubation was aerobic at 37 °C, but when the milieu was anaerobic after an initial decline in population density some cultures

could recover and survive for a long time. Harrison and Carpenter [81] published that the growth rate of *L. monocytogenes* on chicken breast, processed using a moist heating method and then vacuum-packaged or wrapped in an oxygen permeable film and stored at 4 or 10 °C, was different in consequence of the differences in packaging at both temperatures. Berrang et al. [60] observed that a controlled atmosphere storage (lower $O_2$ conc., higher $CO_2$ conc.) lengthened the shelf life of all vegetables (fresh asparagus, broccoli, cauliflower) but populations of *L. monocytogenes* on them increased and the growth rate of the microorganism was not influenced. Grau and Vanderline [116, 117] checked the growth of *L. monocytogenes* on vacuum packaged beef. The microorganisms could grow at 0 and 5 °C. Their growth was only influenced by the temperature, pH of the lean and the type of tissue. Ingham et al. [118] examined the growth rates of *L. monocytogenes* and *Pseudomonas fragi* on cooked chicken loaf stored under air and two modified atmospheres (MA1: 50% $CO_2$ and 10% $O_2$, MA2: 80% $CO_2$ and no $O_2$) at different temperatures. Neither MA1 nor MA2 were effective to inhibit the growth of listeriae, however, their growth rate was influenced. Farber et al. [119] evaluated microbiological quality of various sandwiched and meats packaged under modified atmosphere (higher $CO_2$ conc. and $N_2$ without normal air). They found *Listeria* strains in eight of the 58 lots. Five out of the strains were *L. monocytogenes*. They stated that *Listeria* could at least survive in the modified atmosphere. Wimpfheimer et al. [120] studied the growth of *L. monocytogenes* and competitive spoilage organisms in raw chicken packaged under modified atmospheres ($CO_2$:$N_2$:$O_2$, 72.5:22.5:5) and ($CO_2$:$N_2$, 75:25), as well as in air at different temperatures (4, 10 and 27 °C). The anaerobic modified atmosphere inhibited the growth of both the aerobic plate counts and *L. monocytogenes* at all temperatures. The modified atmosphere could inhibit the aerobic spoilage flora while allowing pathogenic *L. monocytogenes* to increase. Kallander et al. [121] inoculated shredded cabbage with *L. monocytogenes* and stored it in normal air or a modified (70% carbon dioxide and 30% nitrogen) atmosphere at 5 and 25 °C. Under both the normal and the modified atmospheres at 25 °C colony counts increased within 2 d of storage but then decreased to undetectable levels within 6 d of storage. At 5 °C the counts increased gradually, but only by about 1 log, in both atmospheres. In normal atmosphere at 5 °C after 13 d of storage colony counts decreased which coincided with decreases in cabbage pH and development of spoilage. The increased level of $CO_2$ was ineffective in controlling *L. monocytogenes* at 5 °C. At 25 °C cabbage spoilage was rapid and colony counts declined under both atmospheres of storage.

*Tolerance to salts*

One of the eldest methods of food technology is the use of salt. *Listeria* strains could grow at 12% NaCl concentration (0.91 value of available free water – $a_w$ –) and could survive higher concentration (25.5% – $a_w$ 0.75) also for weeks. They could grow in solutions up to 39.4% sucrose ($a_w$ 0.92), too [122–124]. The catalase, superoxide dismustase, haemolysin activities and heat susceptibility of *L. monocytogenes* could be influenced by salt concentrations higher than 2.5% [125]. Shahamat et al. [122] published that $NaNO_2$ had inhibitory effect against *L. monocytogenes* and its activity was dependent on the temperature, pH and NaCl content. We found that only 10 000 ppm $NaNO_2$ killed *L. monocytogenes* in NB at 37 °C. Junttila et al. [123] also studied this question. They used nitrite (120 ppm) and salt (3.0%) at levels commonly used in the meat industry today and at elevated levels ($NaNO_2$ 200 ppm, $KNO_3$ 300 ppm). The lower concentrations were slightly effective, the higher levels of nitrite and nitrate had a good bacteriostatic effect in Finnish fermented sausage. Buchanan et al. [114] observed that sodium nitrite could have significant bacteriostatic activity against *L. monocytogenes*, if it was used in conjunction with a combination of acid pH, vacuum packaging, high salt concentration and adequate refrigeration. The bacteriostatic activity sodium nitrite was highly dependent on the initial pH of the medium. No activity was evident in cultures having an initial pH of 7.5 even at 1000µg/ml. The only exception was anaerobic/pH 7.5 cultures containing >200µ/ml. Sodium nitrite has substantial bacteriostatic activity when the initial pH of the system was 6.0 and it was more effective against *L. monocytogenes* when the incubation temperature was lowered to 5 °C. Raising the level of NaCl from 0.5 to 4.5% also increased the inhibitory effects of sodium nitrite. Notermans et al. [57] observed a temporary inhibitory effect of 25% sucrose in whole eggs.

Shelef and Yang [126] studied the effect of sodium and potassium in TSB. Concentrations higher than 5% delayed growth of 3 *L. monocytogenes* strains. Experiments in sterile comminuted chicken and beef at 35, 20 and 5 °C showed growth suppression by 4% lactate, which increased with decrease in storage temperature. The organism was consistently more sensitive to lactate in beef than in chicken, displaying and extended lag phase of 1-2 weeks at 5 °C. Combination of lactate (4%) with NaCl (3%) or nitrate (140 ppm) did not enhance the effect.

*Effect of relative humidity*

Doyle et al. [127] found that *L. monocytogenes* could survive the process of spray drying of milk (inlet temperature 165 ± 2 °C; outlet temperature 67 ± 2 °C) to a moisture content of 3.6 to 6.4%. A reduction of ca 1 to 1.5 $\log_{10}$ *L. monocytogenes*

occurred during the process. Listeriae could persist for at least 12 weeks in non-fat dry milk held at 25 °C, if sufficient numbers (ca $10^5$ c.f.u./g) were present initially. Farrag et al. [128] observed that storage of contaminated sweetened condensed milk for 6 weeks at 21 °C was accompanied by a decrease in population of *L. monocytogenes*. At 7 °C the decrease was minimal or there was none. In contrast, all listeriae grew well in evaporated milk. The ability of *L. monocytogenes* to survive dehydrated storage at different temperatures (5 and 25 °C) and related humidities (75, 59, 35, 14 and 1%) when it was suspended in different menstrua was studied by Palumbo and Williams [129]. *L. monocytogenes* survived longer when held at 5 °C compared to 25 °C, and when suspended in beef extract, glycerol, Karo syrup, skim milk, and canned milk compared to distilled water. The contribution of relative humidity to survival was less clear, though survival tended to be longer at the lower relative humidities. *L. monocytogenes* was not injured during drying or storage at the various relative humidities.

The above mentioned data indicate that the growth kinetics, the persistence and the heat resistance of *L. monocytogenes* are dependent on the interaction of the type of medium (TSA, TPB, TSB, TSB-YE, TB, NB, Chicken B) or foods (milk, cheese, meat, eggs, shrimp, vegetable, juice), the pH, the kind of acidulants (acetic acid, propionic acid, lactic acid, malic acid, citric acid, tartaric acid, hydrochloric acid) as well as salts (NaCl, $NaNO_2$, $KNO_3$, lactate) and their concentration, the atmosphere (aerobic, anaerobic, modified) and the type of packaging (vacuum, gas), the temperature, the humidity, the length of the exposure, the condition of the foods (fresh meat, cured meat, meat product – frankfurter, liver paste –, fermented meat product – sausage, salami –, cooked meat, raw milk, pasteurized milk, skim milk, fermented milk, milk products, soymilk, fresh liquid whole eggs, albumen, yolks, cooked liquid whole eggs, albumen, yolks, raw carrots, cooked carrots, carrot juice, raw cabbage, cabbage juice, fruit juice), the type of meat (beef, pork, chicken, turkey, shrimp), the quality of meat (fatty, lean), the consistency of meat (minced), and the condition of the strain (prior temperature-effects – freeze injury, heat injury –, prior heat shocking, acid injury).

*Mathematical models for growth characteristics*

Buchanan and Phillips [115] developed an empirical mathematical model that effectively described the effects and interactions of temperature (5–37 °C), pH (4.5–7.5), NaCl (5–45 g/l), sodium nitrite (0–1000μ/ml), and atmosphere (aerobic and anaerobic) on the growth kinetics of *L. monocytogenes*. Evaluation of the model indicated that it can be used to provide reasonable "first estimates" of the impact of food formulation and storage conditions on the growth of *L. monocytogenes*. Cole et al. [98] also studied the effect of temperature, salt concentration and pH on both the

survival and growth of *L. monocytogenes* in factorially designed experiments. Simple predictive models describing the effect of hydrogen-ion and salt concentration on the time for at least a 100-fold increase in numbers of *L. monocytogenes* at 10 and 30 °C were constructed. Their model can be used to determine the nature of the interaction between salt concentration and hydrogen-ion concentration on the growth of *Listeria*. These interactions were found to be purely additive.

*Effect of disinfectants*

Wide range of chemicals have been tested to know their effect on *Listeria* strains. El-Kest and Marth [130] determined D-values of available chlorine during the first 30 s of exposure of *L. monocytogenes* cells. The chlorine concentrations were 0.5, 1.0, 2.0, 5.0 and 10 ppm and the D-values were 61.7, 11.3, 6.7, 4.9 and 4.7 s, respectively. They stated that *L. monocytogenes* responded to chlorine much as did other non-sporeforming bacteria, but highly alkaline solutions of chlorine were virtually ineffective as sanitizing agents [131]. Mustapha and Liewen [62] found that sodium hypochlorite and quaternary ammonium compounds were effective against *L. monocytogenes* at the recommended manufacturer's levels. It was also observed that *Listeria* cells were more resistant on pitted SS surfaces and in the presence of high moisture than in vitro. Frank and Koffi [64] studied the susceptibility of surface-adherent microcolonies of *L. monocytogenes* to benzalkonium chloride, anionic acid sanitizer and heat. Their results demonstrated the ability of *L. monocytogenes* to develop resistance to inactivating agents when exposed to specific growth environments.

Lee and Frank [65] determined the inactivation of *L. monocytogenes* cells adherent to stainless steel by hypochlorite. Eight-day adherent cells were over 100 more resistant than the 4 h adherent cells population when exposed to 200 ppm hypochlorite for 30 s. Adherent cells were heated at 72 °C. Both adherent cell populations were inactivated after 1 min. Detectable numbers of *L. monocytogenes* remained on stainless steel slides after treatment at 65 °C for 3 min when adherent 8 d cells were tested but not when adherent 4 h cells were used.

*Susceptibility to preservatives*

Some authors demonstrated that *L. monocytogenes* was susceptible to sorbic acid and its greatest effect was observed at low pHs (<4.8) and low temperatures [132]. Numbers of *L. monocytogenes* steadily decrease in cheese food containing 0.3% sorbic acid or 0.3% sodium propionate, the latter being acidified to pH 5.0–5.1 with lactic and/or acetic acid [107]. Phenolic compounds (phenolic antioxidant tertiary butylhydroquinone and propyl paraben) were significantly more effective

than potassium sorbate against *L. monocytogenes* in a wide pH range ($\leq 7$) at 35 °C [133]. The effect of 3 antioxidants (the concentration of each was 200 ppm) (butylated hydroxyanisole – BHA –, butylated hydroxytoluene – BHT –, and tertiary butylhydroquinone – TBHQ –) on *L. monocytogenes* was studied by Yousef et al. [134]. They observed that TBHQ was more effective in delaying growth of *L. monocytogenes* than BHA and BHT. At permitted concentrations, TBHQ was likely a barrier to growth of *L. monocytogenes* in foods. These antioxidants might support quality and safety of foods, but fat contents of foods could influence their effects. In our country the permitted level of BHT is 150 ppm and that concentration did not inhibit *L. monocytogenes* [88]. Data of El-Shenawy and Marth [135] clearly indicated that *L. monocytogenes* was susceptible to the action of benzoic acid. Higher concentrations caused complete inhibition or, in addition, partial or complete inactivation of the organism during incubation of 117 h at 21 °C or 78 h at 35 °C. The inhibitory effect of benzoic acid was influenced by temperature and pH.

Beef roasts were pumped by Unda et al. [136] with brines containing sodium chloride, phosphates and acetic acid, sodium lactate, potassium sorbate, or glycerol monolaurin, and cooked in-the-bag once or twice to 62.8 °C. Samples were examined during storage at 2, 5, and 10 °C and after mishandling at 25 °C for survival or inoculated *L. monocytogenes*. Listeriae survived once cooking when surface inoculated and two cookings in roasts inoculated internally. Listeria survival was reduced by lactate and monolaurin in recooked surface-inoculated roasts.

*Antimicrobial agents*

Effects of antibiotics and chemotherapeutics have also been examined. Ampicillin, erythromycin, trimethoprim or rifampicin alone or in combination are generally effective against *L. monocytogenes*. One of the most successful synergistic combination is ampicillin and gentamicin [18]. Carvajal et al. [137, 138] studied the treatment of listeria infection in CNS and septicaemia. They found no differences in the antibiotic treatment of survivors and that of non-survivors, but the mortality rate depended on underlying diseases and predisposing factors. Ampicillin alone was as effective as ampicillin combined with aminoglycoside. Poyart-Salmeron et al. [139] isolated a chloramphenicol, erythromycin, streptomycin and tetracycline resistant *L. monocytogenes* from a patient with meningoencephalitis. The genes conferring resistance to these antibiotics were carried by a 37-kb plasmid, pIP811, that was self-transferable to other *L. monocytogenes* cells, to enterococci-streptococci, and to *Staphylococcus aureus*. The dissemination of resistance to other strains of *L. monocytogenes* is likely.

*Inhibitory substances of plants*

Examination of substances originating from plants is an ancient and fascinating field of research. Smoking of food has been used as a means of preservation and flavouring long ago. Antimicrobial effect of 8 liquid smokes (Firmenich, Liquid Smoke HRG, Aro-Smoke, Solu-Smoke, Charsol, Smoke Oil, Liquid Smoke of Szilas, Fumasol) on pure culture of *L. monocytogenes* in NB at 37 °C was studied by us [140]. Charsol and Liquid Smoke of Szilas were the most effective. Both killed listeriae in 0.5% concentration, and Charsol had partial killing effect in 0.25% concentration, too, after 24 h incubation. Presence of 6% NaCl Liquid Smoke of Szilas killed listeriae in 0.25 and 0.12% concentrations, too. Messina et al. [141] examined the antibacterial effect of Charsol-10, Aro-Smoke P-50, Chardex Hickory, Charsol PN9, Char Oil Hickory. All reduced viable cell number of *L. monocytogenes* in 0.5% concentration. In 0.25% concentration Charsol-10 and Aro-Smoke P-50 exhibited strong antimicrobial effect within 4 h. Beef franks dipped in Charsol-10 liquid smoke were shown to have a >99.9% reduction in *L. monocytogenes* numbers after 72 h of storage. Hartemink and Georgsson [142] published that smoking of fish did not seem to inactivate *Listeria* strains. All fish was smoked in a relative low-temperature smoking process. Woodsmoke was used. The concentrations and the effects of the antimicrobial agents in woodsmoke depended on the type of wood, the size and type of the product to be smoked, the time and concentration of the smoke as well as on the temperature.

Pearson and Marth [143] published that cocoa could inhibit *L. monocytogenes* in broth and this effect could be neutralized by casein. Coffeine and theobromine allowed some growth of *Listeria* strains, however, their lag phases and generation times were lengthened [144]. Later they observed enhanced growth of *L. monocytogenes* at 13 and 30 °C in skim milk containing cocoa, sugar and/or carrageenan as well as with or without agitation [145]. Chung et al. [146] studied the effect of six extracts from Chinese medical plants. Three out of them inhibited the growth of three strains of *L. monocytogenes*. Two of these three extracts were effective in cabbage juice, too, but their inhibitory effect could be diminished by protein. Beuchat and Brackett [147] observed that whole and shredded raw carrots and raw carrot juice reduced the population of *L. monocytogenes*. Cooking destroyed the effect of this unknown component(s).

*Effect of enzymes*

Use of enzymes against microorganisms is a natural way of the protection. Ralovich et al. [24] examined the effect of protamine like protein (0.1 and 1.0 mg/ml), apolactoferrin (0.1 and 1.0 mg/ml) and myeloperoxidase system (0.01, 0.025,

0.05 and 0.1 mg/ml) on *Listeria* strains. Only myeloperoxidase system showed bactericidal activity in cell-physiological concentration. Recently, Payne et al. [148] obseved that lactoferrin inhibited the growth of *L. monocytogenes* at 23 and 46 mg/ml concentrations and apolactoferrin had bacteriostatic effect at 15 and 30 mg/ml levels. Siragusa and Johnson [149] wrote that lactoperoxidase system had a temperature dependent inhibitory effect on the growth of *L. monocytogenes* in both broth and milk. Denis and Ramet [150] described that lactoperoxidase system might be effective not only in both TSB-YE and UHT milk but in dairy products also as a safety factor to assist in inhibition of *L. monocytogenes* 10, 20 or 40 ppm concentrations of lactoperoxidase were investigated. Hughey et al. [151] showed that egg white lysozyme killed or prevented growth of *L. monocytogenes* in several foods. Lysozyme was more active in vegetables than in food of animal origin. For maximum activity in certain foods, EDTA was required in addition to lysozyme. Their results suggested that egg white lysozyme could be useful as a preservative to protect against *L. monocytogenes* contamination in several foods, especially those prepared from fresh vegetables.

*Antagonistic effects of other microorganism*

Competition among microorganisms is a well-known phenomenon. Errebo Larsen [152] found that different species of Gram-negative bacteria, *Streptomyces* and moulds exhibited antagonistic effect to *Listeria* strains. Inhibitory effect of *Pseudomonas* species on *L. monocytogenes* strains was examined by Freedman et al. [153]. Farrag and Marth [154, 155] observed that growth and/or survival of *L. monocytogenes* at low temperatures (7 or 13 °C) was affected by presence of the psychrotrophic *Pseudomonas* spp. in TB. When the experiments were performed in sterile skim milk then *L. monocytogenes* could grow in the presence of the *Pseudomonas*. Freedman et al. [153] found that 4 out of 13 *Pseudomonas* strains showed wide spectrum activity against 8 *L. monocytogenes* strains in Tryptose. BHI and Medium B of King. Schaack and Marth [51, 52, 156] studied survival of *L. monocytogenes* in refrigerated (4 °C) cultured milks and yoghurt. Skim milk containing *L. monocytogenes* were fermented at 21, 30, 37 or 42 °C for 15 h with *Streptococcus lactis, Streptococcus cremoris, Streptococcus thermophilus, Lactobacillus bulgaricus* or *L. bulgaricus* plus *S. thermophilus* (LBST). *L. monocytogenes* survived in all fermentation with *S. cremoris* and *S. lactis* and commonly also grew to some extent but that was inhibited. When *S. thermophilus* was used then no growth only survival was observed during the fermentation. In case of *L. bulgaricus*, listeriae survived only between 9 and 15 h of incubation. After the fermentation, the storage survival of *L. monocytogenes* ranged between 3 d to 37 weeks depending on the fermentor strain, size of inoculum and temperature of fermentation. A yoghurt

fermentation typically lasts 4–6 h at 45 °C. *L. monocytogenes* could grow during the fermentation and then could survive from 1–12 d during refrigerated storage. Papageorgiou and Marth [102, 103] observed listerial survival in Feta cheese and Blue cheese, too. Knöpfel et al. [157] observed that *Carnobacterium carnis* and *Lactobacillus* species (*L. casei, L. plantarum, L. xylosus* and *L. sake*) also exhibited antagonism against *Listeria*. According to Kaya and Schmidt [158], when the germ count of natural bacteria of a minced meat was lower ($5 \times 10^5$/g) then *Listeria* strains ($<10^2$/g) could slightly multiply in the meat at temperature 12 and 20 °C but they could not at 4 and 8 °C. When the germ count of the natural bacteria was high ($10^7$/g) then they inhibited the growth of listeriae at all temperatures (8, 12, 20, 25 °C). Similar phenomenon was observed by Quist et al. [159]. Kaya and Schmidt [158] also found that *Lactobacillus* had a good inhibitory effect on *Listeria* but *Pseudomonas* species exhibited only a mild effect. Shelef [160] studied survival and growth of 3 *L. monocytogenes* strains in the presence of natural microflora in ground beef or liver during storage for weeks at 4 and 25 °C. The number of total aerobs increased but that of *L. monocytogenes* remained unchanged in both products. Farber et al. [161] examined samples from 96 lots of raw-fermented (dry-cured) sausages for the presence of *Listeria* species. Out of 54 samples 5 were positive for *L. monocytogenes* after fermentation, but not after the drying period. Ten out of the other 42 samples were positive for *L. monocytogenes* before fermentation, 5 out of the 10 remained positive after the drying period. In their opinion, it is difficult to make any definitive statement about the effect of starter cultures on the presence of *L. monocytogenes* in the final fermented meat product. Our opinion is similar to this [162]. Wenzel and Marth [163] examined the behaviour of *L. monocytogenes* in the presence of lactic acid bacteria in an agitated medium with internal pH control. The major finding of their study was that all 3 strains of *Listeria* survived and grew to a certain extent in both types of medium with internal pH control during the 30 h incubation regardless to the experimental conditions. Inhibition of listeriae by *S. lactis* and *S. cremoris* was observed but it was never complete. In general, *S. cremoris* was more inhibitory than *S. lactis*. At the end of the incubation the number of listeriae was between $10^3$ and $10^6$ c.f.u./ml.

*Action of bacteriocins*

It was observed by Ralovich [18] that enterocins produced by several strains of enterococci killed sensitive cells of different serotypes of *L. monocytogenes*. The most effective product was enterocin $E_1A$ prepared from *Enterococcus faecium* strain $E_1$. Nisin is a polypeptide bacteriocin produced by *Lactococcus lactin*. It can be digested by proteases, and shows antimicrobial activity against a range of Gram-positive bacteria. As to the inhibitory concentration of nisin on *Listeria* strains, contradictory

results (from 16 IU/ml to 2000 IU/ml) were published [67]. We observed that only 20 000 IU/ml killed *L. monocytogenes* in NB at 37 °C [140].

Monticello and O'Conner [164] found that nisin (100 IU/ml) could decrease only the initial viable cell number of *L. monocytogenes* during the first 2–3 h of treatment but further lysis was not evident. Carminati et al. [165] investigated the effect of bacteriocin like substances produced by *S. lactis* on *L. monocytogenes*. Their results indicated that these substances effectively inhibited in vitro the growth of six *L. monocytogenes* strains. Plate inhibition was confirmed by bactericidal activity in broth cultures. McKay [166] also reported that *E. faecium* strains isolated from cheese and silage samples had antimicrobial action against a wide range of *Listeria* species. Fourteen bacteriocin-producing strains from the genera *Lactobacillus*, *Leuconostoc, Pediococcus* and *Lactococcus* were evaluated by Harris et al. [167] for their ability to inhibit the growth of 8 strains of *L. monocytogenes*. They found that seven strains of lactic acid bacteria were antagonistic to *L. monocytogenes* by producing bacteriocins. The action of these bacteriocins could be eliminated by proteolytic enzymes. Pucci et al. [168] observed that the bacteriocin produced by *Pediococcus acidilactici* PAC 1.0 was inhibitory and bactericidal for *L. monocytogenes* not only in broth cultures but in several food systems including dressed cottage cheese, half-and-half cream and cheese sauce also. Berry et al. [169] published that a bacteriocin-producing *Pediococcus* species was used as a starter culture organism to produce fermented semidry sausage from artificially contaminated meat. The microorganism, in addition to its primary functions (improving the keeping quality, enhancing the flavour and texture of foods) could provide only limited preservative effect against *Listeria*. After the heat treatment (internal temperature was 64.4 °C) and 2 weeks long storage, 9 out of 90 samples were listeria-positive. Harding and Shaw [170] evaluated a new *Leuconostoc gelidum* strain for its ability to inhibit *L. monocytogenes*. Cell-free extract of the strain retained activity after 60 min at 100 °C but was sensitive to protease. Its inhibitory effect was bactericidal and rapid. Recently, Schilliger and Holzapfel [171] and Schilliger and Lindner [172] published that *Carnobacterium* strains produced bacteriocins or bacteriocin-like substances primarily active against other carnobacteria. *L. monocytogenes* was inhibited only by a supernatant that was concentrated tenfold. Lewus et al. [173] isolated 10 strains of bacteriocin-producing lactic acid bacteria from retail cuts of meat. These 10 strains together with 11 other bacteriocin-producing lactic acid bacteria were tested for inhibitory activity against psychrotrophic pathogens, including four strains of *L. monocytogenes*. Eight of the meat isolates had inhibitory activity against all four *L. monocytogenes* strains. Bacteriocin activity against *L. monocytogenes* was found in all of the strains obtained from other sources.

*Irradiation technology*

Convenient food safety and more effective protection of consumers have forced the application of irradiation technology in the food industry. Cobalt 60, caesium 137, electron beam and X-ray irradiators can be used. Stegeman [174] and Tarján [175] studied the effects of gamma-irradiation. The former obtained D-values for *L. monocytogenes* of 0.2 kGy in buffer and 0.38 kGy in minced meat at 4 °C, and 1.06 kGy at −18 °C. Tarján presented that irradiation of *L. monocytogenes* with 2 kGy in culture medium of pork meat paste at room temperature resulted in a 7-log reduction in viable cell counts, although one strain out of the 3 survived a dose as high as 4 kGy. Huhtanen et al. [176] studied gamma-radiation sensitivity of *L. monocytogenes* in culture media or in mechanically deboned chicken meat. Their results suggested that gamma radiation at doses less than 10 kGy could reduce or eliminate listeriae from meat. An applied dose of 2.0 kGy would be sufficient to destroy 4 $\log_{10}$ of listeria. Cultures of cells survived an irradiation dose of 1.5 kGy were not more radiation resistant than unexposed cells. Hashisaka et al. [177] treated listeria-preinoculated ice cream and Mozzarella cheese by gamma irradiation at −78 °C. The calculated $D_{10}$ values were 1.4 kGy for Mozzarella cheese and 2.0 kGy for ice cream. The effective level of irradiation ($D_{12}$) for inactivating *L. monocytogenes* was 16.8 kGy for Mozzarella cheese and 24.4 kGy for ice cream. Farag et al. [178] carried out studies with 3 strains of *L. monocytogenes*. Their data indicated that gamma rays and high-energy electrons were equally effective. For one strain $D_{10}$ values were 0.18, 0.21 and 0.44 kGy in PB, TSB-YE and poultry feed when gamma radiation was used. For the other two strains $D_{10}$ values were 0.25 and 0.46 kGy in TSB-YE. Their results indicated that a relatively moderate pasteurizing dose of 5 kGy would reduce the population level of viable *L. monocytogenes* by 5−10-log cycles in a low-moisture material (poultry feed). The reduction in the high moisture materials would be even greater. Lewis and Corry [179] examined the incidence of *L. monocytogenes* and other *Listeria* spp. in 32 experimentally irradiated (2.5 kGy) and in 25 matched unirradiated raw chickens. Of the unirradiated samples 76% contained *Listeria* (including 56% *L. monocytogenes*) but of the irradiated ones only 34% (all strains were *L. monocytogenes*). Their results indicated that a radiation dose of 2.5 kGy could reduce numbers of *L. monocytogenes* but not eliminated it completely.

*L. monocytogenes* was sensitive to short wave UV energy (100 uW/cm$^2$) with D-values of around 16−18 s for moist cells and 43 s for dry cells [180]

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# COLONIZATION OF INFANT MICE WITH FLAGELLAR VARIANTS OF *CAMPYLOBACTER JEJUNI*

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The role of flagella in the colonization of the intestine by *Campylobacter jejuni* was investigated by challenging infant mice with two flagellated strains and their nonflagellated variants. The intestinal tracts of infant mice were regularly colonized with motile strains, but not by nonmotile variants. Colonization of mice with motile *C. jejuni* occurred with as few as 1000 bacteria per mouse.

*Campylobacter* species are important pathogens of man and animals [1, 2]. These organisms have bipolar flagella which confer a characteristic darting motility. Experimental evidence suggest that flagella have an important role in the pathogenesis of campylobacter infections and contribute significantly to several serotyping schemes [3–5]. Some strains of campylobacters have been shown to undergo both phase and antigenic variations of flagella [6–8]. Phase variation refers to a bidirectional transition between flagellated ($Fla^+$) and nonflagellated ($Fla^-$) phenotypes. When both motility phenotypes ($Fla^+$ and $Fla^-$) of *C. jejuni* are used to infect rabbits or humans only flagellated organisms can be recovered from the faecal samples [6, 9]. Nonmotile variants poorly colonize the intestinal tract of suckling mice and hamster [3, 4, 10]. In addition, passive protection with antiflagellar antibodies has been demonstrated in a suckling mouse model [11].

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We investigated the role of flagella in the colonization of the intestinal tract by human and canine strains of *C. jejuni* by challenging infant mice with $Fla^+$ and $Fla^-$ phenotypes.

## Materials and methods

*Bacterial strains.* Two strains of *C. jejuni* were used in this study. Strain NA167 was isolated from the stools of a child suffering from diarrhoea and strain KE23 from a dog with haemorrhagic enteritis. The wild type strains were actively motile by polar flagella ($Fla^+$). Nonmotile variants ($Fla^-$) were obtained from the wild type population by serial passages from the centre of semisolid agar and identified by the type of growth on motility agar. Nonmotile variants were subcultured at least six times through motility agar to confirm their phenotypic stability. The phenotypes of $Fla^+$ and $Fla^-$ strains were also confirmed by dark field microscopy and flagella staining.

*Media and growth conditions.* Semisolid nutrient gelatin agar (nutrient broth containing 0.8% gelatin and 0.75% agar) was used for the selection of nonmotile variants. Motility agar (thioglycolate medium supplemented with 0.33% agar) was used to confirm motility phenotypes. Preston selective medium (Oxoid) was used for the culture and colony counts of *C. jejuni* from mouse faecal pellets. All cultures were incubated for 48 h at 37 °C in a microaerophilic atmosphere (5% $O_2$, 10% $CO_2$, 85% $N_2$).

*Animals.* Swiss albino mice from a close breeding colony were used in the study. They were 6-8 days old at the beginning of experiments. The infants remained with their dams for the duration of studies. Before the experiments, faeces of each animal was checked for campylobacters.

*Challenge of mice.* Inoculum was prepared by suspending 48 h growth of *C. jejuni* on blood agar into phosphate buffered saline (PBS). The colony forming units (c.f.u.) per ml of inoculum were obtained by diluting the suspension in PBS and plating on blood agar.

Eight groups of 8 infant mice, each group from a single litter, were used for inoculation (Table I). Groups of mice received $10^7$ and $10^3$ c.f.u. of $Fla^+$ and $Fla^-$ phenotypes of strains NA167 and KE23. The inoculum was administered intragastrically in a volume of 0.1 ml with a tuberculine syringe equipped with polyethylene tubing. Colonization of the intestine was examined at 1 week intervals for 4 weeks. At given intervals faecal pellets from each mouse were collected, diluted in PBS and seeded for viable bacterial count onto campylobacter selective agar.

## Results

When mice were challenged with the $Fla^+$ *C. jejuni* strains, all mice were colonized with the organisms recoverable at levels ranging between approximately $10^4$ and $10^6$/g of faeces (Table I). Both inocula ($10^7$ and $10^3$ c.f.u.) of motile strains resulted in the colonization of all mice.

Excretion of motile NA167 continued for 2 weeks after infection in all animals, and at least 4 weeks in a few animals. Motile KE23 was recovered from most of the animals for 2 weeks and for 3 weeks in some of them.

When mice were challenged with $10^7$ and $10^3$ c.f.u. of nonmotile variants of *C. jejuni* (both strains), none of mice excreted the organisms with their faeces.

**Table I**

*Colonization of infant mice by flagella phenotypes of C. jejuni*

| Strain | Phenotype | Inoculum (c.f.u./mouse) | No. of mice | Colonization* (week) 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|---|
| NA167 | Fla$^+$ | $10^7$ | 8 | $8(2\times10)^6$ | $8(8\times10)^5$ | $6(4\times10)^5$ | $2(2\times10)^4$ |
| | | $10^3$ | 8 | $8(5\times10)^5$ | $8(5\times10)^4$ | $5(2\times10)^4$ | $1(6\times10)^3$ |
| | Fla$^-$ | $10^7$ | 8 | 0 | | | |
| | | $10^3$ | 8 | 0 | | | |
| KE23 | Fla$^+$ | $10^7$ | 8 | $8(7\times10)^5$ | $6(6\times10)^4$ | $4(7\times10)^3$ | 0 |
| | | $10^3$ | 8 | $8(9\times10)^5$ | $5(3\times10)^4$ | $2(2\times10)^3$ | 0 |
| | Fla$^-$ | $10^7$ | 8 | 0 | | | |
| | | $10^3$ | 8 | 0 | | | |

*Number of colonized mice (mean c.f.u./g faeces)

The motility phenotypes of inoculated strains did not change in mouse intestine; mice inoculated with Fla$^+$ *C. jejuni* excreted only flagellated organisms.

## Discussion

For the experiments we were able to use variants of *C. jejuni* that remained stable after serial passages in vitro. Although mutagens were not used for the selection the possibility of other phenotypical differences cannot be excluded. We, however, did not detect any other phenotypical differences between the Fla$^+$ and Fla$^-$ variants used.

The results presented in this report make it apparent that the colonization of the intestinal tract of infant mice by *C. jejuni* strains differs strikingly according to their flagellar phenotypes. Bacterial counts after inoculation suggested that nonmotile variants were swept along the intestinal tract like ingested food but wild type strains reached a relatively constant population size and remained for a long time.

Few reports have been presented regarding the relationship between virulence or colonization and the motility of *C. jejuni*. Black et al. [9] inoculated humans with a mixture of Fla$^+$ and Fla$^-$ *C. jejuni* and only Fla$^+$ phenotypes were excreted by the

volunteeers. Newell et al. [4] found that a nonflagellated variant of *C. jejuni* poorly colonized the infant mice intestine but the flagellated strain readily colonized the mice. Morooka et al. [3] studied motility variants by using chemical mutagenesis and found that motility was associated with the ability of *C. jejuni* to colonize the mouse intestinal tract. Caldwell et al. [6] recovered $Fla^+$ organisms from all rabbits challenged with $Fla^-$ variant of *C. jejuni*. Aguero-Rosenfeld et al. [10] used a stable $Fla^-$ variant and determined a significant difference in the ability of flagellated and nonflagellated variants of *C. jejuni* to infect hamsters. Although these studies used different strains and in vivo systems, all reports confirm our findings.

There appeared to be no clear cut dose relationship in our study, the incidence of colonization near to termination and bacterial counts were somewhat greater with higher challenge doses. Colonization of mice with $Fla^+$ phenotypes occurred with as few as $10^3$ c.f.u. per mouse. Newell et al. [4] and Morooka et al. [3] have also found that $10^3 - 3 \times 10^4$ c.f.u. of $Fla^+$ *C. jejuni* are sufficient for the colonization of infant mice.

Colonization by both human and canine isolates was similar but the colonization by the canine strain terminated before that of the human strain. Strain differences in colonization of mice have also been reported by Field et al. [12] and Jesudason et al. [13].

In conclusion, flagellum is found to be a necessary factor for the intestinal colonization of *C. jejuni*.

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# COMBINED EFFECTS OF FLAVONOIDS AND ACYCLOVIR AGAINST HERPESVIRUSES IN CELL CULTURES

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The combined antiviral effects of some flavonoid compounds and acycloguanosine (acyclovir, Zovirax) were studied on the multiplication of herpes simplex virus types 1 and 2 in HEp-2 cells and on pseudorabies (Aujeszky) virus in chick embryo fibroblast cells by the yield reduction method. The flavonoids quercetin, quercitrin (quercetin-3-L-rhamnoside) and apigenin exhibit antiviral activity against these herpesviruses, and acyclovir is currently one of the most effective antiherpetic agents. In these studies, the simultaneous application of flavonoids with acyclovir resulted in an enhanced antiviral activity. A mathematical formula was used to interpret the drug interaction, resulting in FIC (fractional inhibitory concentration) indices. Meaning a synergic interaction, all combinations exhibited synergy, FIC values of 0.6 – 0.8 being commonly observed.

The antiviral effects of certain naturally-occurring flavonoids in cell cultures and in experimental animals were described earlier [1 – 5]. The flavonoids tested were effective against herpesviruses [6]. Most antiherpetic compounds are known to belong in the class of nucleoside analogues and some of them have been licensed for clinical use. We previously studied the antiviral effects of certain flavonoids combined with 5-ethyl-2'-deoxyuridine and interferons on the multiplication of herpesviruses, and an enhanced antiviral activity was observed [7, 8]. The aim of the present study was to investigate the combined antiviral activities of some flavonoids with acyclovir (ACV) against herpes simplex viruses and pseudorabies virus.

## Materials and methods

*Viruses and cell cultures.* Herpes simplex virus type 1 (HSV-1) was isolated from conjunctiva in our laboratory, and herpes simplex virus type 2 (HSV-2) was supplied by Dr. I. Hollós (B. Johan National Institute of Hygiene, Budapest). The pseudorabies virus strain, isolated from pig brain, was

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described previously [9]. HSV-1 and HSV-2 were grown in HEp-2 cells and the infectivity was measured in the same cells by the dilution method in microtitre plates (Linbro, Greiner). The infective titre was expressed as $TCID_{50}$, calculated via the formula of Reed and Muench [10]. The HEp-2 cells were cultured in Eagle's basal medium as modified by Macpherson and Stroker [11], supplemented with 5% calf serum. The pseudorabies virus was propagated in primary chick embryo fibroblast (CEF) cells maintained in Gey's solution, containing 4% Tris-HCl buffer pH 7.6, 5% calf serum and 0.25% lactalbumin hydrolysate. The infectivity of the pseudorabies virus was determined in primary CEF cells by the plaque method and the virus titre was expressed in plaque forming units (PFU).

*Antiviral agents.* Quercetin (Merck), quercitrin (Calbiochem) and apigenin (Carl Roth) were commercial products. Stock solutions were prepared in dimethylsulphoxide (DMSO) and further diluted with culture medium. ACV was purchased as Zovirax (The Welcome Foundation Ltd., London); it was dissolved in sterile deionized water and further diluted in culture medium.

*Assay of antiviral activity.* The antiviral activities of the compounds alone and in combination were investigated in cell cultures by the yield reduction test. Monolayer cultures of HEp-2 cells were grown in 96-well microtitre plates (Linbro, Greiner) and were infected with HSV-1 at a multiplicity of 1 $TCID_{50}$ per cell. After adsorption for 1 h at 37 °C, the inoculum was removed and the cultures were washed twice with Hanks' solution and supplied with Eagle's medium containing the drugs in different concentrations. After incubation for 22 h, the cultures were frozen and thawed and the cell debris was removed by low-speed centrifugation. The virus yield in the supernatant was determined in HEp-2 cells grown in microtitre plates by the dilution method. The antiviral effect on HSV-2 was tested in HEp-2 cells with a similar method. The cells were infected at a multiplicity of 0.5 $TCID_{50}$/cell and were treated with drugs for 25 h, and the virus was then estimated in HEp-2 cells. The effects of the compounds on the pseudorabies virus were studied in secondary CEF cells prepared in microtitre plates, also using the yield reduction assay. Confluent cell cultures were infected with pseudorabies virus at a multiplicity of 0.1 PFU/cell. After an adsorption period of 1 h at 37 °C, the inoculum was removed and the cultures were washed twice with Hanks' solution. The culture medium containing the drugs at different concentrations was added and the incubation was continued for 22 h at 37 °C; the cells were then frozen, thawed and centrifuged. The virus content of the supernatant fluid was titrated by the plaque method in primary CEF cells and the virus titre was expressed as $\log_{10}$ PFU/0.1 ml. Control cultures were in all cases incubated with media without drugs.

*Evaluation of combined antiviral effects of the compounds.* A slight modification of the mathematical formula given by Allen et al. [12] was applied to evaluate the combined antiviral affects. The minimal inhibition concentrations (MIC) of the agents alone and in combination were determined. The concentration of the drug which alone or in combination caused 1-log inhibition of the virus yield was regarded as the MIC value. When necessary, it was calculated by linear regression. The fractional inhibitory concentration (FIC) was expressed by the following formula: MIC of drug in combination per MIC of drug alone. The sum of the FIC values gives the FIC index:

$$\text{FIC index} = \frac{\text{MIC of drug A in comb.}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in comb.}}{\text{MIC of drug B alone}}$$

The FIC index was applied for interpretation of the drug interaction. If the value of FIC index is $<1$, the combination is synergic; if the value is $\simeq 1$, the interaction is additive; if the FIC index is 1.1 – 1.9, the effect is indifferent or partially antagonistic; and if the FIC value is $>2$, the interaction is antagonistic.

*Assay of cytotoxicity.* The toxicities of the compounds alone and in combination were also determined. Monolayers of uninfected HEp-2 cells and secondary CEF cells were treated with culture medium containing the drugs at different concentrations for 24 h at 37 °C. The cell cultures were then investigated by microscopic examination.

The effects of the compounds on the incorporation of a radioactive precursor, $^3$H-thymidine, into HEp-2 cells were likewise studied. HEp-2 cells ($3.5 \times 10^4$ cells/100 μl) were distributed in each well of flat-bottomed 96-well microtitre plates. After incubation for 24 h at 37 °C, a 100 μl aliquot of the medium containing the drugs at different concentrations was added to the wells. Each experimental group contained three parallel microtitre wells. The cultures were further incubated for 20 h at 37 °C an the cells were then pulsed with 2 μCi $^3$H-thymidine. At the end of the incubation period, the cells were washed with 100 μl Hanks' solution and were then trypsinized and harvested onto glass filter paper by using a Skatron semiautomatic cell harvester; the radioactivity was measured in a toluene-based scintillation liquid with a Packard 4530 liquid scintillation spectrometer.

## Results

*Cytotoxicity assay.* The structures of the flavonoids are depicted in Fig. 1. After an incubation period of 24–48 h in medium containing the drugs at different concentrations, no visible alteration was observed by microscopic examination at the applied concentration.

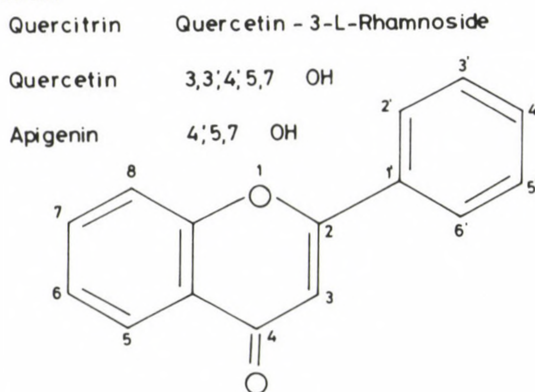


*Fig. 1.* Chemical structures of flavonoids

The effects of the agents on the DNA synthesis of HEp-2 cells were determined via the incorporation of $^3$H-thymidine. With increasing concentration of flavonoids, there was a progressive inhibition of DNA synthesis. The concentrations of the drugs required to reduce the incorporation of $^3$H-thymidine into HEp-2 cells by 50% were considered as minimum toxic dose. In the experiments these concentrations were not applied, only the lower concentrations were used. When the flavonoids were combined with ACV at different concentrations, the inhibition of $^3$H-thymidine incorporation did not change substantially (Table I).

**Table I**

*Inhibition of DNA synthesis of HEp-2 cells in the presence of drugs applied alone or in combination*

| Drug | Concentration (μM) | Inhibition of incorporation* (%) |
|---|---|---|
| Quercetin | 500 | 68.6 |
| | 250 | 31.5 |
| | 100 | 12.2 |
| | 50 | 2.2 |
| | 10 | 0.0 |
| Quercitrin | 1000 | 27.1 |
| | 500 | 11.7 |
| | 250 | 0.0 |
| | 100 | 0.0 |
| | 50 | 0.0 |
| Apigenin | 50 | 67.7 |
| | 20 | 28.6 |
| | 10 | 0.0 |
| | 5 | 0.0 |
| ACV | 1000 | 2.9 |
| | 500 | 0.0 |
| | 100 | 0.0 |
| | 10 | 0.0 |
| Quercetin/ACV | 250/10 | 33.9 |
| | 100/10 | 11.8 |
| | 10/10 | 1.6 |
| Quercitrin/ACV | 500/10 | 18.6 |
| | 250/10 | 8.4 |
| | 100/10 | 1.8 |
| Apigenin/ACV | 20/10 | 12.1 |
| | 10/10 | 0.0 |
| | 5/10 | 0.0 |

*Data are mean values of three parallels

*Antiviral activities of drugs.* The compounds were generally used in 4–5 different concentrations, which were selected on the basis of the cytotoxicity. The concentration scale ranged from concentrations causing an inhibition of the virus yield of at least 1-log to no inhibition produced by the individual substances, and these concentrations were applied in combinations. In all experiments, assays with single drugs alone and in combination were performed in parallel and at least 2 or 3 identical experiments were carried out.

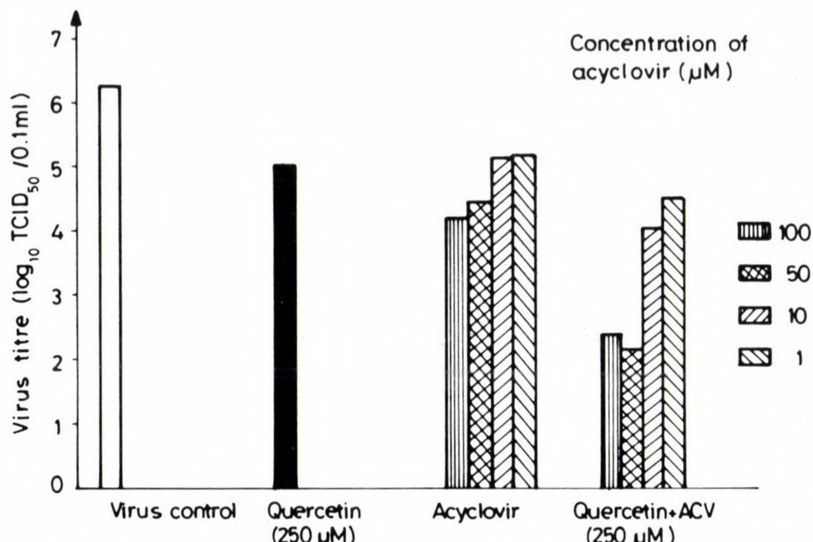


*Fig. 2.* Combined effect of quercetin and acyclovir on the multiplication of herpes simplex virus type 1

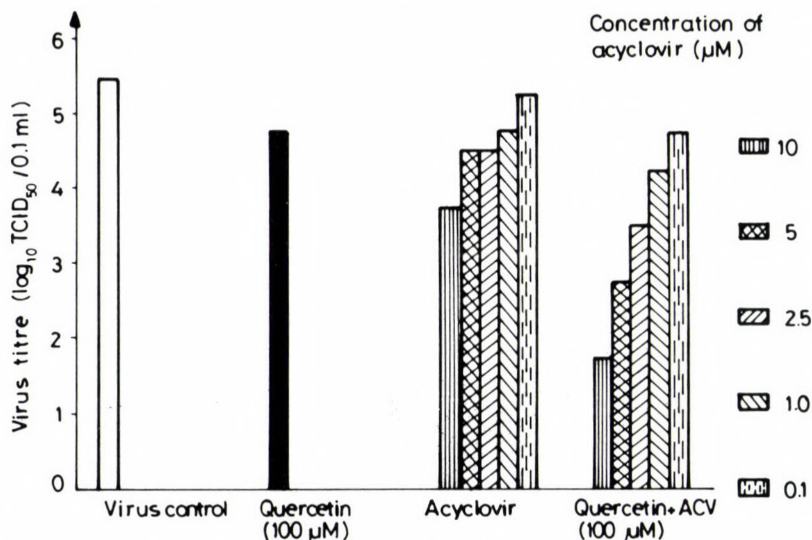


*Fig. 3.* Combined antiviral effect of quercetin and acyclovir against herpes simplex virus type 2

*Combined antiviral effects of quercetin and ACV.* The results of the simultaneous application of quercetin and ACV against HSV-1 are demonstrated in Fig. 2. Depending on its concentration, ACV caused a 1–2-log inhibition of the virus yield. Quercetin at a concentration of 250 μM resulted in a 1.2-log inhibition, and on combined application of the two drugs the antiviral activity increased considerably.

The combined effect of quercetin and ACV on the multiplication of HSV-2 was also tested. Figure 3 shows that the reduction of the virus yield by 100 μM quercetin alone was 0.7 log. The effect of ACV depended on the concentration, 10 μM ACV reducing the virus yield by 1.7 log. When the two drugs were combined, the virus yields were significantly lower than when they were applied alone. The highest concentration of ACV combined with 100 μM quercetin resulted in a 3.7-log inhibition of the virus yield.

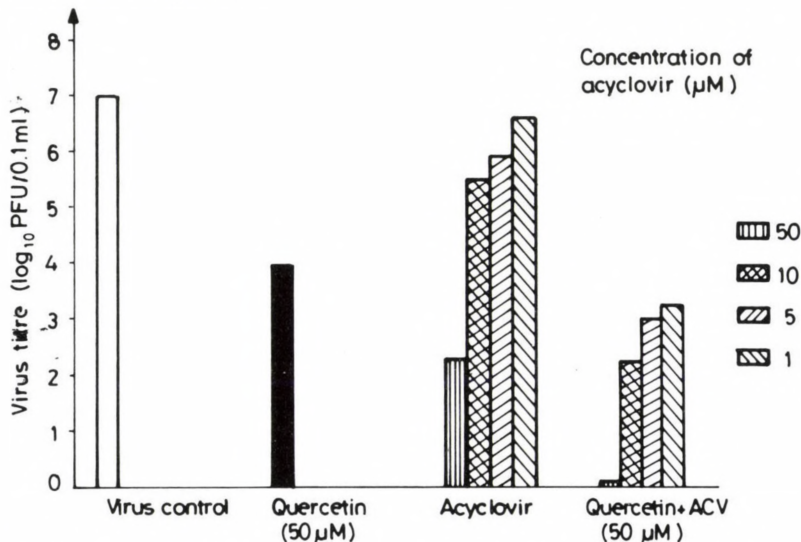


*Fig. 4.* Effect of quercetin combined with acyclovir on the multiplication of pseudorabies virus

The effects of quercetin and ACV applied simultaneously against pseudorabies virus are presented in Fig. 4. Quercetin at 50 μM combined with ACV at different concentrations considerably enhanced the antiviral effect.

*Inhibition of herpesvirus multiplication by quercitrin and ACV combinations.* The effects of the combined application of quercitrin and ACV against HSV-1 and HSV-2 are shown in Fig. 5. Quercitrin at 500 μM caused a 1.6 log inhibition of both virus yields, while ACV reduced the virus yield to an extent depending on the concentration, by 1.8–0.5 logs. The simultaneous application of the two drugs enhanced the reduction of both herpes simplex virus yields.

The effects of quercitrin and ACV combinations on the multiplication of pseudorabies virus were also tested. Quercitrin at 250 μM combined with various concentrations of ACV resulted in greater antiviral activities than in the cases of either quercitrin or ACV alone (Fig. 6).

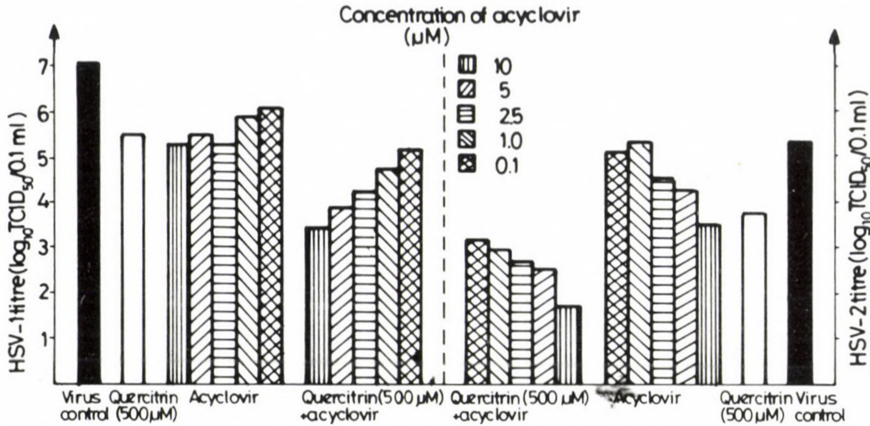


*Fig. 5.* Combined effect of quercitrin and acyclovir on the multiplication of herpes simplex virus types 1 and 2

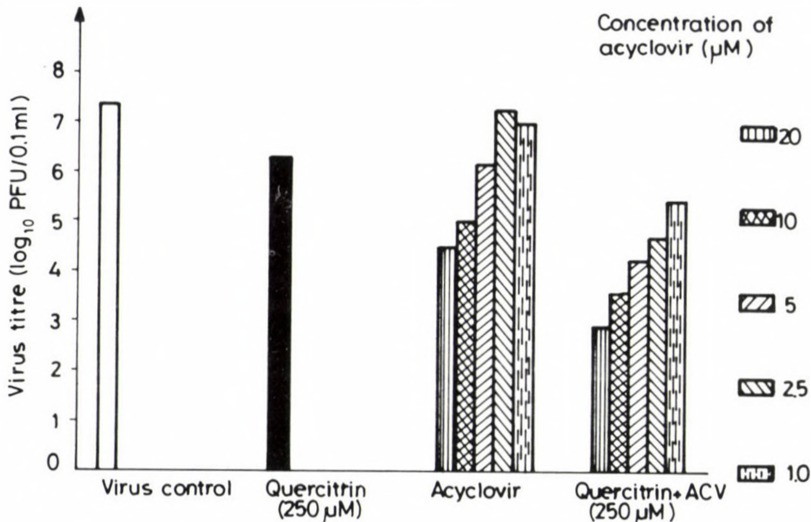


*Fig. 6.* Effect of quercitrin and acyclovir on the multiplication of pseudorabies virus with simultaneous application

*Activities of apigenin and ACV applied simultaneously against herpesviruses.* The combinations of apigenin at 5 μM and ACV at different concentrations were investigated against HSV-2. Apigenin at 5 μM resulted in a 0.75-log inhibition of the virus yield, and ACV itself caused a 2 log or less inhibition, depending on the concentration. The reduction of the virus yield was more pronounced when the two drugs were applied in combination (Fig. 7). A similarly enhanced antiviral effect was

observed when apigenin at 10 μM was applied simultaneously with ACV at various concentrations against pseudorabies virus.

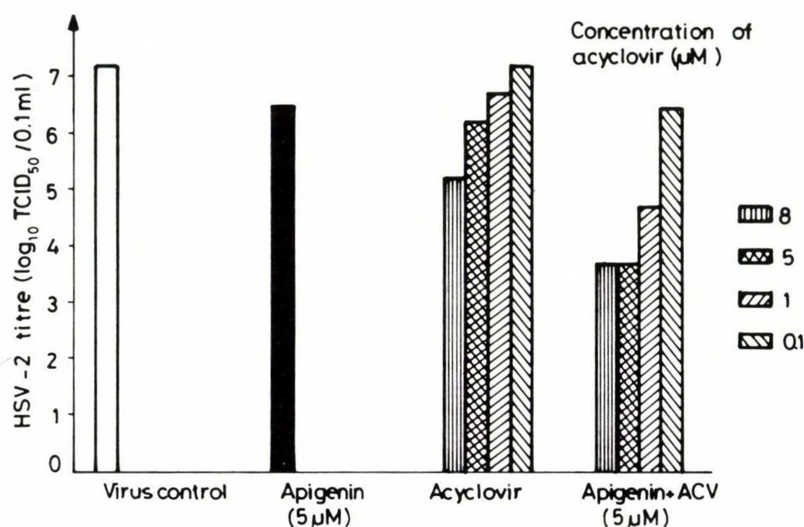


*Fig. 7.* Combined antiviral effect of apigenin and acyclovir on the multiplication of herpes simplex virus type 2

*Evaluation of interactions of flavonoids and ACV.* For interpretation of the drug interactions, FIC indices were utilized. The drug combinations in all cases caused a synergic interaction, with FIC values in the range 0.6–0.85 (Table II). The most effective combinations against HSV-2 were those of quercetin and ACV and of apigenin and ACV, since the values of the FIC indices were 0.6.

**Table II**

*Activities of flavonoids and ACV in combination against herpesviruses evaluated by FIC indices*

| Drugs in combination | Virus | FIC index | Interaction |
|---|---|---|---|
| Quercetin/ACV | HSV-1 | 0.75 | Synergy |
| Quercetin/ACV | HSV-2 | 0.6 | Synergy |
| Quercetin/ACV | Aujeszky | 0.65 | Synergy |
| Quercitrin/ACV | HSV-1 | 0.8 | Synergy |
| Quercitrin/ACV | HSV-2 | 0.8 | Synergy |
| Quercitrin/ACV | Aujeszky | 0.85 | Synergy |
| Apigenin/ACV | HSV-2 | 0.6 | Synergy |
| Apigenin/ACV | Aujeszky | 0.8 | Synergy |

## Discussion

During the past decade, the most successful advances have been made against infections caused by herpesviruses, and several antiherpes drugs have been licensed. However, it has been demonstrated that herpesviruses may acquire resistance to antiviral drugs in tissue cultures and in animal models [13]. In man, the emergence of herpes simplex virus resistance to ACV has been observed most frequently in immunocompromised patients [14]. The combined application of selected antiviral drugs with different mechanisms of antiviral action is on interest, because it may increase efficiency and at the same time the emergence of resistant mutants may be inhibited. Further, the cytotoxicities of the drugs may be reduced if combinations of these agents permit their use in smaller doses [15]. A combination of drugs should be more effective of synergistic if the modes of action are different [12].

The effects of ACV combined with other antiviral substances on the multiplication of herpesviruses have been investigated both in vitro [16 – 19] and in vivo [15, 19 – 22], and in some cases its combinations have also been applied in human therapy [23, 24].

The results of our earlier studies suggested a correlation between the antiviral effects of flavonoids and their intracellular cAMP enhancing activities [25]. This possibility was confirmed by other workers, who demonstrated that intracellular cAMP enhancing compounds such as dibutyryl-cAMP inhibited the multiplication of herpes simplex viruses [26, 27]. The mechanism of the antiviral effect of ACV has been determined. Herpes simplex viruses code for a viral thymidine kinase which is capable of phosphorylating ACV to a monophosphate; this event is essentially absent in uninfected cells. ACV monophosphate is subsequently converted by cellular enzymes to ACV triphosphate, which is a potent inhibitor of the viral DNA polymerase [28]. In our experiments, ACV in combination with flavonoids resulted in a synergic effect, these findings were therefore supporting the assumption that combinations of drugs with different mechanisms of action should be more effective or synergistic [12, 15].

The flavonoids are widespread in nature and some of them have already been applied in clinical trials and therapy [29]. The flavonoids with antiviral activity are known as antipicornaviral agents [30]. However, some derivatives can inhibit other viruses such as herpesviruses, too [6, 31]. A synthetic flavonoid, 4,6-dichloroflavan, has been shown to be a potent inhibitor of rhinovirus replication in vitro [32], but it did not provide protection against rhinovirus type 9 infection in a controlled volunteer trial when it was given orally [33], and a second trial, involving intranasal instillation, was not successful either [34]. It has also been reported that (+/-cyanidanol-3) (+/-catechin) is of therapeutic value in hepatitis caused by hepatitis B virus [35]. The effects of 4,6-dichloroflavan, isoflavan and isoflavene derivatives on

hepatitis A virus infection were recently studied in a monkey cell line. They exerted an inhibitory effect on viral antigen synthesis during hepatitis A virus multiplication [36]. It has also been suggested that some flavonoids should be further studied as potential antiretroviral agents [37].

It is not likely that the flavonoids tested here will be used as antiviral agents, but the results of our studies may be useful for further investigations of combinations with other antiviral agents.

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# IMPACT OF WEATHER CHANGES ON THE GROWTH OF THE UNICELLULAR *BLEPHARISMA UNDULANS* (STEIN)

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The growth of *Blepharisma undulans* (Stein) cells durably treated and not treated with insulin was followed up for three months with special regard a possible impact of meteorological factors on the growth rate. The growth rate of the protozoon was not appreciably altered by weather changes but the growth curves for cells treated and not treated with insulin indicated opposing trends of multiplication under the influence of approaching weather fronts. Warm fronts which have a parasympathic effect and cold fronts which have a sympathetic effect uniformly enhanced the growth rate of the untreated cells and retarded that of the insulin treated cells.

According to practical observations of protozoologist, unicellular organisms occasionally show unexpected inexplicable changes in behaviour (physiological parameters) under laboratory conditions. Since laboratory strains of unicellulars are usually maintained under controlled climatic and other environmental conditions, factors related to the macro-rather than to the micro-environment, above all weather factors, were incriminated for the changes.

Gelei and Szabados [1] demonstrated a close correlation between the flowering of algae and weather fronts. Kiss [2] analyzed 112 blooming periods of algae indigenous in stagnant water over 27 years and observed that flowering was nearly always associated with cyclonal depression and prefrontal weather conditions: he remarked that old Hungarian peasants foretold weather changes on the basis of a blue or green colour change of the water. Biczók [3, 4], too, reported abrupt increases in protozoan populations in connection with weather changes and found

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that some ciliated species (e.g. *Cyclidium glaucoma*) were more sensitive to weather fronts than others (e.g. *Chilodonella unsinata*).

We [5–8] demonstrated earlier that *Protozoa* were able to bind hormones of higher organisms and responded to these by changes in certain physiological parameters. Such behavioural changes persisted over many offspring generations. Thus the unicellulars which had been exposed to a hormone either on a single occasion or chronically, differ from those not exposed at all, owing to alterations in responses and in the second messenger system as well.

With these facts in mind, we followed up for three month the growth of *Blepharisma undulans* (Stein) cells treated and not treated with insulin and analyzed the growth curves with regard to meteorological data recorded during the period of study.

## Materials and methods

*Model cells and their sustenance.* The cultures each of *B. undulans* cells were set up in 10 ml water obtained from the Theodora Spring (composition: (mg/litre): $K^+$ 23; $Na^+$ 45; $NH^+_4$ 0.28; $Ca^{++}$ 57.6; $Fe^{++}$ 6.1; $Mg^{++}$ 0.71; $Sr^{++}$ 3.8; $Cl^-$ 11; $F^-$ 0.84; $Br^-$ 0.1; $SO^{++}_4$ 40; HCO-8 1.7) placing 1 *B. undulans* cell per 10 ml and a single grain of cooked rice into each culture flask. After a cell count of 160–170 was attained, the culture was discarded and a new culture was set up as above. Thus several cultures set up at different times were examined simultaneously for growth performance and there were multiple overlaps on that account.

*The cell counts* were determined daily under a stereomicroscope. Part of the cultures were treated with $10^{-6}$ M insulin (Insulin Semilente, M.C. Novo, Copenhagen, Denmark) during the entire period of counting. The growth rates had been related to the values assessed on the previous day as 1.

*Evaluation.* The numerical values of growth performance were correlated with the medical meteorological data supplied by the National Institute of Meteorology (Budapest). Figure 1 shows the explanation of numbering used in Figs 2A, B and C, which demonstrate the results.

## Results and discussion

As shown in Figs 2A, B and C the rate of cell division was not uniform; there were periods of rapid and slow division on each day and cell death also occurred. Since the growth data were always related to those assessed on the preceding day, the growth curves have ascending and descending portions with plateaus between them. The descending limb indicates a decrease in the rate of division rather than in cell count. The growth curves also make possible the evaluation of trends, more precisely of the behaviour of *B. undulans* cells treated and not treated with insulin.

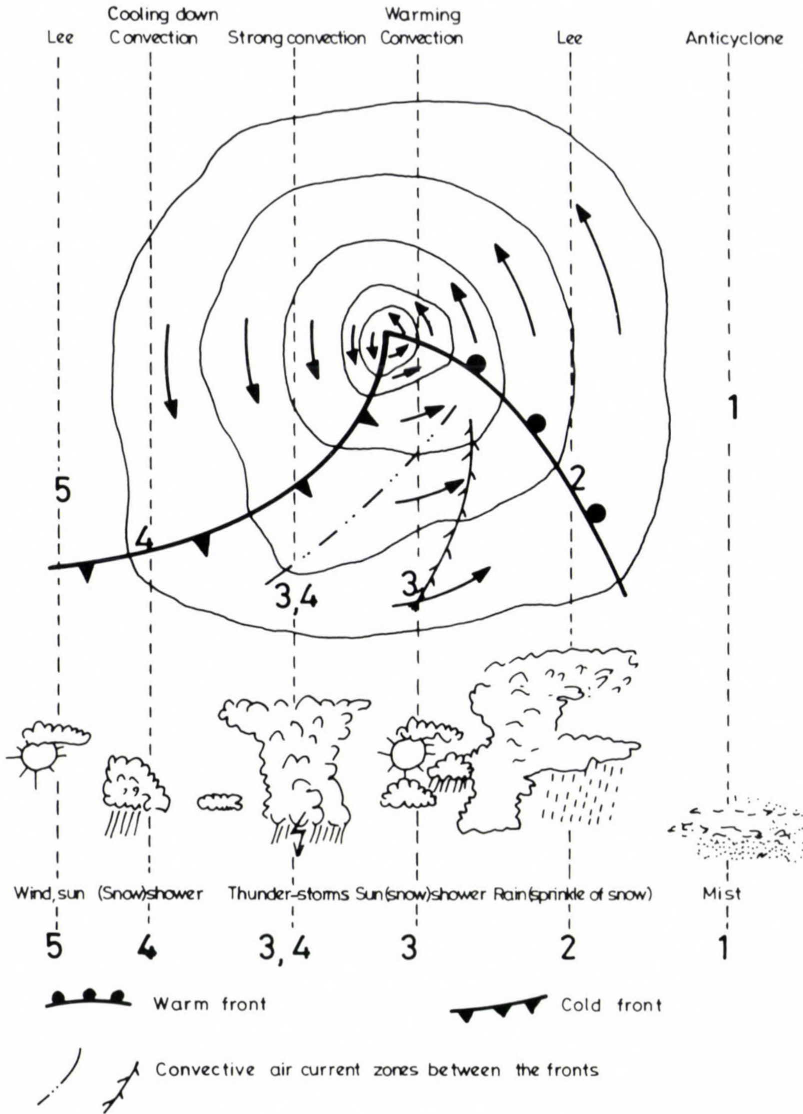
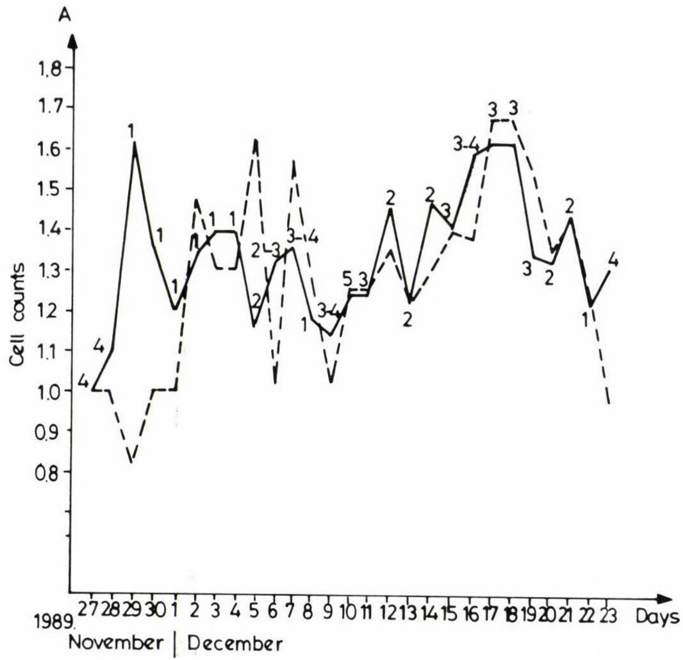
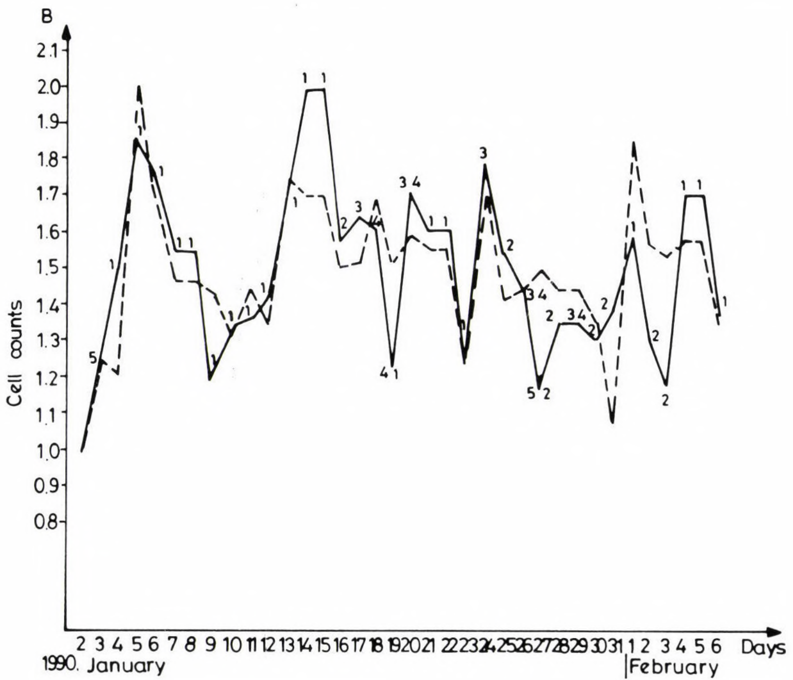



*Fig. 1.* Biologically active atmospherical systems


A
Cell counts
1989.
November | December
Days


B
Cell counts
1990. January
February
Days

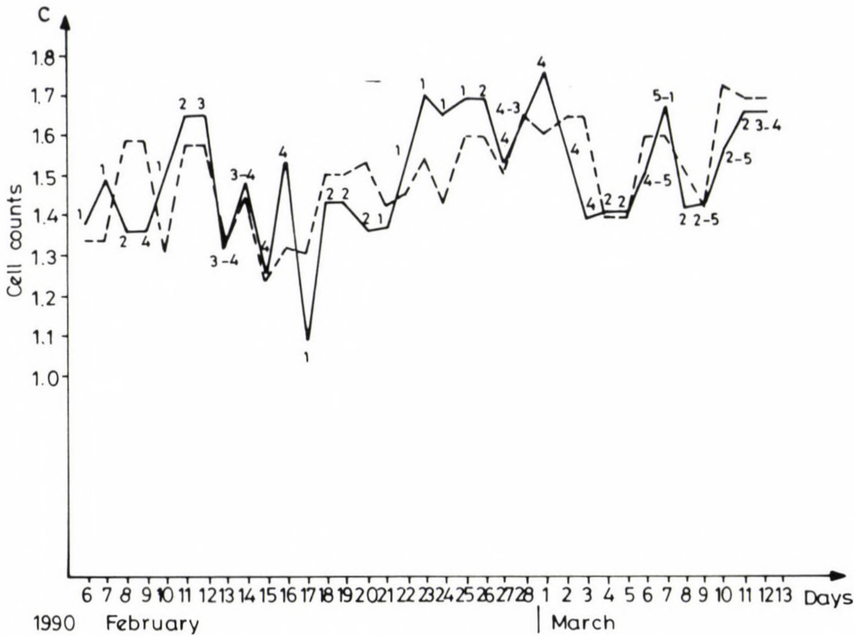



*Fig. 2, A, B, C.* Influence of meteorological fronts on the growth trends of *B. undulans*. ——— Control; – – – – Insulin treated. 1, biologically inactive meteorological situation; 2, warm front associated with strong parasympathetic effects; 3, warm front associated with decreasing parasympathetic and increasing sympathetic effect; 4, cold front associated with strong sympathetic effect; 5, cold front associated with decreasing sympathetic and increasing parasympathic effect

As can be seen, apart from minor differences between the growth intensities of the cells treated and not treated with insulin, the trend of growth was generally similar in both types of cultures. However, with cell counts above 10, the curve for one groups showed an ascending tendency, whereas that for the other a descending tendency, indicating reverse trends of cell growth.

The shape of the growth curves for untreated and insulin-treated cells did not indicate any appreciable influence of the meteorological conditions on the division of *B. undulans*, or at least no regular correlation could be demonstrated on this basis. However, opposite trends of the growth curves for the two groups always coincided with the appearance of weather fronts. Most weather fronts had this effect and a few of them had no influence at all on cell growth either in presence or absence of insulin.

The above experimental observations support the conclusion that weather changes, particularly warm fronts associated with a parasympathic effect and cold fronts associated with a sympathetic effect, also have an influence on the growth trend of *B. undulans*. The influence was uniformly positive in the case of untreated

cells and uniformly negative in the case of insulin-treated ones. Thus we may state that meteorological changes had (generally) an opposite impact on treated and untreated cells, regardless of the cold or warm nature of the approaching weather front. This presents an additional proof for the earlier experimental observation [7] that hormone treatment alters the responsiveness of the cells; under the given conditions it did change the trend of response to weather fronts.

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# THE EFFECT OF KANAMYCIN TREATMENT OF RATS ON THE DEVELOPMENT OF GASTRO-INTESTINAL SYNDROME OF RADIATION DISEASE

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Kanamycin pretreatment through stomach tube eliminates the endotoxin producing Gram-negative bacteria of the intestinal flora and can delay the death of rats suffering from gastro-intestinal syndrome of radiation disease. Endotoxins probably play a significant role in the pathological process of the syndrome.

One of the most radiosensitive tissues is the bone marrow the injury of which leads to the damage of haemotopoiesis (haemopoietic syndrome). In case of higher radiation load a gastro-intestinal syndrome develops. An outstandingly high dose may cause a damage of the central nervous system, too [1].

At the Tshernobil reactor accident the gastro-intestinal syndrome incurable according to our present knowledge caused the death of a great number of people. At high dose irradiation, in the process of the gastro-intestinal syndrome the villi become bare and ulcerated [1]. As an effect of irradiation bile production decreases [2]. The cell-walls of Gram-negative bacteria present in the small intestine contain endotoxin which is released after the destruction of the bacteria. Endotoxin hypersensitivity method made with lead acetate revealed that in gastro-intestinal syndrome endotoxin enters the blood circulation [2–4] causing enteroendotoxaemia contributing to the lethal outcome of the disease. Based on the above finding, it was thought that bile production reduced by ionizing radiation was not enough for the detoxification of releasing endotoxins. Thus, they might enter the blood circulation via the damaged gastric mucous membrane and might evoke endotoxin shock.

In the experiments kanamycin was administered through a gastric tube for decreasing the number of Gram-negative (endotoxin producing) bacteria of the

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intestinal flora. This drug, being less readily absorbed from the gastro-intestinal tract, is injected intramuscularly in human therapy.

## Materials and methods

*Experimental animals.* In the experiment 60 Wistar (LATI, Gödöllő) female rats of 130 – 150 g weight were used. Granulated rat chow (LATI, Gödöllő) and tap water were given ad libitum.

*Kanamycin treatment.* Four days prior to exposure to a radiation dose necessary for evoking gastro-intestinal syndrome, the animals were given kanamycin (100 mg/day).

*Bacteriology.* The total number of aerobic bacteria in the faeces and intestine was determined by using Urea Agar Base (Oxoid CM 53) and Plate Count Agar (Oxoid CM 183), while *Escherichia coli* content was defined on Endo Agar (Oxoid CM 37) medium [5].

On the day of irradiation neither *E. coli* nor *Proteus* as primary endotoxin producers were present in the intestinal content of kanamycin pretreated animals as against control group.

*X-irradiation.* Whole-body irradiation of the animals was made with THX-251 X-ray device at 200 kV, 20 mA, 60 cm focus-body-middle distance 0.5 mm Cu screening. Dose output, 0.488 Gy/min; dose, 8 Gy.

## Results

At first preliminary experiments were made with 40 rats in order to establish the dose of irradiation which in all probability induces intestinal syndrome. This value was found to be 8 Gy, killing the animals in 100% on day 4 – 7. From the 3rd day after irradiation the rats developed diarrhoea. Autopsy proved that the animals died of intestinal syndrome. The majority of dead animals were in state of exsiccosis. The stomach was always full and enlarged, the small intestine swollen and the mucous membrane damaged. The mesenterial lymphatic glands were enlarged. Even the size of coecum extended significantly. Haemorrhages were found all along the whole gastro-intestinal tract.

In the main experiment (see Materials and methods) animals in the first (control) group were exposed to irradiation only. Animals of the second group had been treated with kanamycin for 4 days prior to irradiation and further on till their death. The effect of the treatment is shown in Table I.

At autopsy, rats in the first and second groups exhibited the same characteristic changes as in the preliminary experiment. It seems probable that they died of gastro-intestinal syndrome. The time of survival in the first groups was shorter than that in the second one. The difference shown in survival period in statistically significant.

**Table I**

*Effect of kanamycin treatment on the death of rats following whole-body irradiation*

| Group | No. of animals | Treatment | Death from the day of irradiation onwards |
|---|---|---|---|
| 1 | 10 | X-ray whole body irradiation; dose 8 Gy | between days 4 – 7 100% |
| 2 | 10 | Kanamycin treatment, 100 mg/day started 4 days before irradiation (X-ray whole-body dose 8 Gy) and continued till death | between days 9 – 13 100% |

## Discussion

Based on the experiments it may be supposed that the endotoxins of Gram-negative bacteria play a significant role in the development of gastro-intestinal syndrome [4]. The bacterial endotoxins entering the blood circulation lead to the development of an enteroendotoxaemic shock. If Gram-negative bacteria and their endotoxins are not present at the time of radiation and following it, there is no possibility for the induction of endotoxin shock. When Gram-negative bacteria were eliminated from the intestinal flora by kanamycin pretreatment, the lives of animals were prolonged to a significant extent, however, the treatment is not enough to prevent later death.

It may be assumed that the damage of the haemopoietic system caused by radiation played a decisive role in the later death of the animals. The practical use relies in the fact that by kanamycin treatment time enough would be allowed for the mesenteric mocous membrane to regenerate to some extent, in cases only when the radiation dose is of a lower limit necessary for the induction of the gastro-intestinal syndrome. Accordingly, there might be an opportunity for the "lightening" of the gastro-intestinal syndrome to a haemopoietic one. The latter syndrome is also severe but can be cured in some cases.

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# MULTIPLE ENLARGEMENTS IN THE RIGHT INVERTED TERMINAL REPEAT OF THE DNA OF CANINE ADENOVIRUS TYPE 2

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The Manhattan strain of canine adenovirus type 2 (CAV 2) was examined. Restriction endonuclease analysis and blot hybridization experiments revealed the heterogeneity of the viral DNA. At least 9 unequally expanded species of the viral genome have been recognized. This diversity is caused by different enlargements in the right inverted terminal repeat (ITR) of the virus. The differences between the individual enlargements were shown to be the different multiples of 150 base pairs. Relatedness of CAV 2 DNA to the DNA of bovine adenovirus type 2 (BAV 2) and human adenovirus type 2 (HAV 2) has also been observed during DNA hybridization experiments.

Canine adenoviruses belong to the family *Adenoviriade*. As other members of this virus family their genome is linear, duplex DNA molecule with inverted terminal repeats at both ends which serve as the origin of the viral DNA replication.

Alterations of the adenoviral ITRs have been described in several cases. The enlargements of the right ITR of human adenovirus type 12 [1], 16 [2] and 34 [3] as well as canine adenovirus type 1 [4] were reported. In these studies the viral genome was increased by different numbers of reiterated DNA sequences from the ITR. In our present work we report the presence of similar alterations of the right ITR of CAV 2.

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## Material and methods

*Viruses and tissue cultures.* The Manhattan strain of CAV 2 attenuated by 16 passages in primary dog kidney cells and 30 passages in primary porcine kidney cells was obtained from G.M. Baer and the Toronto strain of CAV 2 was obtained from G. Darai. Viruses were propagated in the canine MDCK cells maintained as monolayer cultures in $Ca^{++}$ free Eagle's MEM supplemented with 10% of fetal calf serum, 0.1 mg/ml streptomycin and 0.5 mg/ml penicillin.

*Purification of viral DNA.* When cytopathic effects were extensive, virus infected cells were pelleted with low speed centrifugation an extracted with 0.4 M NaCl, 5 mg/ml Triton X 100 and 10 mM Tris-HCl, pH 7.5 as described [5]. Virus particles were purified from this extract by equilibrium gradient centrifugation at 30 000 rpm in a Beckman SW 41 Ti rotor for 6 h at 5 °C using preformed CsCl gradients (1.1 mg/ml to 1.4 mg/ml), the complete virus particles were collected, dialyzed and the DNA was prepared as described earlier [6]. In certain cases DNA was prepared directly from the extract made by the Triton X 100 and NaCl treatment of the virus infected cells [6].

*Molecular clones of BAV 2.* The pKMl left subterminal *Bam*HI-*Nco*I fragment (0.03-0.275 map unit), pSB207 *Eco*RI C fragment (0.45 – 0.65 m.u.) and pSB116 *Eco*RI-*Bam*HI fragment (0.65 – 0.84 m.u.) recombinants of the corresponding BAV 2 subtype B fragments [7] were used.

*Restriction endonuclease analysis.* Restriction enzymes were purchased from Pharmacia, Amersham or Reanal (Hungary) and were used according to the instructions of the manufacturers. DNA fragments were separated by horizontal slab gel electrophoresis using 1% agarose gels, an were visualized by UV light illumination after staining with ethidium bromide. In the case of experiment shown in Fig. 1B, 1.2% agarose was used.

*Southern blotting and hybridization.* DNA fragment were transferred to Hybond N+ (Amersham) or Gene Screen Plus (Dupont) membranes by capillary blotting. The membranes were hybridized with different DNA probes labelled with $^{32}P$ dCTP using the random priming technique. Hybridizations were carried out in 50% formamide, 6XSSC, 1% SDS and 10% dextran sulphate at 41 °C for 24 h after prehybridization with the same solution for 1 h [8]. Low and high stringency washings were applied, and the membranes were exposed to X-ray films using intensifying screens (Dupont Cronex) at – 90 °. Low stringency washings were used in the case of hybridizing with the BAV 2 clones.

## Results

*Restriction endonuclease patterns.* The cleavage profiles of CAV 2 DNA generated by *Xho*I, *Bam*HI, *Sal*I, *Bgl*II, *Pst*I and *Sma*I are shown in Fig. 1. Digestion of both the CsCl density gradient purified virion DNA and the DNA prepared directly from the Triton X 100 solution extract with a given enzyme resulted in virtually the same patterns, and revealed the heterogeneity of certain submolar DNA fragments. These fragments comprise at least 6 different DNA populations in the case of *Sal*I, *Bgl*II, *Pst*I and *Sma*I. The size of the heterogeneous fragments generated by different restriction enzymes are summarized in Table I. The difference between the molecular weight of the individual members of the diverse fragments are always the different multiples of approximately 150 base pairs, independently of the type of the restriction enzyme used.

**Table I**

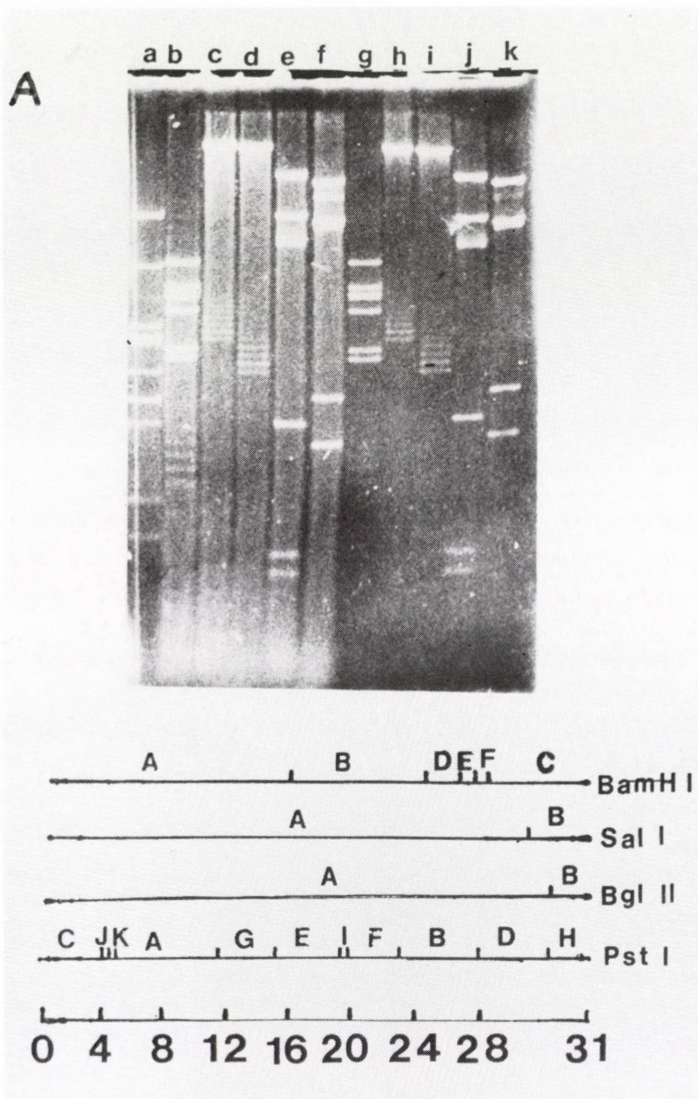
*Heterogeneity of CAV 2 (Manhattan strain) DNA restriction endonuclease fragments*

| Enzyme | Heterogeneous fragment | Length of the heterogeneous fragments |
|---|---|---|
| *Sal*I | B | 3350 3500 3650 3800 3950 4100 |
| *Bgl*II | B | 2750 2900 3050 3200 3350 3500 |
| *Pst*I | H | 1450 1600 1750 1900 2050 2200 2350 2500 2650 |
| *Sma*I | I/J | 350 500 650 800 950 1100 1250 1400 1550 |
| *Bam*HI | C | 6300* |

*Diversity can be seen but it can not be calculated exactly

Since it appeared that this CAV 2 strain is a mutant one, another prototype strain was also examined. Figure 2 shows the cleavage patterns of the DNA of the Manhattan and Toronto strains of CAV 2 generated by *Pst*I. Instead of the ladder like heterogeneity in the *Pst*I digested DNA of the Manhattan strain the unaltered *Pst*I-H fragment can be seen in the Toronto strain DNA. Even the lowest component of the DNA heterogeneity of the Manhattan strain DNA is bigger with approximately 150 bps. than the normal *Pst*I-H fragment of the Toronto strains.

*Verification of the colinearity of BAV 2 and CAV 2 with HAV 2.* The published physical maps of CAV 2 DNA from different studies [9, 10] were contrasting, therefore the validity of these maps were checked in order to localize the observed DNA heterogeneity on the CAV 2 genome. Molecular clones of BAV 2 DNA were used in order to study the polarity of the *Bam*HI fragments of the CAV 2 DNA. The blot of *Bam*HI digested CAV 2 and *Eco*RI digested HAV 2 DNA probed with three different BAV 2 clones can be seen in Fig. 3. These clones hybridized to both CAV 2 and HAV 2 DNA and showed the following order of the *Bam*HI fragments of CAV 2 DNA from left to the right: A, B, D, E, F, C which corresponds to the data of Spibey and Cavanagh [10]. According to their maps the heterogeneous DNA fragments of the Manhattan strain can be localized on the right end of the CAV 2 genome.


A
a b c d e f g h i j k
A
B
D E F
C
BamH I
A
B
Sal I
A
B
Bgl II
C J K A
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E
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F
B
D
H
Pst I
0 4 8 12 16 20 24 28 31

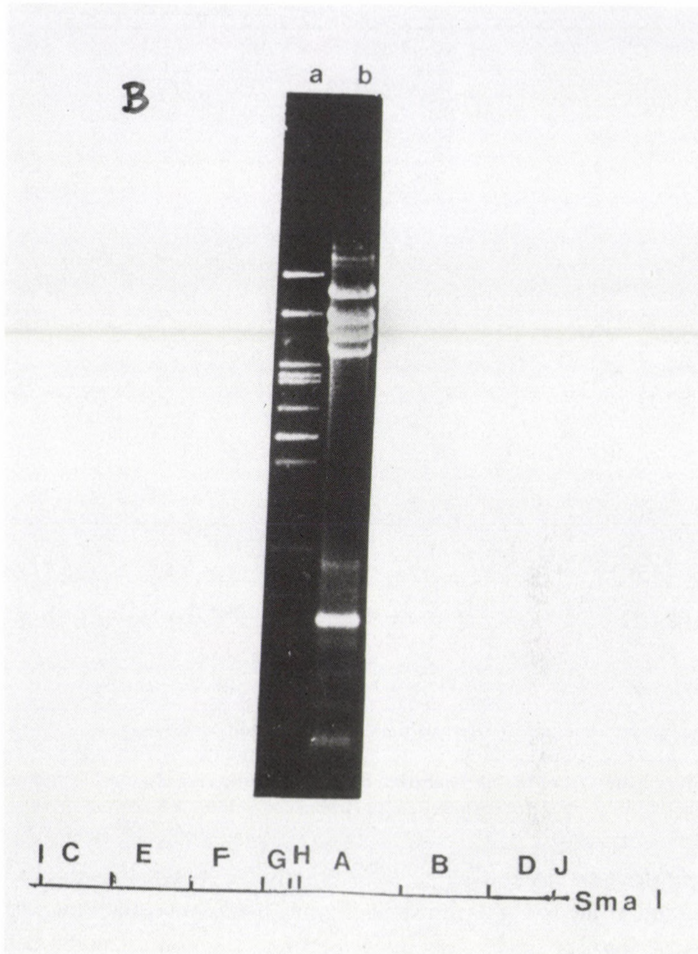


*Fig. 1.* Restriction enzyme analysis of DNA of CAV 2 Manhattan strain. *A*, *Top*. Lane a: HAV 1 DNA cut by *Hind*III as molecular weight control. Lanes b-f: CAV 2 DNA prepared from the Triton supernatants of infected MDCK cells cut by *Pst*I, *Sal*I, *Bgl*II, *Bam*HI, *Xho*I. Lanes g-k: CAV 2 DNA prepared from the ultracentrifuged virions cut by *Pst*I, *Sal*I, *Bgl*II, *Bam*HI, *Xho*I. *Bottom*: restriction maps of CAV 2 [10]. *B*, *Top*. Lane a: HAV 1 DNA cut by *Hind*III as molecular weight control. Lane b: CAV 2 DNA prepared from the ultracentrifuged virions cut by *Sma*I. *Bottom*: restriction map of CAV 2 [10]

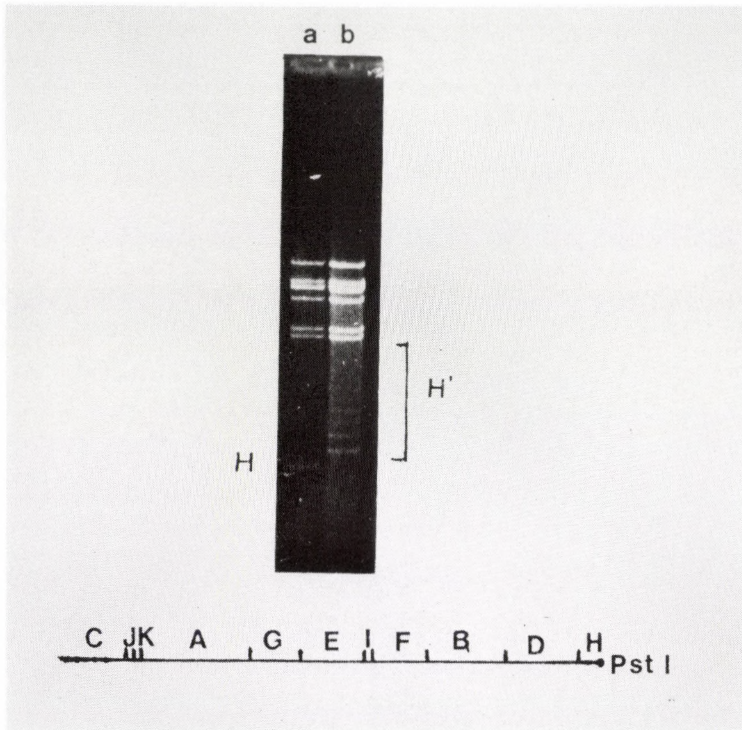


*Fig. 2.* Restriction enzyme analysis of DNA of CAV 2 Toronto and Manhattan strains. *Top.* Lane a: DNA of CAV 2 Toronto strain prepared from ultracentrifuged virions cut by *Pst*I. Lane b: DNA of CAV 2 Manhattan strain prepared from the ultracentrifuged virions cut by *Pst*I. *Bottom*: restriction map of CAV 2 [10]

*Blot hybridization using CAV 2 DNA.* Figure 4 shown the autoradiography of the blot obtained from the gel shown in Fig. 1. after hybridization with the whole CAV 2 DNA. A similar heterogeneity of certain fragments can be seen as in the ethidium bromide stained gel, but because of the higher sensitivity of this method more components of the heterogeneous DNA populations (in the case of *Pst*I at least 9) can be recognized.

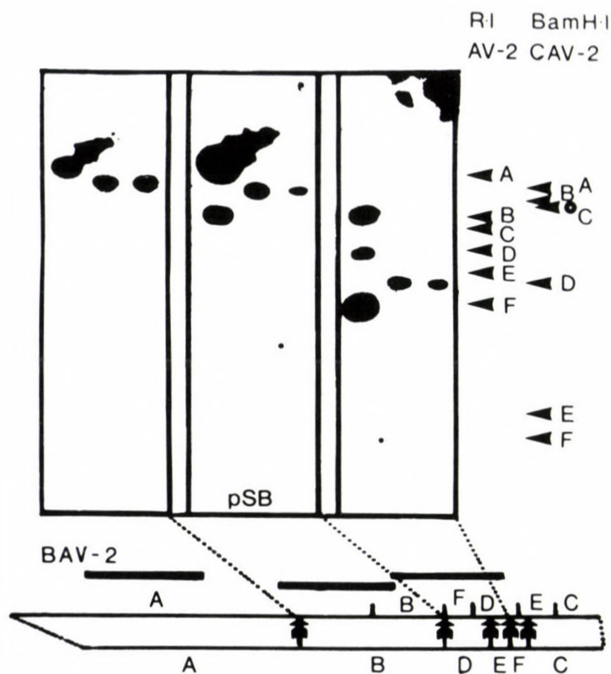


*Fig. 3.* Colinearity of BAV 2 CAV 2 and human AV 2 genomes. Restriction fragments of the DNA of human AV 2 (*Eco*RI) and CAV 2 (*Bam*HI) were blotted to nylon membranes, and hybridized with $^{32}$P labelled cloned (pSB) fragments of BAV 2 DNA (shown by horizontal bars below the photographs). *Arrows*: positions if the ethidium-bromide-stained bands in the original gel. *Vertical arrows*: localization of the radioactive signals along the physical maps of the blotted genomes. *Capital letters*: *Eco*RI-fragments of human AV 2 (upper line) and *Bam*HI-fragments of CAV 2 (lower line) DNA. The same blot was hybridized with all three BAV 2 probes upon subsequent washing of the filter

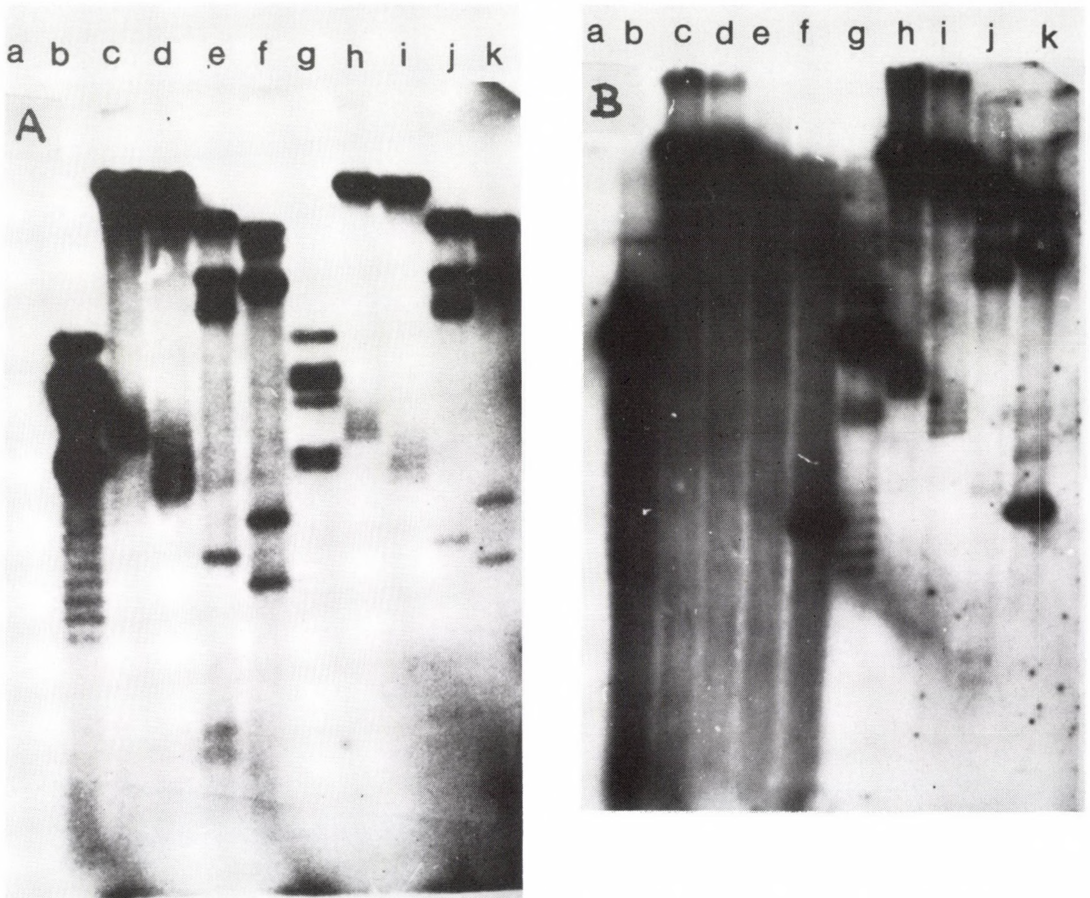


*Fig. 4.* Hybridization of $^{32}P$ labelled CAV 2 probes with CAV 2 genome. *A*. Hybridization of $^{32}P$ labelled CAV 2 full length DNA with Southern transfers of HAV 1 DNA as molecular weight control (lane a), of CAV 2 Manhattan strain DNA prepared from the Triton supernatants of infected MDCK cells (lanes b-f), and of CAV 2 DNA prepared from the ultracentrifuged virions (lane g-k). DNAs were digested by the following enzymes lane a: *Hind*III; lanes b, g: *Pst*I; lanes c, h: *Sal*I; lanes d, i: *Bgl*II; lanes e, j: *Bam*HI; lanes f, k: *Xho*I. *B*. Hybridization of $^{32}P$ labelled *Eco*RI-C fragment of CAV 2 DNA was used in the experiment shown in *A*

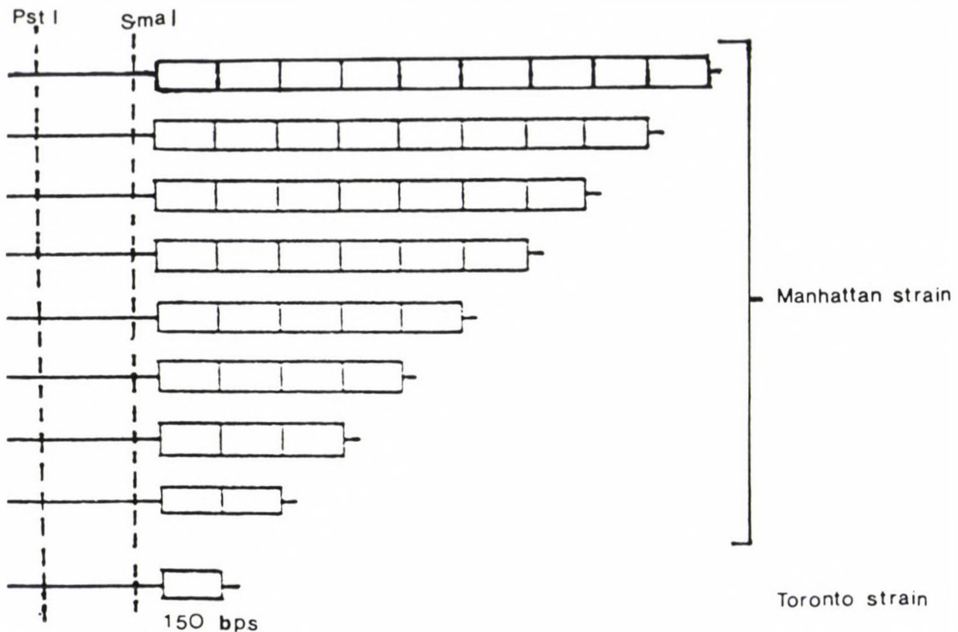


*Fig. 5.* The proposed model for the altered right terminus of CAV 2 Manhattan strain

## Discussion

In this paper we described an altered strain of CAV 2 with a heterogeneously increased genome. This enlargement can be localized to the right end of the viral DNA based on the known physical maps [10].

Sequence data are available for the leftmost 11.3% of the CAV 2 genome [11]. There is a *Sma*I site at nucleotide 180, and because of the nature of the ITR and can expect the same site at the right end of the viral DNA. Accordingly, the published physical map of CAV 2 [10] shows two terminal *Sma*I fragments with the same size. Since cutting the DNA of our CAV 2 strain results in the heterogeneity of this small terminal *Sma*I (Fig. 1B), the observed diversity of the genome is presumably caused by different insertions in the right ITR.

Mutant adenoviruses possessing amplified right ITRs have been reported previously [1–4]. In these studies the viral genome was increased by different numbers of tandemly reiterated DNA sequences from the ITR. Our data suggest a probably similar variation in the DNA of the CAV 2 strain studied by us.

The proposed model for the right terminus of our CAV 2 strain can be seen in Fig. 5. It is important to note that the differences between the size of the individual members of the heterogeneous terminal fragments are always the multiples of 150

base pairs, which indicates the insertion of a repeating sequence with this size into the right ITR in numbers varying from 1 to at least 9. The enlarged viral DNAs were encapsidated into viable virions as heterogeneity of the genomes of CsCl density gradient purified virions was observed. The upper limit of the possible enlargement of the genome is probable determined by packaging restriction. In our case the increase of the genome size is about 1350 base pairs. This is 4% of the CAV 2 genomes, which is similar to the maximum enlargement of the mutant HAV 34 genome [3].

The mechanism of the genesis of the probable insertions into the ITR are not known, but as in the above cited cases [1–4], illegitimate recombination and subsequent DNA replication can be proposed as a possibility to generate tandemly reiterated DNA sequences [10].

It is interesting that other vaccine strains of CAV 1 [4] and CAV 2 [10] have been reported to carry aberrant right termini. Nevertheless, the aberration found in the latter case was not the same as in ours according to the restriction profiles. It is possible that as in these cases the high multiplicity of infection in the nonhomologous tissue culture during attenuation could result in the development of the observed enlargements of the genome.

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# MODIFIED SPOT HYBRIDIZATION TEST USING BIOTINYLATED DNA PROBE

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A modification that simplifies the spot hybridization technique is described for using biotinylated DNA probes. Plasmid EWD299 having LT gene insert, labelled with biotin either by nick translation or using photobiotin was used as DNA probe for the specific detection of enterotoxigenic *Escherichia coli*. A simple protocol has been described for easy lysis of test samples by boiling in distilled water followed by detergent treatment and was found to be as efficient as the lysis using lysozyme and protease. Three different solid supports namely DEAE-cellulose paper, nitrocellulose paper and nylon membrane were also compared for their suitability in this spot hybridization test. Nitrocellulose paper was found to give better colour signal with the photobiotinylated DNA probe.

DNA hybridization technique has emerged as a popular and powerful tool for specific detection of nucleic acid sequences in test samples because of its specificity and sensitivity. DNA probes are extensively being used in microbiology [1, 2]. In the present study we have used biotinylated plasmid EWD299 containing the sequence coding for heat labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* (ETEC) for detection of bacteria which have the genetic potential for this toxin production by spot hybridization. Biotinylated DNA probes have been described earlier for ETEC detection by dot blot hybridization using purified DNA from the test samples [3]. The method reported here is simple and allows the use of biotinylated probes for the detection of ETEC by spot hybridization without any enzymatic treatment unlike the cumbersome colony hybridization [4], still retaining the specificity and sensitivity for detection. Labelling of plasmid with biotin has been done by enzymatic method of nick translation and photochemical method using a photoprobebiotin. Three different solid matrices viz. DEAE-cellulose paper, nitrocellulose paper and nylon membrane were also evaluated for their use in this spot hybridization test.

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## Materials and methods

*Preparation of plasmid. E. coli* C600 strain harbouring plasmid EWD299 was obtained from the kind courtesy of Professor W.E. Hill, Food and Drug Administration, USA. This plasmid constructed originally from pBR 313 and p307 has the heat labile toxin gene flanked by HindIII and HincII sites. HindIII digest generates 850bp fragment that contains B subunit and one third of A subunit of LT. Isolation of plasmid EWD299 was done as previously described by Birnboim and Doly [5].

*Labelling of plasmid by nick translation.* Plasmid was labelled with Biotin-7-dATP using BRL Nick Translation Kit (Bethesda Research Laboratories, USA) as per the manufacturer's instructions.

*Labelling of plasmid with photobiotin.* Plasmid was labelled with Photoprobebiotin (Vector Laboratories Inc., USA) as per manufacturer's instructions.

Sensitivity of the above labelled DNA probes were tested by diluting the probe and spotting different concentrations of the diluted DNA on the solid matrices before processing for colour development as mentioned below.

*Solid supports.* DEAE-cellulose paper (Whatman DE81), Nitrocellulose paper Type SCN (Advanced Microdevices Pvt., Ltd., Ambala, India) and Nylon membrane (Sigma Chemical Co., USA) were used as solid supports for immobilizing test samples.

Three different protocols as described below were used for processing the bacterial samples prior to the spot hybridization. One ml aliquot of bacterial cultures grown overnight in Luria broth was centrifuged.

(A) The pellet was suspended in 100 μl of sterile distilled water and the cells were lysed by keeping for 5 min in boiling water bath. Cell debris removed by centrifugation and supernatant boiled for 5 min and cooled over ice before spotting.

(B) The pellet in this protocol was suspended in Tris (25 mM pH 8.0) EDTA (10 mM) glucose (50 mM) containing lysozyme (2 mg/ml), kept in ice for 15 min followed by removal of debris by centrifugation. Supernatant was treated with proteinase K (200 μg/ml) at 37 °C for 10 min and extracted with phenol:chloroform (1:1) twice for 5 min each. Aqueous layer was collected, boiled for 5 min and cooled over ice before spotting.

(C) the pellet was suspended in 50 μl of sterile distilled water and kept for 5 min in boiling water bath. Equal volume of 20% SDS was added to this and kept at room temperature for 5 min. After the removal of cell debris by centrifugation, crude nucleic acids in the supernatant were extracted with equal volume of phenol:chloroform (1:1). Aqueous layer was collected, boiled for 5 min and cooled over ice before spotting on filters.

Paper strips were cut and washed in sterile distilled water and then soaked in 1 M ammonium acetate for 10 min. On these wetted strips 10 μl of sample prepared by the above-mentioned protocols were spotted. The strips were then air dried and baked at 80 °C in vacuum oven for 2 h. Prehybridization of these strips was done in small plastic bags as described by Maniatis et al. [6]. Hybridization was done by 10 μg of the biotinylated probe (10 ml of hybridization buffer for 10x10 $cm^2$ strips for 6 h). After hybridization strips were washed in 2x Standard Saline Citrate (SSC) (1x SSC is 0.15 M Sodium Chloride and 0.015 M Sodium Citrate) and 1% Sodium Dodecyl Sulphate (SDS) for 30 min at 42 °C followed by 2 washes of 10 min each in 1x SSC, 1% SDS at 42 °C followed by 0.1x SSC, 1% SDS at 68 °C for 30 min followed by 2x SSC for 5 min. Washed strips were air dried and baked for 5 min and then colour development was done using Streptavidin-Alkaline Phosphate conjugate (BRL) with 5-bromo-4-chloro-3-indolyl phosphate substrate with nitro blue tetrazolium for colour production. Development of blue dots indicate hybridization.

## Results

Results obtained for the detection of bacteria with and without LT gene using biotinylated DNA probe with the three protocols described as A, B and C are given in Fig. 1. Using the biotin labelled plasmid EWD299, there was no non-specific binding when samples were processed as per protocols B or C. Results obtained from the protocols B and C are quite comparable. Biotin labelling of the probe DNA by photoprobebiotin was found to be better than by nick translation. Probe concentration of as low as 0.8 pg could be visualized by photobiotin labelling whereas a concentration of 0.08 ng probe could be seen in the case of nick translated probe (Fig. 2). Hybridization of different concentrations of test culture immobilized on three different solid supports viz. DEAE-cellulose paper, nitrocellulose paper and nylon membrane was done using 200 ng of photobiotin labelled probe per 10 ml of hybridization buffer and the results are shown in Fig. 3.

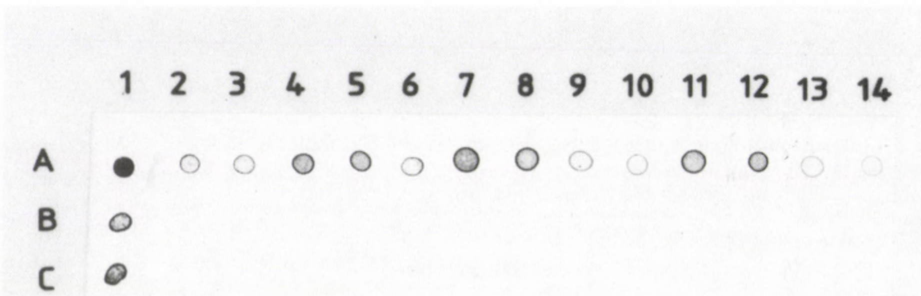


*Fig. 1.* Comparison of different sample preparation protocols (A, B and C) for detection of ETEC using biotinylated probe in strain containing LT gene (1) and strains not having LT genes (2 to 14). 1 – *E. coli* C600/EWD299/, 2 – *E. coli* JC411, 3 – *E. coli* CRC-603, 4 – *E. coli* C600, 5 – *E. coli* K724, 6 – *E. coli* W310, 7 – *E. coli* K12, 8 – *E. coli* 1100, 9 – *E. coli* W1485, 10 – *E. coli* B, 11 – *E. coli* 58-161, 12 – *E. coli* AB301, 13 – *Shigella dysenteriae* type 1, 14 – *Salmonella typhi*

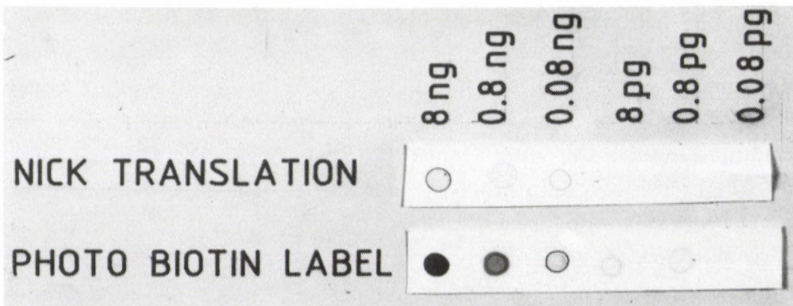


*Fig. 2.* Sensitivity of biotinylated DNA probes labelled by nick translation and photoprobe biotin. Target DNA concentrations (immobilized on the solid support) are also given above

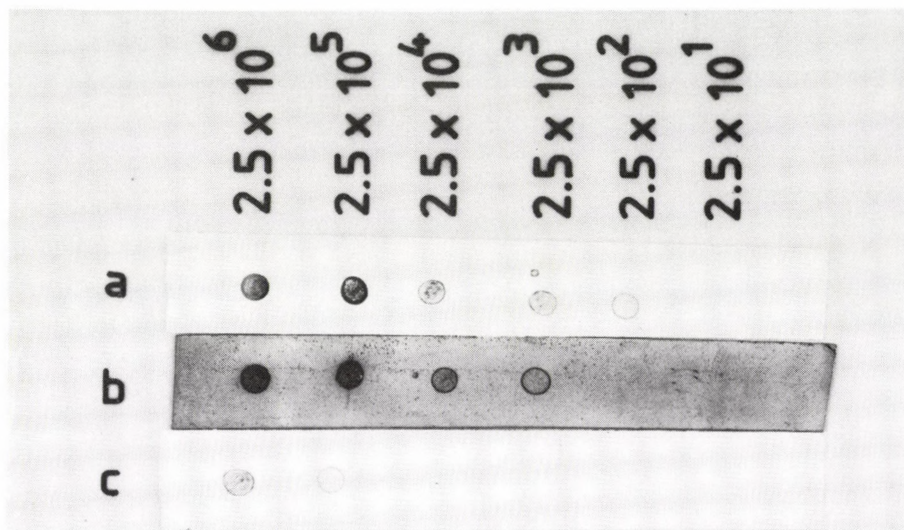


*Fig. 3.* Comparison of three different solid supports viz., a – Nitrocellulose paper, b – Nylon membrane and c – DEAE-cellulose (Whatman DE-81) paper for spot hybridization. Number of cells spotted on the support given above

## Discussion

Protocol C was found to be the best among the three protocols because of its simplicity and specificity and also it can have wider applications in the routine clinical investigations. Higher non-specific background colour development in the case of samples prepared by Protocol A indicates the need for deproteinization while using the biotinylated DNA probes unlike radiolabelled probes. Complete absence of intracellular factors which may bind to streptavidin decides the specificity of biotinylated probes. Intracellular biotin, a prosthetic group bound covalently to enzymes involved in one carbon transfers, leads to non-specific binding of streptavidin and hence the appearance of false positive signal. So it becomes obligatory to get rid of contaminating proteins from DNA preparations or else purified DNA needs to be used [7]. When large numbers of samples are to be screened, preparation of purified DNA becomes tedious. The protocol C mentioned in this study can conveniently be used in clinical laboratories without employing any enzymatic lysis procedures. Simple hypotonic lysis with distilled water aided by subsequent detergent treatment makes this procedure rapid. Deproteinisation by

phenol:chloroform is enough without elaborate DNA purification steps for efficient use of biotinylated probes.

On comparing suitability of different solid matrices for protocol C suggested in this study, nitrocellulose membrane gave very satisfactory result. DE81 paper did not find an application in this present investigation because of its low efficiency of binding as well as fragility of paper. Although nylon membrane gave comparable results to nitrocellulose paper, yet the high background colour produced using this paper interferes with the clarity of signal. Using nitrocellulose paper even as low as $2.5 \times 10^2$ bacterial cells were clearly visualized.

Labelling of probe DNA was found to be better with photobiotin than by the classical nick translation technique. Photobiotin is a photoactive analogue of biotin which forms stable linkages with single and double stranded nucleic acids and proteins. Hence it can also label single stranded DNA molecules yielding more biotinylated probe. Use of photobiotin is advantageous over nick translation because this method was found to be simple, efficient, rapid, and reliable. Moreover, labelled nucleic acid is easily purified from free photobiotin and the progress of labelling can be visually monitored as the labelled nucleic acid is red in colour. Main disadvantage in nick translation is the degradation of single stranded DNA by DNAse I with increased time of incubation which is overcome by this photochemical coupling. Biotin labelled probes have been found by us to be quite stable and are able to give a strong colour signal even after 10 months of labelling.

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# DISTRIBUTION OF AFLATOXIN-PRODUCING MOULDS AND AFLATOXINS IN DAIRY CATTLE FEED AND RAW MILK

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Distribution of aflatoxigenic moulds and aflatoxin $B_1$ in Yugoslav dairy cattle feeds as well as the presence of aflatoxin $B_1$ and $M_1$ in raw milk, was tested. The experiments were carried out through three years (in all seasons). Samples were taken from state and private farms in Vojvodina. Feeds were contaminated in 83–100% with moulds. Fungi of *Aspergillus flavus-oryzae* group were present permanently and the highest incidence of them was noticed during the third research year. Aflatoxin $B_1$ was not found in the first year, but malt spent grains used for cows' feeding in summer of the second research year was contaminated with it (50.0 μg/kg). The same feed and pelleted sugar beet pulp were contaminated with aflatoxin $B_1$ in winter, spring and summer of the third research year (5.0 to 16.0 μg/kg). Aflatoxin $B_1$ and $M_1$ were not found in raw milk through three-years investigations.

Members of *Aspergillus flavus-oryzae* group are widespread fungi isolated from various animal feeds, which can produce high toxic metabolites, aflatoxins.

The high incidence of the *A. flavus-oryzae* group in animal feeds has been reported by several authors [1–4]. Moreno et al. [5] reported that of the 32 animal feed samples, 27 (84%) contained *A. flavus* and 21 (66%) of them had at least one aflatoxin-producing isolate.

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Lactating animals consuming contaminated feeds transform primary aflatoxins to the other forms and excrete them into milk. Aflatoxin $B_1$, the most commonly found aflatoxin, is metabolized to aflatoxin $M_1$ [6].

The present paper describes recent progress in distribution of aflatoxigenic moulds and aflatoxin $B_1$ in Yugoslav dairy cattle feeds and the possible finding of aflatoxins residue in raw milk.

## Materials and methods

Contamination of dairy cattle feeds, originated from four state and four private farms in Vojvodina, with moulds and aflatoxin $B_1$ was examined. The investigations were carried out through three years, in all seasons. At the same time, raw milk samples, taken from the same farms, were analyzed on aflatoxin $B_1$ and $M_1$ presence.

Determination of mould species was performed according to Raper and Fennel [7], Booth [8], Ellis [9] and Pidoplicko and Milko [10]. Qualitative and quantitative determination of aflatoxin $B_1$ in feeds was carried out by thin-layer chromatography (TLC) as described by Balzer et al. [11] and aflatoxin $B_1$ and $M_1$ in milk according to Stubblefield et al. [12]. Pure aflatoxin $B_1$ (A 6636) and $M_1$ (A 6438) were supplied by Sigma Chemical Co., USA.

## Results and discussion

Results of three-years investigations of fungal distribution in dairy cattle feeds indicate high level of contamination in all research periods. One can notice from Fig. 1. between 83% (fall – third research year) and 100% (summer and spring – second research year) samples tested were contaminated with moulds.

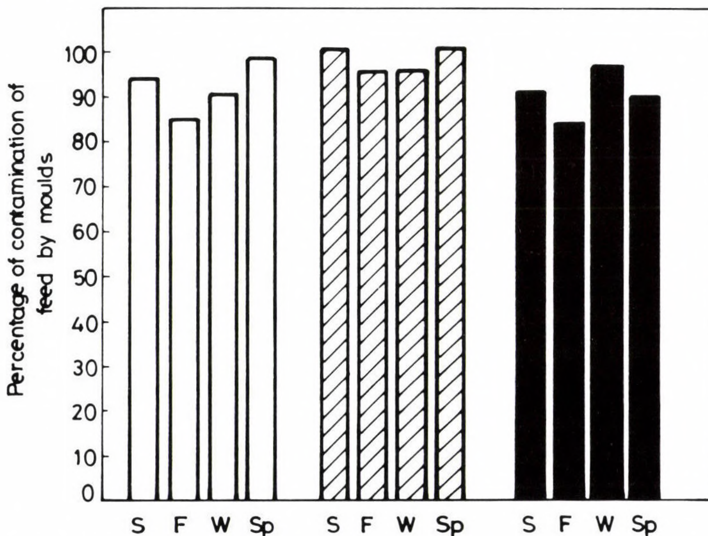


*Fig. 1.* Contamination of feeds with moulds. Open columns: first research year; shaded columns: second research year; solid columns: third research year; S = summer, F = fall, W = winter, Sp = spring

Mycological analyses showed that *Aspergillus* species were rather frequent and they had a significant share in mycopopulations isolated from feeds. It was established that from 30 (in winter) to 58% (in spring) moulded feed samples were contaminated with fungi of genus *Aspergillus* during the first research year (Fig. 2) Moulds of the *A. flavus-oryzae* group were present permanently. In summer, fall and winter only *A. flavus*, as a member of this group, was isolated. In spring, *A. parasiticus* was noticed as a contaminant, too. *A. flavus* was isolated from various feed samples, such as concentrate, dried and fresh alfalfa, hay, corn stover and pelleted sugar beet pulp. But it is necessary to point out that the concentrate was constantly contaminated with *A. flavus* at high level.

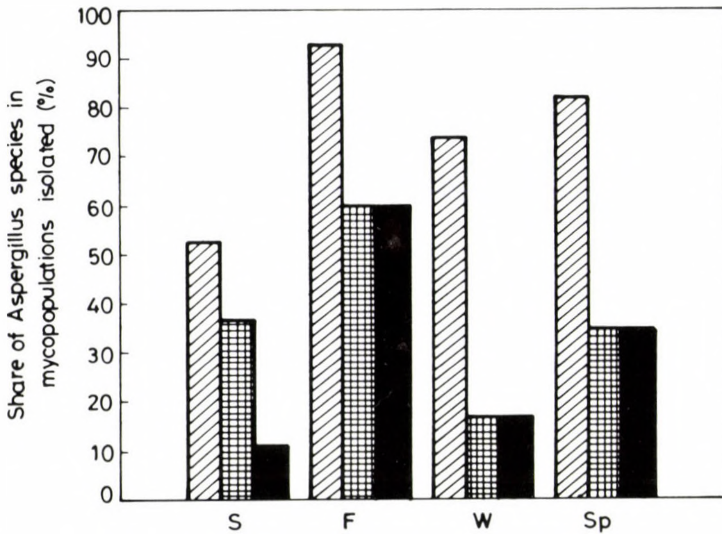


*Fig. 2.* Share of *Aspergillus* species in mycopopulations isolated from moulded feed samples during the first research year. Shaded columns: *Aspergillus* spp.; hatched columns: *A. flavus-oryzae* group; solid columns: *A. flavus*; S = summer, F = fall, W = winter, Sp = spring

Although aflatoxigenic fungi were very frequent, aflatoxin $B_1$ was not found during the first research year. Aflatoxin $B_1$ was detected in malt spent grains (50.0 μg/kg) used for dairy cattle feeding in summer of the second research year. From the same feed *A. flavus* was isolated as well. In fall of the same year aflatoxin $B_1$ was found in hay, bur at lower concentration (trace). From these samples various non-aflatoxigenic moulds were isolated (*Alternaria alternata, Cladosporium cladosporioides, Fusarium semitectum, Fusarium solani, Mucor circinelloides* and *Rhizopus nigricans*). In winter and spring of the second research year aflatoxin $B_1$ was not found more, although *A. flavus* was very common in various feeds (Fig. 3), especially in concentrate.

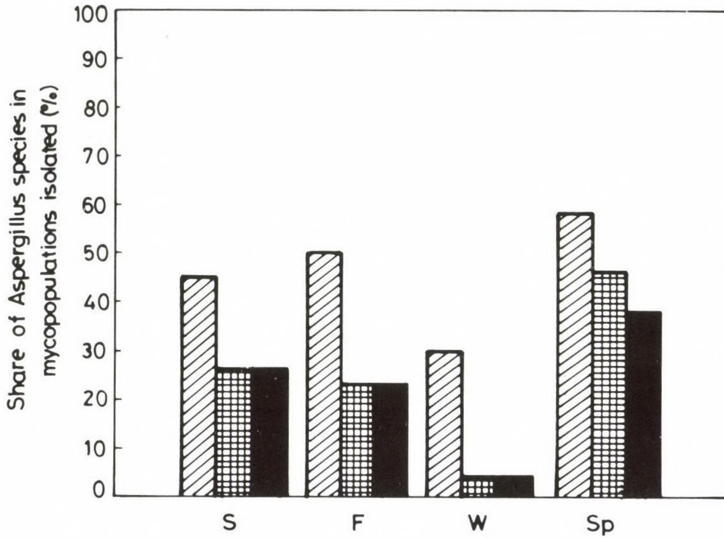


*Fig. 3.* Share of *Aspergillus* spp. in mycopopulations isolated from moulded feed samples during the second research year. Shaded columns: *Aspergillus* spp.; hatched columns: *A. flavus-oryzae* group; solid columns: *A. flavus*; S = summer, F = fall, W = winter, Sp = spring

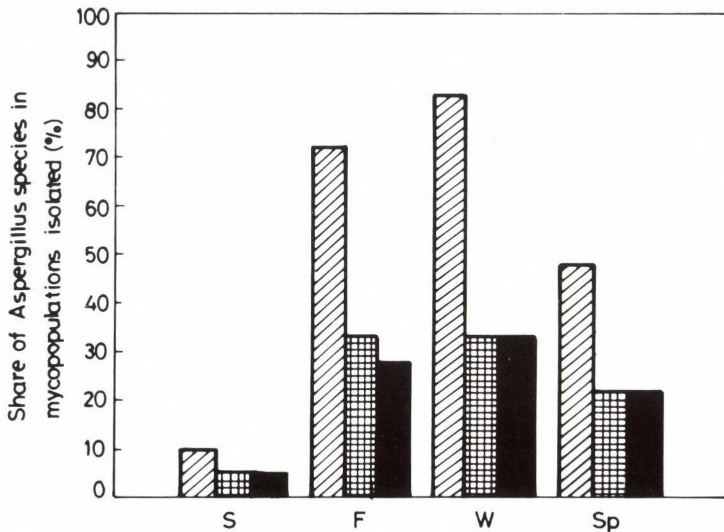


*Fig. 4.* Share of *Aspergillus* spp. in mycopopulations isolated from moulded feed samples during the third research year. Shaded columns: *Aspergillus* spp.; hatched columns: *A. flavus-oryzae* group; solid columns: *A. flavus*; S = summer, F = fall, W = winter, Sp = spring

The highest distribution of *Aspergillus* spp. in mycopopulations isolated from dairy cattle feeds was noticed during the third research year (Fig. 4). They were the most frequent in the fall and spring period, when they were isolated from even 93 and 82% feeds contaminated with moulds. At the same time, fungi of the *A. flavus-oryzae* group were the most frequent, too.

It was found that some samples of malt spent grains and pelleted sugar beet pulp were contaminated with aflatoxin $B_1$ in all season of the third research year, except in fall. Concentrations detected ranged from 5.0 to 16.0 $\mu$g/kg.

None of raw milk samples tested was contaminated neither with aflatoxin $B_1$ nor with aflatoxin $M_1$ during three-year investigation.

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# PRODUCTION AND REGULATION OF A THERMOSTABLE PROTEASE BY *PSEUDOMONAS* SP. B45

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A *Pseudomonas* sp. produced an extracellular thermostable protease which required induction by peptone. Growth of the organism and the production of protease was optimum at 30 °C. The enzyme was subjected to catabolite repression by glucose. Both chloramphenicol and rifamycin completely abolished protease production indicating de novo synthesis of the enzyme. Leucine, lysine, histidine and glycine enhanced the protease production considerably and they were the most effective when added during the active period of production. Glucose repression could not be relieved by addition of leucine.

Microbial proteases are amongst the most important of the commercially produced enzyme. They have diverse application such as in the food, pharmaceutical, leather, detergent and brewing industries. The genus *Bacillus* has played a major role in this development [1]. Among others *Vibrio* [2], *Chromobacterium* [3], *Micrococcus* [4], *Aeromonas* [5], *Serratia* [6] and *Pseudomonas* [7 – 11] are prevalent. The genus *Pseudomonas* is a versatile group involved in various detoxification mechanisms, leaching, production of metabolites, enzymes, etc. A *Pseudomonas* strain isolated in our laboratory from tannery backyard soil has the capacity to produce enormous amount of thermostable protease in both solid and liquid media. Application of this enzyme for various purposes is being explored including the hydrolysis of the gelatin layer from spent photography and X-ray film, hydrolysis of fleshings and trimmings obtained as a solid waste from tannery and for unhairing of raw skins, bating, etc. The organism could grow on waste chrome shaving and liquefy it completely. Importance of this enzyme in various applied fields led us to investigate the production and study of its regulation. Details of this study is presented in the present communication.

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## Materials and methods

*Organism*. Strain B45 was isolated from soil. It was identified as a *Pseudomonas* sp. proteolytic by gelatin liquefaction and casein hydrolysis plate tests. It was routinely maintained on yeast extract malt extract agar slants.

*Medium*. This contained (g/litre): peptone, 15.0; $K_2HPO_4$, 2.0; $MgSO_4 \cdot 7H_2O$ 0.1; $CaCl_2$, 0.1; $ZnSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$ traces adjusted to pH 8.0. Five ml of the medium were inoculated with a loopful of the organism and incubated at 30 °C for 24–28 h in an Universal Orbital shaker (B. Braun, West Germany) at 140 rpm. The growth of the organism was monitored by measuring the optical density of the culture at 600 nm. At the end of the incubation period, the cells were separated by centrifugation at 8000 *g* for 10 min and the supernatant served as the enzyme extract.

*Assay of enzyme activity*. Proteolytic activity was measured by casein digestion. The reaction mixture containing 1 ml of 2% casein, 1.9 ml of 0.01 M Tris-HCl buffer pH 8.5 and 100 μl of enzyme was incubated at 65 °C for 30 min. The reaction was stopped by the addition of 2 ml of 5% trichloroacetic acid. An enzyme blank was always included. The undigested casein was separated by filtration. The absorbance of the TCA soluble fragments was measured at 280 nm and compared with a tyrosine standard.

One unit of enzyme activity is that amount of enzyme which is required to liberate 1 mg of tyrosine under assay conditions.

Various physiological parameters were evaluated for their effect on enzyme production to optimise conditions for production. The effect of growth temperature (15 °C to 45 °C), various organic nitrogen sources (soybean meal, peptone, yeast extract, casitone, gelatin, beef extract, casein, albumin, malt extract) at a final concentration of 1.0%, different concentrations (0.1 to 4.0%) of the best inducing nitrogen source (peptone), pH level of the production medium, phase of growth at 30 °C and 37 °C, various carbon sources (D-glucose, sucrose, D/-/ fructose, lactose, starch, dextrin, maltose) at final concentration of 1.0 to 3.0% and various amino acids (L-cystine, DL-α-alanine, DL-tryptophan, DL-methionine, L-lysine, L-leucine, L-cysteine, L-tyrosine, DL-serine, DL-aspartic acid, L-glumatic acid, L-histidine, L-arginine, glycine), effect of inhibitors (chloramphenicol, rifamycin) on enzyme synthesis were studied.

Sugars and amino acids used in this study were autoclaved separately at 10 lb pressure for 10 min and added to the medium.

*Fractionation of peptone*. Peptone (2 g) was dissolved in 5 ml of 0.01 M $NH_3$-$NH_4Cl$ buffer (pH 8.5) and layered on a Sephadex G-25 column (45 x 1.5 cm). Samples (10 ml) were eluted with the same buffer at a flow rate of 60 ml/h. The absorbance of the fractions was measured at 255 nm. The samples was found to be eluted in two peaks (I and II). Fractions under the same peak were pooled, dialyzed against two changes of distilled water at 4 °C and lyophilized. One per cent of lyophilized samples of peaks I and II were added to the production medium and their inducing effects were compare to that of the control, 1% unfractionated peptone.

*Temperature profile and thermal stability of the enzyme*. Reaction mixtures of the enzyme assay were incubated at different temperatures ranging from 4 to 90 °C or 30 min and enzyme activity was determined to study the temperature profile of the enzyme. To study the stability of enzyme, 100 μl enzyme in 1.9 ml of assay buffer was preincubated at various temperatures mentioned above for 30 min, followed by substrate addition and subsequent assay as described above.

All the results are the average of two independent trials.

## Results

Study on the stability of the crude enzyme revealed that it is a thermostable enzyme and retained its activity even at 65 °C (Fig. 1). This is similar to the characteristics of enzymes from other *Pseudomonas* sp.

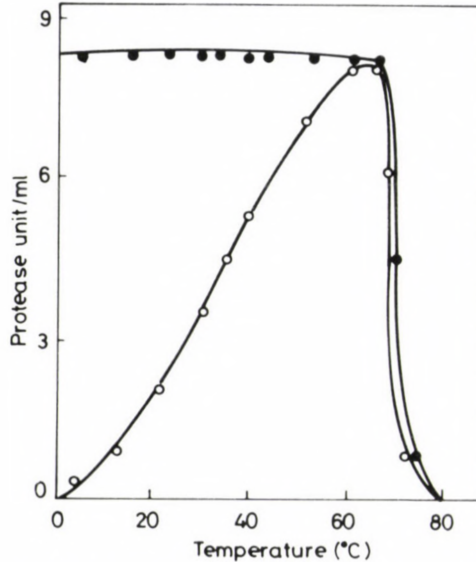


*Fig. 1.* Temperature profile and thermal stability of the enzyme. Enzyme activity at different temperature (o), enzyme stability profile (•)

**Table I**

*Effect of various organic nitrogen sources on the production of protease by Pseudomonas sp. B45**

| N-source | Protease unit/ ml of the culture filtrate |
|---|---|
| Soybean meal | 1.17 |
| Peptone | 6.0 |
| Yeast extract | 5.0 |
| Casitone | 4.8 |
| Gelatin | Nil |
| Beef extract | Nil |
| Casein | 4.0 |
| Albumin | Nil |
| Malt extract | 2.0 |

*The culture was grown at 30 °C for 28 h

It was observed that no enzyme secretion occurred in a minimal medium (glucose/10 g/litre) and $NaNO_3$ (1 g/litre) added instead of peptone. Hence various organic nitrogen sources were evaluated for their ability to induce production (Table I).

In order to arrive at the optimal concentration of the inducer for maximal production, varying concentrations of peptone from 0.1 to 4% were tested for their influence on extracellular protease synthesis. Table II shows that 1.5% peptone induced maximum activity. Higher concentrations proved to be inhibitory.

Table III indicates the effect of addition of 1.5% peptone cultures of *Pseudomonas* at different times after inoculation. It is seen that maximum production occurred when peptone was added at zero time. When peptone was added up to 12 h after inoculation, there was a marginal decrease in enzyme synthesis. If added after 12 h of inoculation there was a steep fall in enzyme production.

**Table II**

*Effect on different concentrations of peptone on the production of protease by Pseudomonas sp. B45**

| Concentration of peptone (%) | Protease unit/ml of culture filtrate |
|---|---|
| 0.1 | 0.44 |
| 0.5 | 2.70 |
| 1.0 | 5.88 |
| 1.5 | 8.45 |
| 2.0 | 6.48 |
| 2.5 | 5.85 |
| 3.0 | 5.75 |
| 4.0 | 3.87 |

*The culture was grown at 30 °C for 28 h

Since peptone served as a strong inducer of proteolytic activity, an attempt was made to identify the inducing factor in peptone. For this purpose peptone was fractionated in a Sephadex G-25 column and two major peaks were obtained (Fig. 2; Peak I-fractions 6 to 12 and Peak II-fractions 13 to 30). It was observed that the fraction corresponding to Peak I caused only a small increase in enzyme synthesis while the fraction corresponding to Peak II increased the activity by 28% over the control which was the unfractionated peptone (Table IV).

**Table III**

*Effect on addition of 1.5% peptone at different period of incubation on protease production by Pseudomonas sp. B45**

| Addition of peptone at (h) | Protease unit/ml of culture filtrate |
|---|---|
| 0 | 8.5 |
| 3 | 7.49 |
| 6 | 7.78 |
| 9 | 7.76 |
| 12 | 7.05 |
| 15 | 4.78 |
| 18 | 3.13 |
| 21 | 2.47 |
| 24 | 2.45 |

*The culture was grown at 30 °C for 28 h

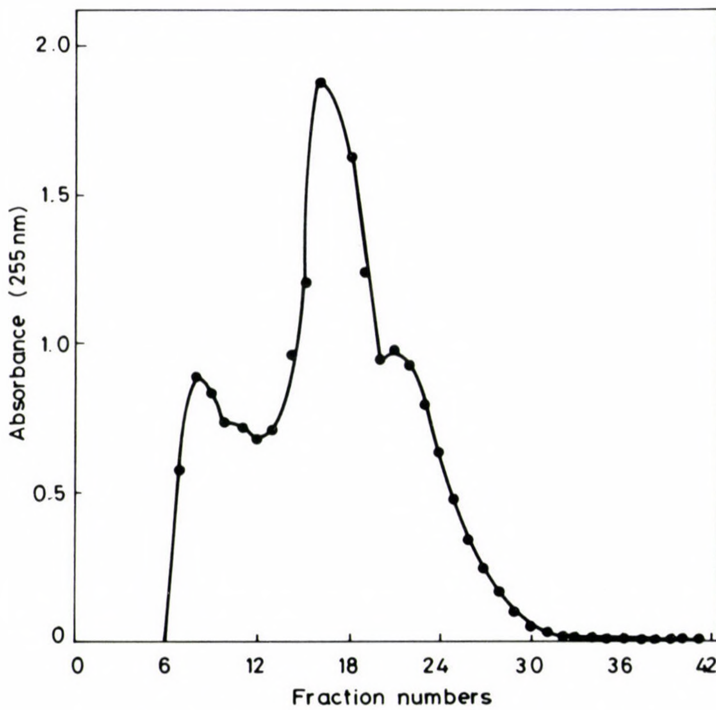


*Fig. 2.* Gel filtration of peptone on Sephadex G-25

Table IV

*Effect of fractionated peptone on incubation of protease synthesis*

| Inducer | Protease activity (%) |
|---|---|
| Peptone (unfractionated) | 100.0 |
| Peak I (fractions 6–12) | 3.88 |
| Peak II (fractions 13–30) | 128.7 |

Protease production by this organism has been studied at different growth temperatures from 15 °C to 45 °C. It is seen that a considerable amount of enzyme was produced at growth temperatures ranging from 23 °C to 37 °C with a maximum at 30 °C, hence all the experiments were set up at 30 °C unless stated otherwise.

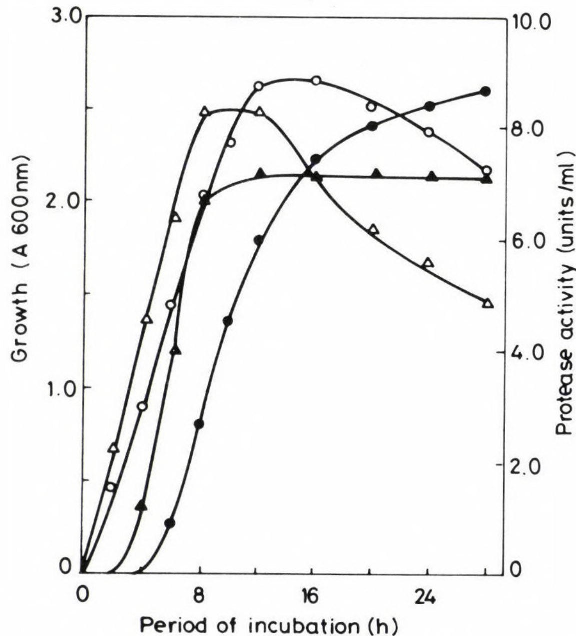


*Fig. 3.* Growth pattern of *Pseudomonas* sp. at 30 °C and 37 °C and protease production at different periods of growth. Growth at 30 °C (o), 37 °C (∧); protease production at 30 °C (•), 37 °C (∧)

A detailed growth study coupled to production of the enzyme has been carried out at 30 °C and 37 °C at different periods of growth. These results are presented in Fig. 3. It can be clearly seen from this figure that this organism grows very rapidly at 37 °C, attaining the stationary phase at the end of 8 h of growth. At 30 °C, the log phase was longer, the stationary phase being reached after 12 h. In addition, it was

seen that the final cell density was higher at 30 °C. For both the temperatures tested the synthesis of extracellular protease occurred during the active phase of growth. The productivity was higher at 30 °C than at 37 °C. At 37 °C the production of the enzyme ceased as soon as the culture reached the stationary phase, while at 30 °C productivity continued during the stationary phase but at a reduced rate.

The effect of chloramphenicol and rifamycin in enzyme synthesis was studied to determine the nature of the protease synthesis. When both chloramphenicol and rifamycin were added separately at various time intervals to actively secreting cells (Fig. 4) it was observed that the inhibition of protease synthesis was prompt and almost complete irrespective of the time of addition of the antibiotic.

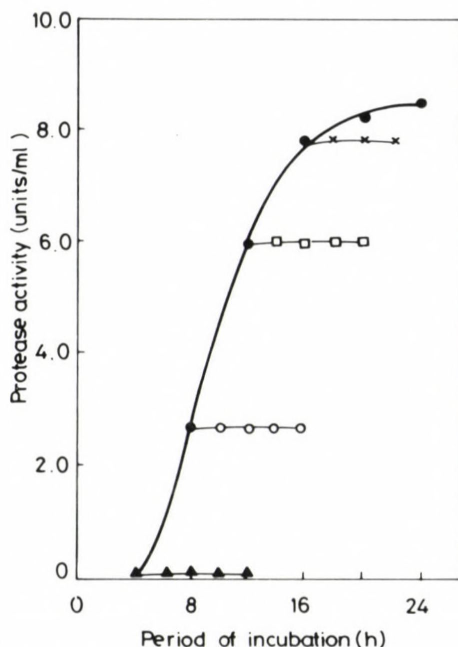


*Fig. 4.* Effect of chloramphenicol on protease production. No addition (•); chloramphenicol (100 μg/ml) was added at 0 h (▲); 7 h (o); 12 h (□); 16 h (x)

Table V indicates the effect of the addition of various amino acids on protease synthesis. Cysteine, cystine and aspartic acid, severely repressed protease production while it was moderately influenced by arginine and alanine. Leucine, histidine, glycine and lysine had a strong, positive influence on enzyme production. When these four amino acids were added together, no cumulative effect could be observed indicating that their stimulatory activities are not additive.

**Table V**

*Effect of different amino acids (0.1%) on the production of protease*

| Amino acid | Protease activity (%) |
|---|---|
| Control (no addition) | 100 |
| L-Cystine | 9.6 |
| DL-α-Alanine | 126.0 |
| DL-Tryptophan | 107.0 |
| DL-Methionine | 114.0 |
| L-Lysine | 140.0 |
| L-Leucine | 153.0 |
| L-Cysteine | 8.65 |
| L-Tyrosine | 100.0 |
| DL-Serine | 100.0 |
| DL-Aspartic acid | 39.5 |
| L-Glutamic acid | 100.0 |
| L-Histidine | 150.0 |
| L-Arginine | 126.3 |
| Glycine | 140.0 |

Amino acids were sterilized separately and added to the peptone medium at final concentration of 0.1%. Culture was incubated at 30 °C for 28 h

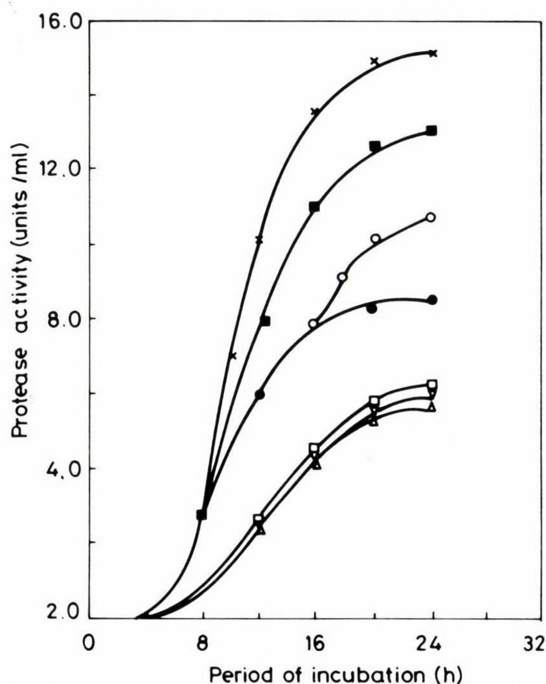


*Fig. 5.* Effect of leucine (0.1%) glucose (1.0%) added at different stages of growth on the production of protease. No addition (•); leucine added at 0 h (▪); 7 h (x); 16 h (o). Growth medium containing only glucose (Δ); leucine added to glucose medium at 7 h (□); 16 h (∇)

Addition of leucine caused the maximal increase in enzyme production. When leucine was added at 0.7 and 16 h of growth (Fig. 5), it was observed that productivity almost doubled when leucine was added during the active phase of growth and it had little influence when added at 16 h (stationary phase).

The pH of the medium played and important role in the production of extracellular protease by this organism. The production of the enzyme was greatly affected at pH 6.7 and below. From pH 7.2 there was and increase in enzyme production which continued till pH 9.0 with a peak at pH 8.5. Thereafter there was a pronounced decrease in enzyme synthesis.

Various carbohydrates were tested for their influence on protease production. Table VI indicates that while sucrose, lactose and fructose had no influence on enzyme synthesis, glucose severely repressed production of the enzyme. Starch increase enzyme synthesis at all the concentrations tested while maltose showed an increase at 1 and 2% levels.

**Table VI**

*Effect of different carbon sources on the production of protease by Pseudomonas sp. B45*

| C-source added in peptone medium | Protease activity (relative to control*) concentrations of | | |
|---|---|---|---|
| | 1.0% | 2.0% | 3.0% |
| Glucose | 67.0 | 4.7 | 0 |
| Sucrose | 100.0 | 100.0 | 100.0 |
| Fructose | 100.0 | 100.0 | 100.0 |
| Lactose | 100.0 | 97.8 | 57.0 |
| Starch | 129.0 | 143.4 | 107.0 |
| Dextrin | 118.0 | 100.0 | 100.0 |
| Maltose | 136.0 | 120.0 | 100.0 |

Amino acids were sterilized separately and added to the peptone medium at final concentration of 0.1%. Culture was incubated at 30 °C for 28 h
*100% activity

## Discussion

Although the production of thermostable extracellular proteases by *Pseudomonas* is known, not much information is available on the regulation of its production. Our studies on the production of a thermostable protease by a *Pseudomonas* sp. isolated from soil indicate that it is not a constitutive system in this organism. It requires the presence of organic nitrogen sources for elaboration of

enzyme activity. All organic nitrogen sources do not serve the purpose. The organism is very specific with respect to the nature of the inducer. In this instance peptone elicited maximum enzyme synthesis. When the concentration of peptone was increased in the medium it was found that at 1.5% maximum yield was obtained and at higher concentration synthesis was repressed probably due to feed back inhibition.

Peptone was fractionated into Peak I and Peak II fractions in order to study the nature of the inducing factor. Peak II fraction, comprising the lower molecular weight fraction caused maximum induction. It may be that because the protein inducers are large molecular weight proteins and cannot enter the cell due to permeability barriers, the lower molecular weight polypeptides or peptides associated with the inducer will enter the cell and trigger enzyme synthesis which then indicates the breakdown of the polymeric inducer present outside the cell. Mckellar [12] has reported on the inducing ability of a low molecular weight fraction of skimmed milk in *Pseudomonas fluorescens*. Jaffa [13] noted the importance of small molecular weight compounds in the induction of proteases *Pseudomonas aeruginosa*.

Studies with the transcriptional and translational inhibitors indicate that protease synthesis is a "de novo" process in this organism and does not involve the release of performed protein molecules.

In certain bacteria like *Bacillus megaterium* the presence of amino acids inhibits the synthesis of proteases [14]. On the other hand in *Aeromonas proteolytica* the presence of amino acids or peptides is essential for the protease formation [15]. Long et al. [16] reported that protease synthesis in *Vibrio alginolyticus* was sensitive to repression by a number of carbon sources including glucose and a number of amino acids but not histidine. In our studies it was seen that leucine, histidine, glycine and lysine had a promoting effect on enzyme synthesis. Our results (Fig. 5) further suggested that leucine may be involved in the secretion of the enzyme through the membrane.

Extracellular proteases require the presence of a signal peptide for secretion. In a study on the signal peptide sequences from several procaryotic and eucaryotic systems, Pertman and Halvorson [17] indicated that in most cases positively charged lysine and arginine usually proceeded the hydrophobic core region of the signal peptide and in the hydrophobic core region, alanine and leucine are most abundant followed by the C-terminal leader peptide where a positively charged histidine is available. All the amino acids indicated above as being part of the signal peptide are stimulatory for protease synthesis by this organism, indicating a possible involvement of these amino acids in the secretion of protease by this strain. Addition of chloramphenicol 1 h after the addition of leucine completely inhibited enzyme synthesis (result not shown) indicating that leucine may be involved in post-transational steps.

The presence of glucose repressed enzyme synthesis indicating the presence of a catabolic like repression phenomenon. Since glucose is readily metabolized by the cell for rapid growth, protease synthesis is switched off in its presence. It was observed that the presence of leucine could not relieve the repression due to glucose (Fig. 5).

The present study provides some insights regarding the regulation of extracellular protease synthesis in a *Pseudomonas* sp. Further studies are required to characterize and comprehend the role of the inducing factor, leucine and glucose on enzyme synthesis at the molecular level.

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# BACTERIOCIN-LIKE ANTAGONISM IN *YERSINIA ENTEROCOLITICA*

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A total of 121 *Yersinia enterocolitica* O3 isolates from patients with gastroenteritis and 37 *Y. enterocolitica* reference strains with different O antigens were tested for bacteriocine production and sensitivity. By using cross-streaking method strains belonging to serogroups of O5; O7,8; O7,13; O11; O11,23; O13,27; O17; O19,8 and O34 produced bacteriocin-like substances. None of the *Y. enterocolitica* O3 strains produced bacteriocin-like material and most of them were uniformly sensitive against the bacteriocin-like material produced by strains of serogroups O7,8; O7,13; O13,27 and O19,8. By sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) significant differences were demonstrated in the whole cell protein patterns of *Y. enterocolitica* reference strains belonging to different serogroups in the range of 33–47 kilodalton (kDa). Out of the ten examined bacteriocin-like material producer strains only one strain harboured a plasmid of about 60 megadalton (MDa).

The genus *Yersinia* originally included *Y. pestis, Y. pseudotubercolosis* and *Y. enterocolitica*. Strains previously termed "atypical *Y. enterocolitica*" or "*Y. enterocolitica*-like organism" have been classified into separate species as *Y. intermedia, Y. kristenseni*, *Y. frederikesenii*. Now there are 11 species recognized within the genus [1, 2]. Since the 1960s the importance and worldwide spread of *Y. enterocolitica* as a causative agent of human infections has increased. Enterocolitis is the most common clinical manifestation affecting usually young children and older adults. *Y. enterocolitica*-associated pseudoappendicular syndrome is known and mostly diagnosed in older children and young adults.

In several countries *Y. enterocolitica* has surpassed shigella and rivals salmonella as a causative agent of acute bacterial gastroenteritis. Reactive arthritis

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and erythema nodosum may develops after *Y. enterocolitica*-associated enterocolitis [3]. Recently, *Y. enterocolitica* sepsis associated with the transfusion of contaminated erythrocytes has been reported [4, 5].

There are more than 50 serogroups of *Y. enterocolitica* but only five of them are associated with human diseases. These are: O1,2a,3; O3; O5,27; O8 and O9 [6]. According to the revised scheme of Wauters et al. [7], there are six biogroups and five of them are regarded as human pathogens: IB; 2; 3; 4 and 5.

Pathogenic *Y. enterocolitica* strains belonging to serogroups of O3 and O9 have been isolated in Europe, Canada, and Japan. Earlier, serogroups of O8 and O5,27 were the dominant in the United States but since the mid 1980s serogroup O3 become predominant [8, 9]. In Hungary and in the neighbouring countries serogroup O3 is the most common [10 – 12].

Epidemological investigation of *Y. enterocolitica* O3 has been hampered by the limited availability of a suitable phage typing system whereas most of the strains belong to the same phage type. On the basis of a nationwide typing survey 93% of the strains, isolated in Hungary, belonged to phage type VIII [12].

Plasmid and protein profile analysis can be used for the differentiation of strains. In a study [13] epidemic *Y. enterocolitica* O3 strains harboured only a virulence plasmid of 40 – 50 MDa. These results suggest the necessity of further analysis by restriction enzymes. Molecular subtyping (ribotyping) was successfully used for the separation of *Y. enterocolitica* in a recent study [14].

Production of bacteriocins as a specific marker has been widely used in the characterization of bacteria [15]. Bacteriocins have been reported for *Y. pestis* [16], *Y. pseudotuberculosis* [9], *Y. intermedia* [17], and *Y. enterocolitica* [18].

The aim of this study was to screen the bacteriocin producing ability of strains representing all the available serogroups of *Y. enterocolitica* and to find potential indicators for developing a typing scheme. Furthermore, we wanted to evaluate its importance in differentiation of strains belonging to serogroup O3 and determine the plasmid profile and the whole bacterial cell protein pattern of selected strains.

## Materials and methods

*Bacterial strains.* Fifty strains from Nógrád County and further 71 strains of *Y. enterocolitica* O3 from different parts of Hungary were isolated from stool samples of patients with gastroenteritis during the period of 1987 – 1990. The used *Yersinia* reference strains were provided by the Department of Bacteriology, and by the Hungarian Collection of Medical Bacteria, "B. Johan" National Institute of Hygiene, Budapest (Table I)

*Media.* Tryptone soy broth (Oxoid) and tryptone soy agar (Oxoid) supplemented with 5% (v/v) defibrinated blood.

*Screening of bacteriocin-like antagonism.* The cross-streaking method was performed as described by Gillies and Govan [19], Govan and Gillies [20], Brandis and Smarda [21] and modified by Cafferkey et al. [18].

*Plasmid DNA analysis* was performed with ten bacteriocin-like material producer strains by the method of Birnboim and Doly [22] and analyzed by electrophoresis through 0.8% (wt/v) agarose gel.

*Whole bacterial cell protein analysis* was performed with the above-mentioned strains by the method of Laemmli [23]. Proteins were separated by electrophoresis through an SDS – 12.5% (wt/v) polyacrylamide gel.

**Table I**

*List of Yersinia reference strains*

| HNCMB* | Dep. Bacteriology** | Serogroups |
|---|---|---|
| 98003, 98010, 98014, 98015, 98021 | | O1 |
| 98022 | | O1,2a,3 |
| 98008 | | O2 |
| 98023 | | O2a,2b,3 |
| 98001, 98006 | | O3 |
| 98016 | | O4 |
| | 96 | O4,32 |
| 98012 | | O5 |
| 98017 | | O6 |
| | 102 | O6,30 |
| 98018 | | O7,8 |
| 98024 | | O7,13 |
| 98007, 98019 | | O8 |
| 98002, 98025, 98026 | | O9 |
| 98028, 98029 | | O10 |
| 98027 | | O10K |
| 98030 | | O11 |
| | 105 *Y. kristensenii* | O11,23 |
| | 841 | O11,24 |
| 98031 | | O12,25 |
| | 103 | O12,26 |
| | 553 | O13,27 |
| 98032 | | O14 |
| 98033 | | O15 |
| 98034 | | O16 |
| | 867 *Y. frederiksenii* | O16,29 |
| 98035 *Y. intermedia* | | O17 |
| | 846 | O18 |
| | 842 | O19,8 |
| | 1110 | O21 |
| | 1647 | O25,35 |
| | 7100 | O27 |
| | 1501 | O34 |

*Hungarian National Collection of Medical Bacteria

**Department of Bacteriology, B. Johan National Institute of Hygiene

## Results

*Production of bacteriocin-like antagonism*. Altogether 121 *Y. enterocolitica* O3 wild type isolates were tested against each reference strain and none of them had any inhibitory effect, i.e. the strains did not produce any bacteriocin-like material. Reference strains of *Y. enterocolitica* serogroup O5; O7,8; O7,13; O11; O13,27: O16; O19,8 and O34 as well as *Y. kristenseni* O11,23 and *Y. intermedia* O17 strains exerted bacteriocin-like antagonism examined in the same system. None of the producer strains was sensitive against its own active substance.

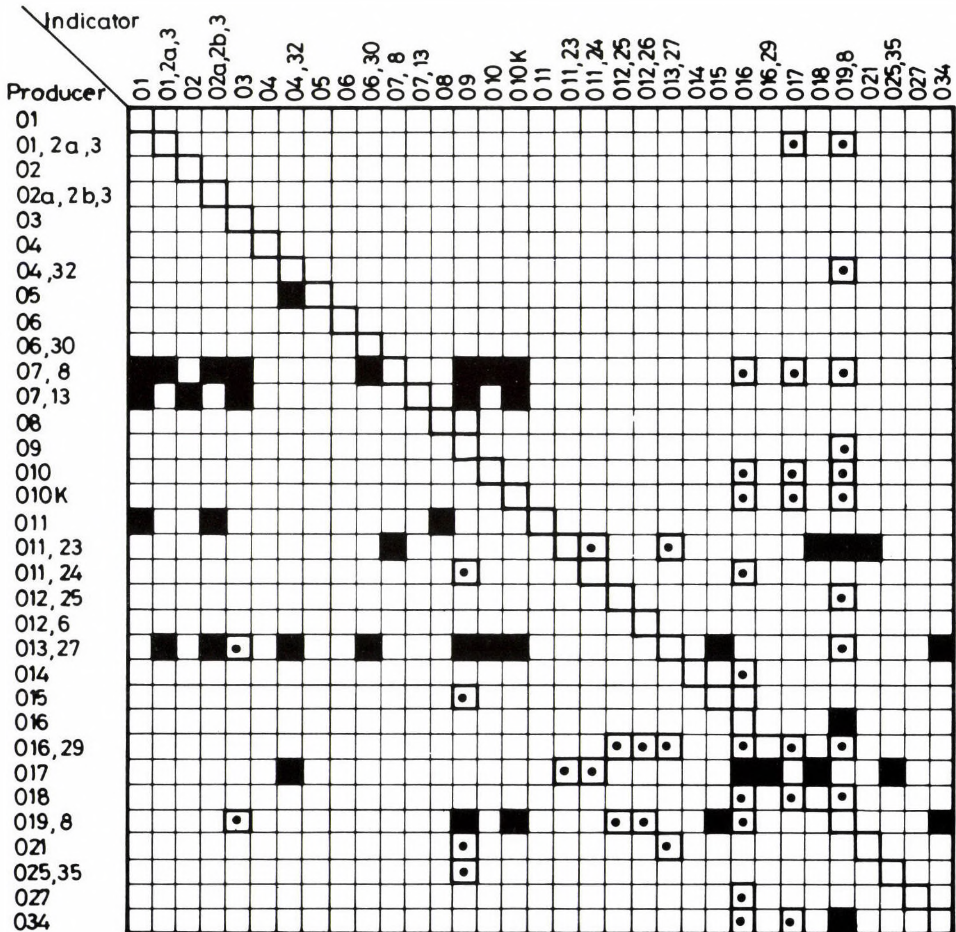


*Fig. 1.* Inhibition spectra of bacteriocin-like materials in *Y. enterocolitica* strains belonging to different serogroups. Solid squares, total inhibition; dotted squares, partial inhibition; open squares, negative

The results of the bacteriocin-like antagonism are summarized in Fig. 1. The inhibitory zones were usually clearly defined. Colonies which had grown within the inhibition zone were retested and did not show any further resistance.

When the bacteriocin-like producer strains were stabbed into solid media they gave very characteristic inhibition zones. The zones looked like the zones caused by colicin production. Typical inhibitory zones are demonstrated in Fig. 2.

In broth culture after UV irradiation or mitomycin C induction, a titre as high as 1:64 could be reached. A typical titration is shown in Fig. 3. We have never seen any phage plaque in the active substances.

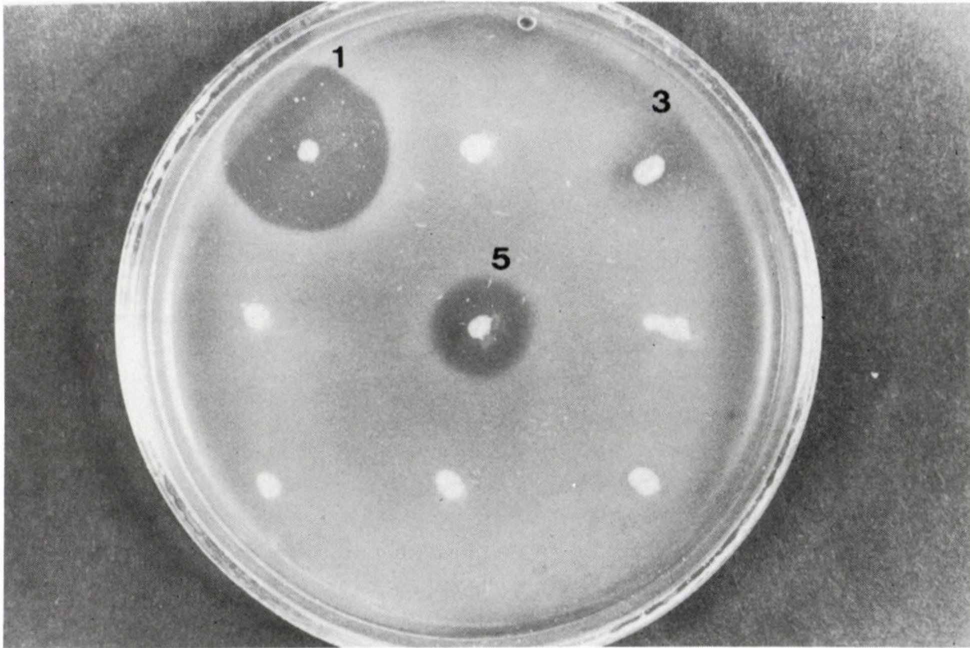


*Fig. 2.* Bacteriocin-like inhibition around the colony developing after stab-inoculation: left to right (1) O7,8; (3) O19,8; (5) O7,13. Indicator: *Y. enterocolitica* O3

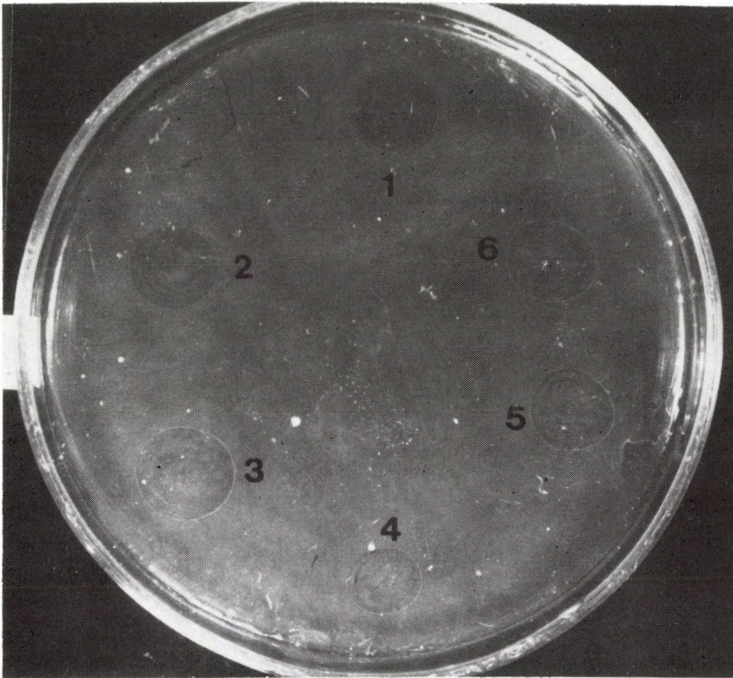


*Fig. 3.* Titration of filtrate lysate of *Y. enterocolitica* O7,8 and O3 indicator lawn. (1) concentrate; (2) 1:2; (3) 1:4; (4) 1:8; (5) 1:16; (6) 1:32 dilutions

All but five of the wild-type *Y. enterocolitica* strains were sensitive against the bacteriocin-like substance producing strains of serogroups O7, 8; O7,13; O13,27 and O19,8 (Fig. 4). Broth culture samples were treated with trypsin, but the trypsin treating did not reduce the activity of the selected samples.

Ten bacteriocin-like substance producer strains belonging serogroups were selected for further characterization.

*Plasmid profile analysis*. Out of the ten examined strains only strains 842 belonging to serogroup O19,8 harboured a plasmid of about 60 MDa.

*Whole bacterial protein analysis*. Comparing the total protein patterns of the selected reference strains significant differences could be seen in the range 33 – 47 kDa (Fig. 5).

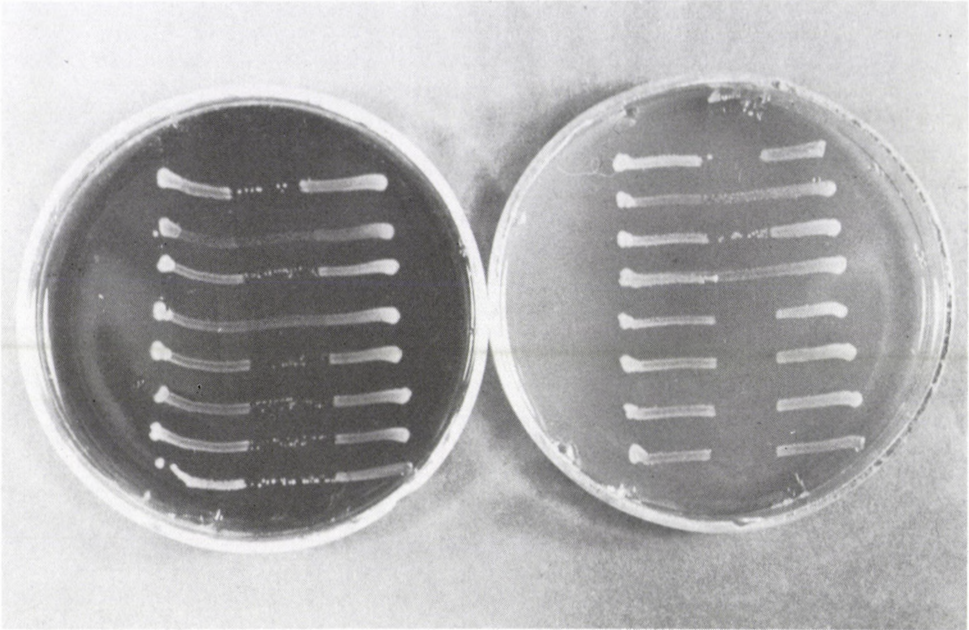

*Fig. 4.* Sensitivity of *Y. enterocolitica* O3 strains to the active substance of O7,8 serogroup. Left: killed by chloroform; right: killed by UV

## Discussion

In the 1960s Hamon et al. [24] studied the bacteriocin producing ability of *Y. enterocolitica* strains. According to their study only one out of 189 strains produced lethal material. Later Cafferkey et al. [18] demonstrated bacteriocin-like antagonism with a higher frequency among *Y. enterocolitica* wild-type strains several of which belonged to *Y. enterocolitica* O3. In contrast, in our study none of the Hungarian *Y. enterocolitica* O3 strains produced any inhibitory material. Furthermore, almost all (116/121) of the strains proved to be uniformly sensitive against the inhibitory materials produced by strains belonging to other serogroups.

Using the bacteriocin-like antagonism for typing strains of serogroup O3 we demonstrated that the method has little or limited value in differentiating between *Y. enterocolitica* O3 strains, because the examined strains were almost uniformly sensitive to the inhibitory material produced by strains belonging to other serogroups.

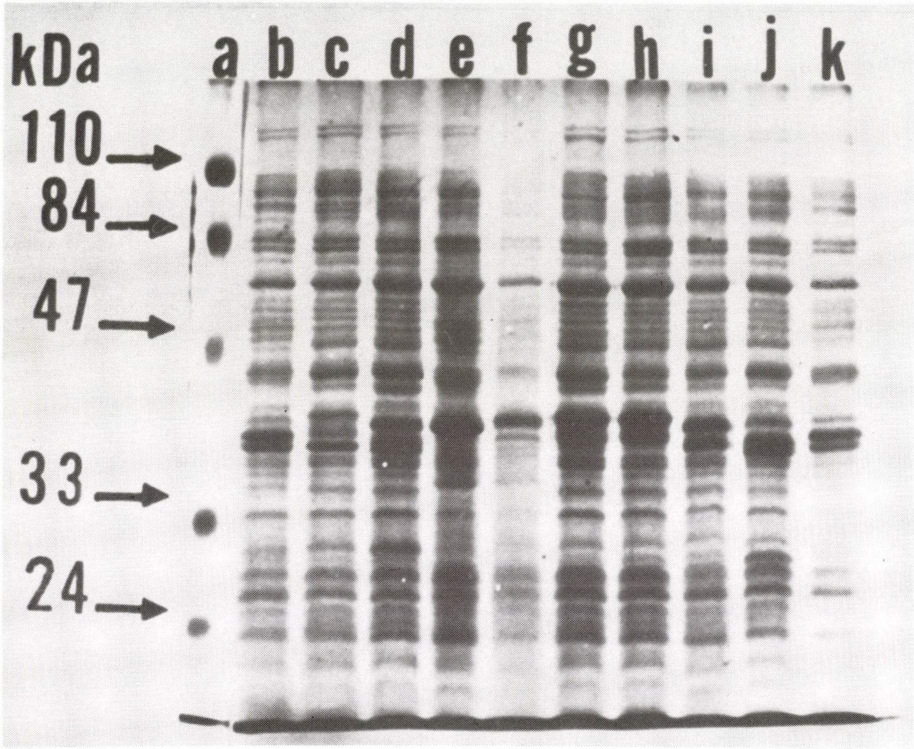


*Fig. 5.* SDS-PAGE of whole cell proteins of *Y. enterocolitica* strains. Lane a: Prestained SDS-PAGE standard (Bio-Rad Laboratories); b: O11 (98030); c: O7,13 (98024); d: O10 (98028); e: O17 (3953); f: O17 (955); g: O19,8 (842); h: O13,27 (533); i: O5 (124); j: O7,8 (106); k: O11,23 (105). The gel was stained with Coomassie-blue

All of the bacteriocin-like materials exhibited a narrow spectrum and did not have any effect against the other members of the family *Enterobacteriaceae* even not against *Y. pseudotuberculosis* serogroups I – V.

Examining the nature of the active bacteriocin-like material earlier in an electron microscopy study, Hamon et al. [24] detected a hardly visible structure that looked like a phage tail. In our lysates after a period of 24 h ultracentrifugation at 160 000 *g*, electron microscopically neither incomplete nor defective phage could be detected. Analysing these samples by SDS-PAGE no prominent protein could be demonstrated. The trypsin digestion did not reduce the bacteriocin-like activity of selected samples. In the future some bacteriocin-like substance will be characterized by SDS-PAGE after ammonium-sulphate precipitation. Additionally, we are plaining to compare the protein patterns of *Y. enterocolitica* O3 isolates.



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# A 4-KILOBASE CONGO RED BINDING PLASMID DNA FRAGMENT OF *SHIGELLA DYSENTERIAE* 1 SUPPRESSES THE GROWTH AND CELL DIFFERENTIATION IN *ESCHERICHIA COLI*

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(Received January 28, 1992)

A 4-kilobase congo red binding plasmid DNA fragment of pCAT 120 of *Shigella dysenteriae* 1 was transferred to an *Escherichia coli* K12 strain by transformation. Transformants were unable to grow in any liquid broth medium. Electron microscopic studies revealed that the transformants grown on tryptic soy agar were associated in clusters after cell division. Normal cell separation among the transformants in comparison with recipient *E. coli* K12 was only observed when the growth medium was supplemented with sterile culture filtrate of the recipient strain. An unknown factor(s) required for cell separation located on the chromosome was suppressed by a 4-kb congo red binding plasmid DNA (pCAT 120) fragment of *S. dysenteriae* 1.

*Shigella dysenteriae* 1 isolated from an epidemic [1, 2] in West Bengal (1984) contained six plasmids ranging in size from 120 to 2.5 kb [2]. An ampicillin (Amp) resistant determinant was located in the 120-kb plasmid (pCAT 120 [3]) and the smallest 2.5-kb plasmid encoded for streptomycin resistant character of our *S. dysenteriae* 1 isolates [4].

The essential virulence property of enteropathogenic bacteria depends on the ability to penetrate and multiply within the colon [5, 6]. The invasiveness of *Shigella* species has been associated with the presence of a large 120–140 MDa plasmid [7–10]. Maurelli et al. [11] and Sasakawa et al. [10] demonstrated that the loss of

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congo red binding ability ($Crb^+$) of *Shigella flexneri* 2a consistently accompanied loss of virulence, and the majority of them had some deletions in the plasmid. Maurelli et al. [11] also reported that a few new deletion derivative plasmids originated from the 140-MDa plasmid of *S. flexneri* 2a.

A 4-kb $Crb^+$ plasmid DNA fragment of pCAT 120 of *S. dysenteriae* 1 was transferred to an *E. coli* K12 strain by transformation [3]. The transformants produced a 130-kDa outer membrane protein (OMP) and the synthesis of four OMP (20 to 16 kDa) was totally inhibited [3]. In this study, we like to examine the growth pattern and cell structure of the transformants under electron microscope.

## Material and methods

*Organisms. S. dysenteriae* 1 strains were isolated from epidemic cases admitted to the Infectious Diseases Hospital, Calcutta. Recipient strain of *E. coli* K12 KL318 (*pro*-48, *trp* A9605, *his*-85, *met* E70, *trp* R55, *lac*I22, *azi*-9, *nal*A19, *rps*L171, λ-) was obtained through the courtesy of Dr. S. Palchaudhuri of Wayne State University. Transformant pET4 ($Crb^+$) was constructed in our laboratory [3].

*Growth media. S. dysenteriae* 1, *E. coli* K12 KL318 and transformant were cultured on bactotryptic soya agar (TSA) or in broth (TSB) (Difco). Transformants were also grown in TSB or on TSA supplemented with culture filtrate (1:1, v/v) of the recipient (*E. coli* K12 KL318) grown at 37 °C for 18 h with constant shaking. Several media including nutrient broth, brain-heart-infusion, minimal M9 [12] medium, etc. were examined for the growth of the transformant (pET4).

*Electron microscopy.* All the bacteria grown on TSA or in TSB medium supplemented with culture filtrate of the recipient strain were fixed with 3% glutaraldehyde and post-fixed with 1% buffered $OsO_4$. Another set of bacteria were also grown on only TSA and fixed in the similar way. The fixed bacteria were dehydrated with ascending concentration of ethanol and embedded in Araldite MY753. Ultrathin sections were cut in a LKB ultramicrotome and examined under a Philips Electron Microscope (Model 420 T).

## Results

Recipient *E. coli* K12 KL318 was a plasmidless strain whereas the transformant (pET4 [3]) contained only a 4-kb plasmid DNA (pCAT 120 [3]) fragment of *S. dysenteriae* 1. The growth pattern of *S. dysenteriae* 1 and recipient *E. coli* K12 KL318 were similar in TSB and on TSA media. The transformant failed to grow in any liquid media including TSB. However, poor growth rate was obtained on TSA. On a medium supplemented with sterile culture filtrate (1:1) of the recipient *E. coli* K12 KL318 there was a normal growth rate. The transformant exhibited a reduced rate of growth when bacto-agar was added to the liquid medium.

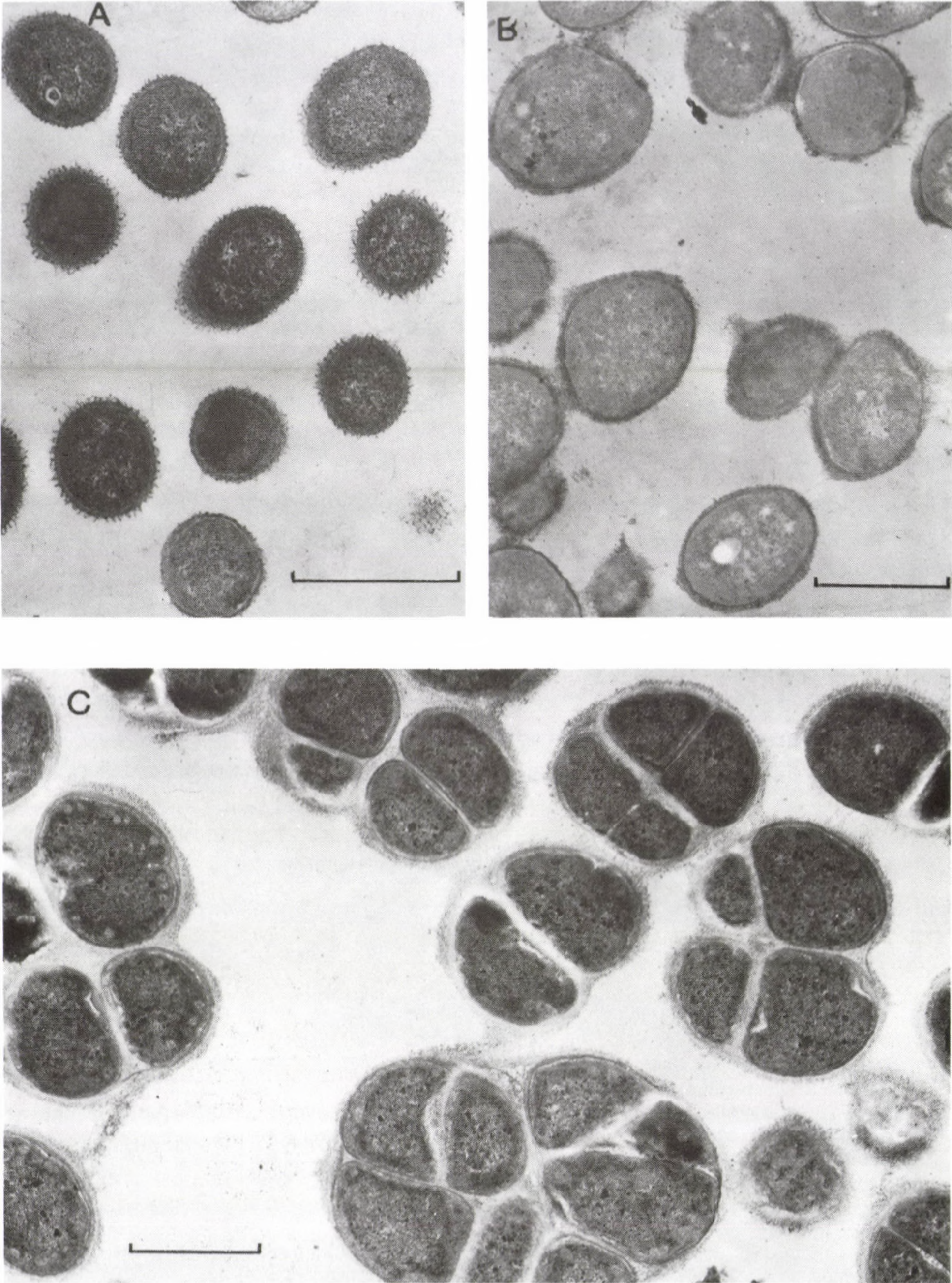


*Plate 1.A*: ultrathin section of the recipient strain *E. coli* K12 KL318 grown on TSA medium. *B*: ultrathin section of the *E. coli* K12 KL318 carrying a 4-kb $Crb^+$ plasmid DNA (pCAT 120) fragment of *S. dysenteriae* 1 (transformant) grown on TSA medium supplemented with sterile culture filtrate of the recipient strain. *C*: ultrathin section of the transformant showing the cells in cluster form grown only on TSA medium. Bar represents 100 nm

Normal cell division was observed for the transformant, however, 4 to 8 cells always remained in a cluster (Plate 1, C). They failed to separate from each other after cell division and remained within a common sheath. The wild type strain showed normal separation (Plate 1, A). The transformant also showed proper cell separation when they were grown in TSB or on TSA medium supplemented with culture filtrate of the recipient *E. coli* K12 KL318 (Plate 1, B).

## Discussion

The transformant showed poor growth rate on TSA medium but it failed to grow in TSB or any other liquid medium used in this study. At least a solid support was required for their growth (poor). Culture filtrate isolated from the recipient strain was used as unknown growth promoting factor(s) which was synthesized by *E. coli* K12.

A 4-kb $Crb^+$ plasmid DNA (pCAT 120) fragment suppresses the growth promoting factor(s) of the recipient *E. coli* K12 KL318. Normal growth rate was observed among transformant after addition of culture filtrate of the recipient strain. However, culture filtrate of *S. dysenteriae* 1 did not support the growth of the transformant.

Cell separation after cell division was inhibited among transformant cells by a 4-kb $Crb^+$ plasmid DNA (pCAT 120) fragment of *S. dysenteriae* 1. We had already reported that the transformant could not produce four low mol wt. OMP (20 to 16 kDa) and also synthesized a 130 kDa OMP [3]. The role of these above-mentioned outer membrane proteins in relation to cell suppration is not yet clear to us. A 4-kb $Crb^+$ plasmid DNA fragment of *S. dysenteriae* 1 acts as suppressor genes for growth and normal cell separation (Plate 1, C) of the recipient *E. coli* K12.

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# THE INFLUENCE OF CELL CULTURE AND STORAGE CONDITIONS ON HIV-1 INFECTIVITY AND FUSOGENIC ACTIVITY

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We have previously demonstrated that acidic medium inhibits the replication of HIV-1. The present study was designed to examine the effects of other growth conditions and infection of fibroblasts by coculture with HIV infected lymphoid cells. Several lymphoblastoid cell lines normally grown in RPMI-1640 were grown in Eagle's MEM. These cells supported virus replication to higher titres than did RPMI-1640. Peak viral titres were achieved within 24–48 h after newly infected or chronically infected cells were placed in fresh medium. When virus was stored in liquid medium either frozen or at higher temperatures, virus titres were retained for several months while frozen but decreased upon storage at 4 °C or higher. If cells were passaged after trypsinization in $Ca^{++}$-depleted medium, then a decreased susceptibility of cells for HIV-1 by 2 $\log_{10}$ at 24 h post infection was observed. Infectivity of cell-free and cell-associated HIV-1 was measured using syncytium formation, reverse transcriptase activity and p24 antigen. No fusion between HIV-1 infected $CD4^+$ lymphoblasts and $CD4^-$ fibroblasts was observed but

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HIV-1 infected lymphoid cells, even in the absence of syncytium formation, exerted a strong toxic effect on fibroblasts. This study extends previous findings that medium acidity was inhibitory to virus replication and survival. Thus, conditions for study of HIV must be well controlled in buffered medium so that misleading results are not obtained regarding virus multiplication and possibly regarding transmission to and pathogenesis in $CD4^-$ cells.

Environmental conditions influencing the integrity of human immunodeficiency virus (HIV-1) are important factors in the spread of this virus and the acquired immunodeficiency syndrome (AIDS), [1–5]. Two essential steps are required for the entry of HIV-1 into susceptible cells: first, binding of virions to the CD4 receptor on cell membranes, and next, fusion of viral and cell membranes. Both processes are mediated by viral envelope glycoproteins: transmembrane gp41 is thought to mediate fusion. Glycoprotein gp120, attached non-covalently to gp41, is responsible for binding to the host cell receptor, CD4. High affinity binding of gp120 to CD4 is postulated to trigger a conformational change in gp120 that reveals a fusogenic domain of gp41 [1].

As is common for all enveloped viruses, the presence of higher protein concentration in solutions contributes to survival of infective HIV-1 particles [6], but deglycosylating factors present in both healthy human and fetal bovine sera can interfere with gp120–CD4 binding in vivo and in vitro [1]. The interacting viral and cellular molecules may be removed or modified by enzymatic processes [7] or altered temperatures, as has been observed using several temperature sensitive mutants of other enveloped viruses [8]. Cations and other substances may alter the membrane charge [5, 7]. During cultivation of cells, acidification of the medium could protonate one or more of the strategic groups in the fusion protein, which is followed by a less stable folding of hydrophobic moieties [4, 9–11] or alteration of glycoprotein contacts [11] and decreased infectivity of the virus.

Following infection of cells by HIV-1, cell-cell fusion, as syncytium formation, is observed in vivo among peripheral blood mononuclear cells (PBMC) and in vitro among established cell lines. This phenomenon has been reproduced by mixing infected cells with non-infected ones expressing CD4 antigens [7]. In general, HIV-1 is not able to induce syncytia and subsequent cell death in the same cells of conventional cultures, contrary to results seen for many enveloped viruses. However, syncytium formation in lymphoid cells in the presence of non-lymphoid cells might occur in humans infected by HIV-1, causing syncytia formation in the $CD4^+$ population and cytotoxicity to the $CD4^-$ cells. Formation of hybridomas between retrovirus carrying lymphocytes and other cell types is reported [12]. Both cell-free HIV-1 and leukocytes carrying and/or producing virus particles can be transmitted into several parts of the host's body [13], and differences in pH and other conditions might determine the outcome of infection [9]. A model has been established, in order to examine relevant changes during in vitro cultivation and storage of extracellular

particles and virus producing cells, that may provide information relevant to in vivo susceptibility to HIV-1 infection.

## Materials and methods

*Cells and viruses.* Suspension cultures of H9 cells (obtained from Dr. R. C. Gallo, NIH, Bethesda, MD, USA, and from Dr. R. A. Weiss, Institute for Cancer Research, London, UK) were maintained by regular passages twice weekly into fresh RPMI-1640 (Bio-Whittaker, Walkersville, MD, USA, of Gibco, Paisley, UK) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS, Gibco), 2 mM L-glutamine (Gibco), 20 mM HEPES buffer (Sigma, St. Louis, MO, USA) and 40 mg/l gentamicin (Gibco). For infection, $1 \times 10^6$ cells in 0.5 ml medium and 0.5 ml of a highly lymphotropic HIV-1 stock, multiplicity of infection (m.o.i.) approximately 1, of either the IIIB or RF strain ([14], obtained from Dr. R. C. Gallo and Dr. D. Aw, Department of Bacteriology, University of Edinburgh, Edinburgh, UK, respectively) were mixed at 37 °C for 2 h, then their volume was adjusted to 20 ml. Infected cultures producing HIV-1 continuously were designated H9/IIIB or H9/RF, and they were maintained by twice weekly passage. Supernatant fluids from 24 h old cultures obtained by low speed centrifugation served as cell-free virus stocks, and aliquots were frozen at −20 °C for 4 h, then transferred to −70 °C. The pelleted H9/IIIB cells served as cell-associated HIV-1. Due to a lack of cytopathogenicity, the proportion of virus producing cells was determined by direct immunofluorescence with anti-p24 monoclonal antibodies (Sera-lab, Sussex, UK). Thirty to forty percent of the cells were positive, which varied slightly from one experiment to the next [9].

Suspension cultures of other leukemic cell lines, CEM (from Dr. D. Aw), and CEM-ss ("supersensitive", from Dr. R. C. Gallo) and two HTLV-I transformed lines, C8166 and MT-4 (both from Dr. D. Aw) were maintained in the same manner as H9 cells. Several attempts were made to utilize commonly used products to optimize conditions. For C8166 cells, FBS was replaced by newborn calf serum (NBCS, Gibco) in some experiments. To facilitate forming cell aggregates and possible adherence to the plastic flasks (Costar, Cambridge, MA, USA), $Ca^{++}$ content of RPMI-1640 medium was doubled to 0.2 g/l using $CaCl_2 \cdot 2H_2O$. The cell lines also were maintained in either Eagle's minimal essential medium (MEM) (Media Department, Institute of Virology, Glasgow) complemented with 10% FBS and antibiotics, with or without tryptose phosphate broth, or in a 1:1 mixture of Eagle's MEM and RPMI-1640 without HEPES buffer. Cultures were infected in the same way and virus production in the CEM/IIIB, CEM/RF, CEM-ss/IIIB, C8166/RF and MT-4/RF cultures was monitored through a single replication cycle of lytic infection. Viability of cells was determined using trypan blue dye (Gibco) exclusion [15]. Dead cells were pelleted through Lymphoprep (Nycomed, Oslo, Norway) by centrifugation at 2000 rpm at 4 °C for 25 min, and subsequently washed and resuspended in fresh medium.

*Trypsinization of virus producing cells.* Instead of dispersing cell clusters by pipetting, H9, H9/RF, CEM, C8166 cultures were split by trypsinization in $Ca^{++}$ depleted medium (0.05% trypsin and 0.02% Versene, Flow Laboratories, Irvine, UK) at 37 °C for 5 or 10 min, then the cells were centrifuged in 100% FBS, and finally resuspended in fresh medium [7]. At 24 h after trypsinization, noninfected C8166 and CEM cells were exposed to HIV-1/RF at an m.o.i. = 1. An adherent subline of CEM-cells, AdCEM ([16], obtained from Dr. D. Aw) was prepared by similar trypsinization but in $Ca^{++}$-enriched RPMI-1640 or Eagle's MEM. Production of floating cell aggregates was prevented by lowering FBS content to 1 or 2%. Non-heated and heated sera (56 °C, 30 min) were used equally. BHK-21, C-13 cells were maintained by conventional trypsinization [17]. AdCEM ($2 \times 10^4$) or BHK-21 ($4 \times 10^4$) cells in 150 μl complete Eagle's MEM or RPMI-1640 were seeded into all wells of 96 well tissue culture plates

(Nunclon, Roskilde, Denmark). The next day upon reaching confluency, the cultures received 50 μl medium containing $2 \times 10^4$ C8166 or CEM cells infected 2 h before with HIV-1/RF at different multiplicities. Other wells of BHK-21 cells received 50 μl cell-free virus at different m.o.i. Cytopathic effect was scored using a light microscope.

*Quantitation of HIV-1 infectivity*

(a) *Syncytium induction.* Residual infectivity of HIV-1 in H9/IIIB and CEM-ss/IIIB cultures, after storage or acid treatment was titrated by transferring 50 μl supernatant fluid or acidified H9/IIIB cells resuspended in 50 μl fresh RPMI-1640 medium onto monolayers of CEM-ss cells ($5 \times 10^4$/well) in 96 well flat bottomed microtitre plates previously coated with 100 μg poly-L-lysine (PLL, 70 000 – 150 000 mol wt, Sigma) per ml [18]. On subsequent days of incubation at 37 °C the number and size of developed syncytia (as infectious units) were monitored using a light microscope. After the formation of the initial group of syncytia, secondary syncytia were sometimes observed beginning 3 – 4 days after the first syncytia.

Yield of H9/RF, CEM/RF, C8166/RF, MT-4/RF, AdCEM/RF and HIV-1/RF infected BHK-21 cells as well as some H9/IIIB and CEM/IIIB cultures was quantitated on 24 h old C8166 cell suspensions in microwells (Nunclon) using $2 \times 10^4$ cells/well in 150 μl Eagle's MEM by adding 50 μl virus suspensions [15]. Cells were monitored twice daily for the development of syncytia.

(b) *Reverse transcriptase assay.* Concurrent with syncytium induction, RT activity was measured [19]. Six μl samples of CEM-ss supernatant fluids described in section (a) were added to 20 μl reaction mixtures containing 0.0625 *M* Tris-HCl, pH 8.3; 0.125 *M* NaCl; 0.0075 *M* $MgCl_2$; 0.25% NP-40; 0.0125 *M*-dithiothreitol; 0.005 mg/ml oligo-$dT_{12-18}$; 0.05 mg/ml poly-rA (Pharmacia, Uppsala, Sweden); 0.63 μCi of $^3$TTP (96 Ci/mmol) and incubated at 37 °C for 105 min in Greiner (Frickenhausen, Germany) U-bottom microtitre plates. The reaction was stopped by addition of 150 μl 0.1% Na-pyrophosphate, 15 m*M* NaCl and 0.1 mg/ml bovine serum albumin, and after cooling to 4 °C 20 μl 60% cold trichloroacetic acid was added for 30 min. The precipitated nucleotides were transferred to LKB DEAE filtermat paper with an LKB cell harvester and radioactivity was counted in an LKB (Wallac) 1205 Betaplate scintillation counter.

(c) *p24 antigen determination.* Thirty μl samples were removed from the supernatants of PLL-bound CEM-ss cultures and subsequently diluted to 1:20 – 1:1000. These dilutions were assayed using the "HTLV-III Antigen EIA Kit" for sandwich solid phase enzyme immunoassay (Abbott, Abbott Park, IL, USA [20]). Quantitation was based upon standards supplied with the kit.

*Electron microscopy studies.* C8166 cell suspensions ($1 \times 10^7$) in Eagle's MEM infected with HIV-1/RF in a 1:1 ratio were mixed with 8% glutaraldehyde and placed into a 15 ml conical centrifuge tube, spun at 2000 rpm for 5 min and left at 4 °C overnight. Supernatant fluids were replaced by paraglutaraldehyde, and after 120 min at 4 °C fluid was replaced by 0.1 *M* cacodylate rinse, pH 7.4 and the pellets were gently loosened from the tube bottom. Samples were kept at 22 °C for 15 min followed by overnight treatment at 4 °C. Pellets were then fixed with osmium tetroxide, dehydrated and embedded in Araldite. Thin sections were stained with uranyl acetate and lead citrate.

## Results

*The effect of medium composition on cell cultures and HIV-1 infectivity.* C8166 and MT-4 cells were successfully cultivated in Eagle's MEM (data not shown). Cell aggregates became more dense upon continued culture. $Ca^{++}$-enrichment of RPMI-1640, as well as full Eagle's MEM or mixed RPMI-1640/Eagle's MEM facilitated

adherence of individual C8166 and MT-4 cells (which normally grow suspended in the medium) to the bottom of the plastic wells. Attempts to passage such adherent cells by removing them using a rubber policeman or trypsinization did not result in a stable cell line capable of forming a cell monolayer. No attachment of cells to plastic surfaces in any other cultures infected by HIV-1 was observed.

The cell division rate of CEM/RF cells exceeded that of noninfected CEM by 20–25%, although total cell viability did not show any difference in Eagle's MEM. Addition of tryptose phosphate broth, as a protein source, to RPMI-1640 did not facilitate cell growth. Cultivation of the CEM line was found to be best in RPMI-1640, but AdCEM cells were grown equally well in $Ca^{++}$-enriched RPMI-1640, or in a 2:1 mixture of RPMI-1640 and Eagle's MEM. A lower FBS content in different media did not prevent formation of floating cell aggregates. No difference in cell growth and virus production was observed using NBCS or heated or non-heated FBS (data not shown).

*The effect of time in culture on HIV-1 production.* H9 cells chronically infected by either the IIIB or RF strain of HIV-1 did not show any cytopathic effects. However, titration of supernatant fluids for infectious virus and confirming quantitation by RT activity from PLL-bound indicator CEM-ss cultures showed the highest titre of cell-free HIV-1 at 24 h after chronically infected cells were diluted into fresh medium (Table I). It then dropped gradually on subsequent days. Direct measurement of RT activity from culture supernatant fluids showed the opposite trend, which reflects the steady release of viruses from increasing numbers of cells in a culture, although there was a loss of infectivity. Virus of the RF strain reached the highest titre in H9 cells grown in Eagle's MEM (Table II), while IIIB virions lost their infectivity more rapidly in aging cultures (Table III).

Using the same HIV-1/RF stock for titration, the virus did not grow in AdCEM or BHK-21 cells. MT-4 cells exhibited slightly less sensitivity (smaller syncytia formed) than did C8166 or CEM cells. HIV-1/IIIB was produced in the same magnitude in CEM ($6.5 \times 10^6$ IU/ml) or CEM-ss cultures ($2.5 \times 10^6$ IU/ml). Particles from the RF strain were more resistant than IIIB to culture ageing, based upon morphologic examination using electron microscopy (Figs 1 and 2). CEM-ss/IIIB suspension cultures exhibited gradual development of syncytia until day 3, these subsequently collapsed as the cultures aged (Table III). The latest time at which secondary syncytia appeared due to additional cells becoming infected by HIV produced in the cultures, was at 5 days. Inoculation of C8166 or MT-4 cells resuspended in Eagle's MEM at high m.o.i. resulted in the fastest development of cytopathic effects. Secondary syncytia occurred occasionally until day 7. Small syncytia in the cell aggregates may have been missed since they were overgrown by dividing cells. When these cell aggregates were resuspended in fresh medium, the syncytia re-developed.

**Table I**

*The effect of culture aging on HIV-1 production by H9/IIIB cells*

| Days after passage[a] | Direct RT activity[b] (cpm × $10^3$/ml) | Assays on PLL-bound CEM-ss cells (96 h) Titration (IU × $10^3$/ml)[c] | RT activity (cpm × $10^3$/ml) |
|---|---|---|---|
| 1 | 1,731 ± 224 | 800.0 ± 70 | 4,592 ± 773 |
| 2 | NT[d] | 20.0 ± 5 | NT |
| 3 | 2,628 ± 385 | 4.7 ± 0.9 | 804 ± 15 |
| 4 | 3,438 ± 185 | 0.7 ± 0.1 | 519 ± 41 |
| 5 | NT | 0.6 ± 0.04 | NT |
| 6 | NT | 0.2 ± 0.04 | NT |

[a] Cells chronically infected with HIV-1/IIIB were cultured for the number of days indicated after being placed in fresh RPMI-1640 medium. At the indicated day an aliquot of cell-free supernatant was removed and either tested directly for RT activity or placed on CEM-ss cells

[b] RT was assayed for 105 min

[c] IU – 1 infectious unit represents one syncytium of 7 or more cells

[d] NT = not tested

**Table II**

*Maximal production of HIV-1/RF in different cell cultures*

| Days post infection | H9[a] | C8166 | Cell lines BHK-21 (IU × $10^4$/ml) | CEM | AdCEM | MT-4 |
|---|---|---|---|---|---|---|
| 1 | 80.0[b] | 2.0 | 0 | 0.03 | 0 | 0.45 |
| 2 | 150 000.0 | 0.85 | 0 | 300.0 | 0 | 156.0 |
| 3 | 25 000.0 | 0.25 | 0 | 120.0 | 0 | 2.0 |
| 4 | 60 000.0 | 0.40 | 0 | 1.0 | 0 | 0.7 |
| 5 | NT | 0.02 | 0 | NT | 0 | 0.03 |
| 6 | 330.0 | 0.025 | 0 | 50.0 | 0 | 0.04 |

[a] H9, C8166, and BHK-21 cells were grown in Eagle's MEM; CEM and AdCEM cells were grown in RPMI-1640, and MT-4 cells were grown in a mixture of Eagle's MEM and RPMI-1640. Cultures were infected (m.o.i. = 1) 24 h after passage in fresh medium

[b] Virus titre is given in infectious units/ml, as measured by an assay on C8166 cells

NT = not tested

**Table III**

*The effect of time in culture post infection on HIV-1 produced by CEM-ss/IIIB cells*

| Days after passage | Microscopic observations | Culture medium | | Assays on PLL-bound CEM-ss cells (72 h) | |
|---|---|---|---|---|---|
| | | pH | Direct RT activity[a] (cpm × $10^3$/ml) | Titre (IU × $10^3$/ml) | RT activity (cpm × $10^3$/ml) |
| 1 | Small cell clumps, few syncytia | 7.4 | 216 ± 9 | 1.1 ± 0.1 | 529 ± 113 |
| 2 | Cell clumps, many small, medium and large syncytia | 7.4 | NT | NT | NT |
| 3 | Large cell clumps, very large syncytia | 7.4 | 684 ± 67 | 2600 ± 500 | 3837 ± 296 |
| 4 | Large cell clumps, large or collapsed syncytia | NT | NT | 200 ± 30 | NT |
| 5 | Dense cell clumps, old collapsed, few new syncytia | 7.1 | NT | 42 ± 5 | NT |
| 6 | Grainy cells and collapsed syncytia | 6.7 | NT | 1.3 ± 0.2 | NT |
| 7 | Grainy cells, medium yellow | 6.4 | NT | 0.6 ± 0.1 | NT |
| 8 | Very grainy cells, collapsed syncytia as dark bodies | 6.4 | NT | 0.4 ± 0.1 | NT |
| 10 | Cell clumps and syncytia lysed | 5.4 | NT | Not detected | NT |

[a] Cells were cultured in RPMI-1640 medium. RT was assayed after 105 min

NT = not tested

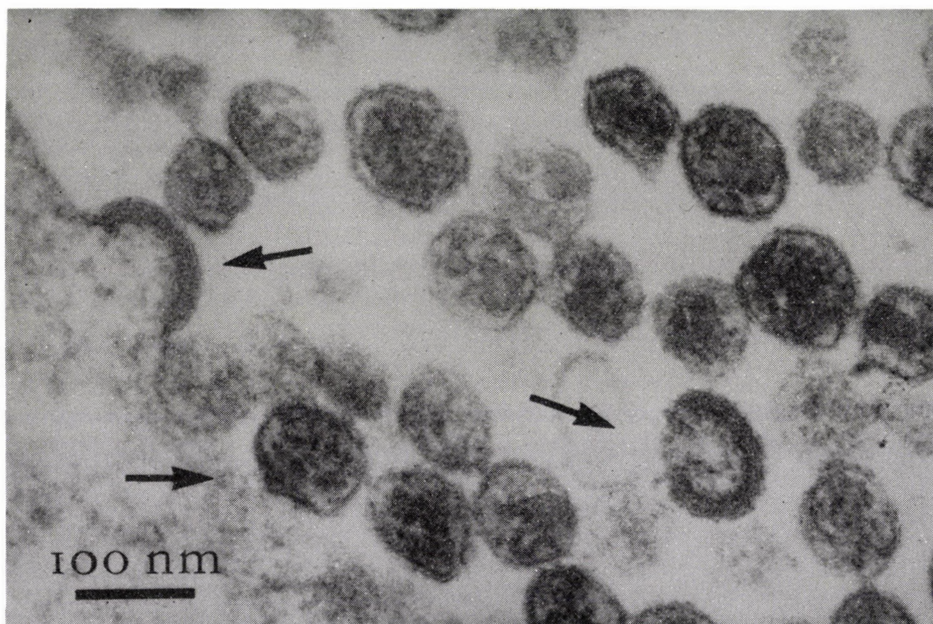


*Fig. 1.* Electron micrograph on the budding process (arrows) and virions with different maturity released from 4-day-old C8166/RF cultures

*Storage temperature affects virus infectivity.* Storing culture supernatant fluids obtained from HIV-1/RF infected CEM or H9 cells below −70 °C did not significantly affect the infectious titre over a period of 214 days (Table IV). Likewise, supernatant fluids from HIV-1/IIIB infected cultures of CEM-ss cells retained infectivity and RT activity (data not shown). This enzyme also appeared to be resistant to repeated freezing-thawing. Both relatively short incubation (15 days) in a closed vial left in an ordinary refrigerator at 4 °C and a shorter incubation (4 days) at a steady pH 7.4 at 37 °C resulted in losing infectivity without loss of RT activity (Table V). There was no difference in retaining infectivity of the same HIV-1 strain prepared and stored in different media.

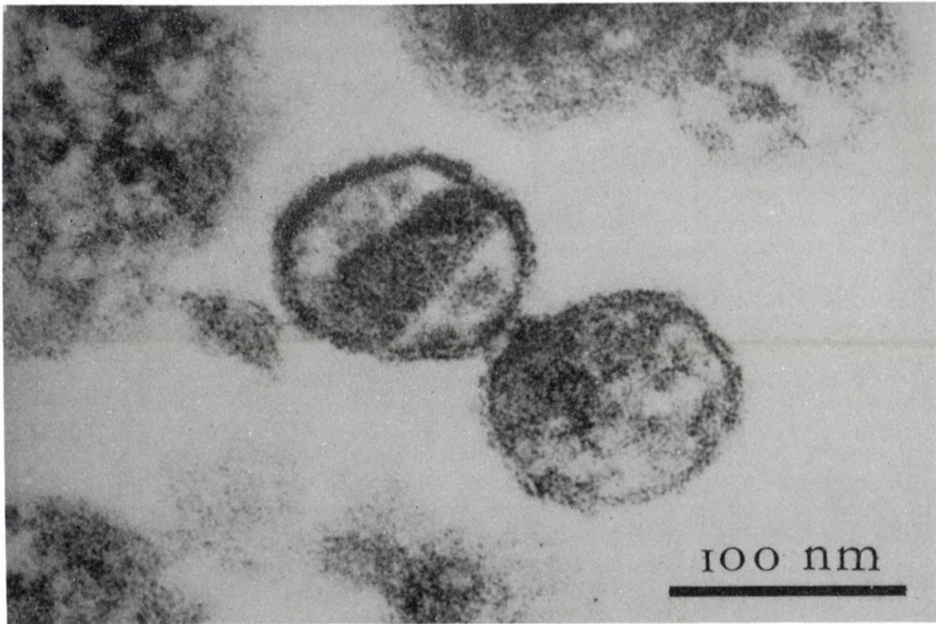


*Fig. 2.* High magnification of typical HIV-1 particles preserved well in the Eagle's MEM of 4-day-old C8166/RF cells

**Table IV**

*Stability of HIV-1/RF after storage for different amounts of time at −70 °C*

| Storage in days | Cell lines CEM Eagle's MEM | CEM RPMI-1640 | H9 Eagle's MEM | H9 RPMI-1640 |
|---|---|---|---|---|
| 0 | 0.4[a] | 200 | 25 000 | 400 |
| 96 | 0.3 | 250 | 10 000 | 250 |
| 175 | 0.7 | 100 | 15 000 | 55 |
| 214 | 0.3 | 40 | 15 000 | 150 |

[a] Virus titre is given in infectious units × $10^4$/ml; virus was collected 3 days post infection

**Table V**

*The effect of different storage temperatures on HIV-1/IIIB*

| Supernatant fluid source/ storage conditions[a] | Direct RT activity[b] (cpm × $10^3$/ml) | Assays on PLL-bound CEM-ss cells (72 h) Titre (IU × $10^3$/ml) | RT activity (cpm × $10^3$/ml) |
|---|---|---|---|
| H9/IIIB cells[c] | 1731 ± 224 | 800 ± 70[d] | 4592 ± 773 |
| −70 °C, 12 days | 1522 ± 59 | 1500 ± 200 | NT |
| CEM-ss/IIIB cells[c] | 216 ± 9 | 1.1 ± 0.1 | 529 ± 113 |
| 37 °C, 5% $CO_2$, 4 days | 212 ± 10 | None detected | NT |
| CEM-ss/IIIB cells | | | |
| 3 days post infection[d] | 684 ± 67 | 2600 ± 500[e] | 3837 ± 296 |
| 4 °C, 15 days | 766 ± 59 | 0.1 ± 0.01 | NT |
| −70 °C, 36 days | 696 ± 29 | 1600 ± 100 | 3447 ± 7 |
| −70 °C, 40 days | NT | 1600 ± 200 | 3606 ± 310 |
| −70 °C, 70 days | 680 ± 41 | 1000 ± 200 | 3185 ± 489 |

[a] Supernatant fluid was collected 24 h after cells were placed in fresh RPMI-1640 medium. Fluid was either tested immediately or stored as indicated

[b] RT was assayed for 105 min

[c] Tested without storage

[d] Indicator cells were infected for 96 h before collection of supernatant fluids to test for virus and RT activity

[e] Indicator cells were infected for 72 h before collection of supernatant fluids to test for virus and RT activity

*The effect of trypsinization on virus production.* After trypsinization in $Ca^{++}$-depleted medium for 5 or 10 min, H9, CEM and C8166 cells formed normal clusters by the next day, but they consisted of fewer cells than their non-trypsinized counterparts. As judged microscopically, effects of trypsinization disappeared by day 2 and cell viability was unaltered. Infecting H9, C8166 or CEM cultures 24 h after trypsinization in RPMI-1640 medium or titrating trypsinized H9/RF virus producer cultures resulted in a 1.5−2 $\log_{10}$ reduction in virus production for all cell types (Table VI).

*The effect of HIV-1 and HIV-1 infected cells on other cell types unable to support growth of this virus.* HIV-1 is unable to replicate in AdCEM cells or BHK-21 cultures (Table II). However, concentrated virus suspensions (m.o.i. ≥ 10) in either RPMI-1640 or Eagle's MEM (pH 7.1−7.4) exerted very strong toxic effects on these cells at 24 to 48 h p.i. Cell growth was retarded, cells became grainy, smaller, and rounded up, but they remained attached to the plastic. Co-cultivating freshly infected

CEM/RF or C8166/RF cells onto BHK-21 monolayers resulted in similar toxicity, but the effect was dose dependent and more evident, even in the case of low m.o.i. (Table VII). CEM/RF cells damaged BHK-21 cells at a lower m.o.i., than C8166/RF. No direct interaction was observed between the infected CD4 positive lymphoid cells and the smaller, dying BHK-21 cells. Interestingly, syncytium formation by the lymphoid cells did not occur in mixed cultures during the 11 days of observation.

**Table VI**

*The effect of trypsinization of cells 24 h prior to infection on HIV-1 production*[a]

| Titre[b] | H9 | Cell lines<br>CEM<br>(Infectious units × $10^3$/ml) | C8166 |
|---|---|---|---|
| Non-trypsinized | 150 | 100 | 450 |
| Trypsinized | 3.5 | 6 | 5 |

[a] Cells were treated with trypsin to disperse clusters, cultured for 24 h in fresh RPMI-1640, then infected at an m.o.i. = 1 HIV – 1/RF

[b] Supernatants collected 3 days post infection

**Table VII**

*Toxic effects of HIV/RF infected cells on BHK-21 cell cultures*

| M.O.I. | Percent[a] | CEM/RF Changes | Percent[a] | C8166/RF Changes |
|---|---|---|---|---|
| 10 | 100 | Complete cell death | 95 | Cells rounded up, |
| 5 | 95 | Cells rounded up, | 75 | normal cells |
| 1 | 50 | normal cells contain | 50 | contain cytoplas- |
| 0.1 | 25 | dark grains | 10 | mic inclusions |
| 0.01 | 10 | Some cells contain | 5 | Retarded growth |
| 0.001 | 5 | cytoplasmic grains | 5 | Subconfluent |
| 0.0001 | 5 | Retarded cell growth | 0 | monolayer |
| 0 | 0 | Very dense, heavy populated cultures of spindle cells | | |

[a] 2 × $10^4$ CEM/RF or C8166/RF cells were added to 4 × $10^4$ BHK-21 cells in Eagle's MEM

## Discussion

The current study has demonstrated that the trypsinization of HIV-1 infected cells, storage temperature, strain of cells infected, and length of time cells are cultured after infection, all contribute to the quantity of infectious virus produced. The type of medium (RPMI-1640 or Eagle's MEM) or the type of serum (FBS or newborn calf, heated or non-heated) used had little to no effect on cell growth but did affect virus yield.

It is likely that the peak titres of infectious virus seen at 1–2 days after addition of cell-free virus or passage of chronically infected cells into fresh medium were related to the pH of the culture medium. The better virus production in Eagle's MEM appears to be related to the slower decrease in pH in this medium. Previous results from these laboratories have indicated that virus production is diminished as the pH of the medium becomes acidic [9]. This is consistent with studies that indicate that HIV-1 does not survive well in an extremely acidic environment [21–23]. The effects of low pH have been related to the loss of virus integrity, which is vital to the ability of the virus to enter susceptible cells and spread disease [24–26]. In the case of HIV-1 the envelope gp120, which is required for binding to target cells, is fragile and frequently shed resulting in loss of infectivity [5, 10, 14, 27–29), while gp41 retains integrity after acetic acid treatment [30]. It is possible that the acidic pH (5.4–6.7) used in this study was responsible for increased shedding of gp120 and loss of ability to bind to susceptible cells [31, 32]. This is consistent with the loss of infectivity of cell-free but not cell-associated virus placed in medium at acidic pH and then restored to neutral pH, since the cells infected with virus could synthesize new gp120 once restored to neutral pH. Furthermore, this is supported by the observation that RT activity was unaltered by an acidic pH environment, since RT activity does not require an intact virus enveloped [4, 20, 33].

Another observation supporting the importance of medium pH was inhibition of virus accumulation in the presence of *Mycoplasma* in the cell cultures (data not shown). These bacteria were shown to inhibit virus multiplication, consistent with previous studies which indicated that *Mycoplasma* cause rapid acidification of medium and inhibit RT activity [34]. Since these organisms are noted to increase the acidity of medium through metabolism [34, 35] it is likely that they also inhibit replication via a pH effect. While this may seem to be in contrast to reports that *Mycoplasma* can increase cell death by HIV-1 [34], it is likely that the cofactor effect in vitro of the bacteria requires a buffer that does not allow significant acidification of the medium.

The effect of trypsinization on cell susceptibility to HIV-1 is likely also to be related to expression of either membrane receptors or viral glycoproteins on the cell surface. Trypsin is known to damage CD4 structure [7], and $Ca^{++}$-depletion inhibits

cell fusion [5]. Once surface molecules have had time to be resynthesized, the cells recover their susceptibility to infection. It is interesting to note that even 24 h after trypsinization the cells still did not replicate virus to the same extent as non-trypsinized cells. Since the effect is a quantitative one it is possible that fewer CD4 receptors were still expressed by the cells at this time, resulting in the observed decrease in virus replication.

Virus production in H9/RF cells cultured in Eagle's MEM was considerably greater than any other cell, medium and virus strain combination. It appears that the contribution of the medium in supporting greater viral replication was related to the nutrients and buffering of the medium [6, 33, 36]. Since metabolism resulted in more rapid acidification of cultures in RPMI-1640, the cells produced less virus. Whether the production of more virus in H9 cells as compared to CEM, CEM-ss, MT-4 or C8166 cells was also related to acidity of the medium used to grow the cells or related to other phenomena, such as the number of cellular receptors for HIV-1 per cell or rate of cell metabolism, is undetermined. Likewise, it is unclear why the RF strain of HIV-1 replicated to hither titre than IIIB under similar conditions.

The failure of HIV-1 to grow in fibroblasts co-cultured with lymphoid cells is not surprising, since the former lacks appropriate cell receptors. However, there are reports of lymphocyte-fibroblast fusion [37] in which transfer of virus from one cell type to the other is reported [12]. Such hybrids are reported to produce prostaglandins and cytokines not typical of normal lymphocytes [12]. Fusion did not appear to occur here and there was no evidence of HIV-1 infection of fibroblasts. Rather, the fibroblasts demonstrated signs of cytotoxicity which seemed to be mediated by soluble substance(s). It is possible that the HIV-1 infected lymphoid cells, which are known to produce tumour necrosis factor [38, 39], were responsible for the cytotoxicity via this mediator. Since HIV-1 infected cells do not fuse with cells of other tissues, virus spread cannot account for the destruction of these tissues in infected hosts. Wasting of body tissues does occur in AIDS patients and this has been attributed to increased levels of tumour necrosis factor/cachectin. Thus, the studies with HIV-1 infected lymphoid cells and BHK-21 or AdCEM cells may serve as a useful model for studying pathogenesis of non-infected tissues in the presence of this virus; especially when trying to evaluate microenvironmental effects on virus replication and spread.

These data make it clear that a well defined system is required in order to maximize virus titres when growing HIV-1. It appears that most culture related effects on virus replication are pH related, although some may be attributed to receptor numbers on particular cell lines. Furthermore, the pH effects noted, in this and a previous study [9], may explain why some investigators fail to get yields of infectious virus from specimens that are positive by RT or p24 assays.

Thus, these studies extend previous findings that implicate environmental conditions as highly significant for the propagation of HIV in vitro [9]. This supports the concept that microenvironmental conditions may influence sites and modes of HIV transmission in vivo. If these in vitro results are extrapolated to humans, the data may help explain the frequency of particular infectious routes for HIV-1.

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# THE MOUSE LIGATED INTESTINAL LOOP ASSAY FOR THE STUDIES ON ENTEROINVASIVE *ESCHERICHIA COLI*

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Enteroinvasive *Escherichia coli* exhibited a positive reaction in the mouse intestinal loop assay except for noninvasive mutant strains. These mean values of fluid weight per gut length of mouse loops inoculated with enteroinvasive *E. coli* were significantly higher than that given by brain heart infusion broth. Oedema and swelling in all positive loops, increased bacterial cell numbers within intestinal loops were observed.

In vitro and in vivo experimental models have been applied for the studies on various virulent bacterial strains. The ligated intestinal loop assay in rabbits was first reported by De and Chatterjee [1] as a new model for the investigation of infection. This method was performed in mice by Punyashthiti and Finkelstein [2]. Their method has been mainly used for the detection of enterotoxigenic strains but not invasive organisms. With a modification of their technique, we found this assay was available for the demonstration of the invasive ability of *Shigella flexneri* [3]. And we also reported positive reaction in this assay with nonenterotoxigenic and nonhaemolytic strains of *Staphylococcus aureus* [4]. This paper is concerned with results using enteroinvasive *Escherichia coli*, which are important cause of diarrhoeal disease and dysentery in infants in developing countries.

## Materials and methods

*Bacterial strains.* Enteroinvasive strains of *E. coli* O124 S, O136, O112, O28 A and O29 were used in this study. A noninvasive rough mutant strain of *E. coli* O124 R and a noninvasive smooth mutant strain of *E. coli* O28 N were used for negative controls of the experiments. Also, an enteroinvasive strain of *S. flexneri* 2b 17-A and a noninvasive smooth mutant strain of *S. flexneri* 17-N were used for positive and negative controls, respectively. Table I shows the characteristics and virulence of these strains.

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*Animals.* An ICR:JCL strain of male mice weighing approximately 30 g, inbred for a long period time in our laborato:y, was used throughout the experiments. More than 3 mice were used for each group.

*The mouse intestinal loop assay.* Primarily, mice were treated with intraperitoneal administration of cyclophosphamide (one dose, 100 mg/kg 3 days previously) and untreated control mice were used for the comparison of the experiments. The mouse intestinal loop assay was carried out as described by Punyashthiti and Finkelstein [2]. The mice were deprived of food for 15–18 h before an operation. Anaesthesia was done by intraperitoneal injection of Nembutal sodium solution (50 mg/kg) for 10 min before the operation. Under anaesthesia, the abdomen was opened about 5 mm and the loops were isolated by silk ligatures from approximately 8 cm distal to the stomach. Two 2 cm loops separated by an interloop of 1 cm were made in a mouse. A 0.2 ml of the broth culture of the strain that were cultured in brain heart infusion broth (B. H. I. Broth; Difco) for 18 h at 37 °C, containing approximately $3 \times 10^8$ organisms, was injected into the lumen of one loop by means of a tuberculin syringe fitted with a 27-gauge disposable needle. The peritoneum and the outer layer of the abdomen were closed with bond, respectively. The mice, deprived of food and water, were kept in the cage at room temperature for the duration of the experiments. Then they were killed under chloroform anaesthesia. Inflammatory changes of the ligated intestinal loops were macroscopically observed and then the weights of fluid in the loop per the gut length were calculated. Punyashthiti and Finkelstein [2] reported that results were regarded as positive, if the ratio of fluid to length was 50 or more mg per cm when the animals were killed 8 h later. In our experiments, inflammatory changes of the ligated intestinal loops were also evaluated 18 h after the operation and the method of Punyashthiti and Finkelstein [2] was used for evaluation.

The lumen of the loops was washed several times with 0.067 M phosphate-buffered saline (pH 7.2) to remove adherent organisms from the surface of the mucosa. And then the loop was homogenized with a homogenizer (Iuchi Co., Ltd., Tokyo, Japan) in B. H. I. Broth, and viable cells within the ligated intestinal loops were counted by the regular pour plate count method.

## Results

Using intestinal loops of untreated mice, the results were shown as in Fig. 1. *E. coli* O124 S, O136, O112, O28 A and O29 caused inflammation in 3 out of 10 loops (3/10; 33.3%), 2/5 (40.0%), 2/5 (40.0%), 1/5 (20.0%) and 1/5 (20.0%), respectively. Using intestinal loops of mice treated with cyclophosphamide, *E. coli* O124 S, O136, O112, O28 A and O29 exhibited a positive reaction in 7 out of 9 loops (7/9; 77.8%), 3/5 (60.0%), 3/5 (60.0%), 4/5 (80.0%) and 3/5 (60.0%), respectively (Fig. 1). Swelling and oedema was observed in these positive loops. These positive rates were about 2 or 3 times as high as those of untreated mice.

The mean values of fluid weight per gut length are shown in Table II. These values in the case of enteroinvasive strains of *E. coli* were as high as the value obtained with the enteroinvasive strain of *S. flexneri* 17 A in untreated mice. These values were significantly higher than that given by the B. H. I. Broth control. Using intestinal loops of treated mice, these values were extremely higher than those of untreated mice. In particular, the mean fluid weight/gut length of loops from treated

**Table I**

*Characteristics and virulence of bacterial strains*

| Characters | *E. coli* | | | | | | | *S. flexneri* | |
|---|---|---|---|---|---|---|---|---|---|
| | O124 S | O124R | O136 | O112 | O28 A | O28 N | O29 | 17-A | 17-N |
| Colony type | smooth | rough | smooth | smooth | smooth | smooth | smooth | smooth | smooth |
| Absorption of Congo red | + | ++ | + | + | + | - | + | + | - |
| Gas from glucose at 37 °C | - | - | + | + | + | + | + | - | - |
| $H_2S$ from TSI | - | - | - | - | - | - | - | - | - |
| Motility | - | - | - | - | - | - | - | - | - |
| IPA | - | - | - | - | - | - | - | - | - |
| Serény test | + | - | + | + | + | - | + | - | - |
| Invasion of cultured cells | + | - | + | + | + | - | + | - | - |
| Suckling mouse assay | - | - | - | - | - | - | - | - | - |
| Toxigenicity to HeLa, Vero, Y-1 cells | - | - | - | - | - | - | - | - | - |
| Agglutination (0.85% saline) | - | + | - | - | - | - | - | - | - |

## Table II

*The mean values of fluid weight (mg) per gut length (cm) for the intestinal loop assay in untreated mice and mice treated with cyclophosphamide (CY) 3 days previously*

| Strains | Broth | *E. coli* | | | | | | | *S. flexneri* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | O124 S | O124 R | O136 | O112 | O28 A | O28 N | O29 | 17-A | 17-N |
| Untreated | 12.7 | 47.8** | 18.3 | 43.6** | 65.9* | 41.9** | 20.5 | 40.7** | 57.1** | 17.6 |
| Mice | ±4.1 | ±21.7 | ±8.4 | ±18.3 | ±69.7 | ±20.9 | ±4.0 | ±24.2 | ±34.6 | ±6.3 |
| (n=) | 10 | 10 | 9 | 5 | 5 | 5 | 3 | 5 | 10 | 12 |
| Treated | N.T. | 115.7** | 19.4 | 72.9** | 76.8** | 114.5* | 25.6 | 49.0** | 132.2** | 27.3 |
| with CY | | ±103.2 | ±8.4 | ±54.1 | ±45.7 | ±120.8 | ±5.9 | ±16.7 | ±116.2 | ±10.0 |
| (n=) | | 9 | 3 | 5 | 5 | 5 | 3 | 5 | 10 | 5 |

** Significance ($P < 0.01$) between the test group and the broth control
* Significance ($P < 0.05$) between the test group and the broth control

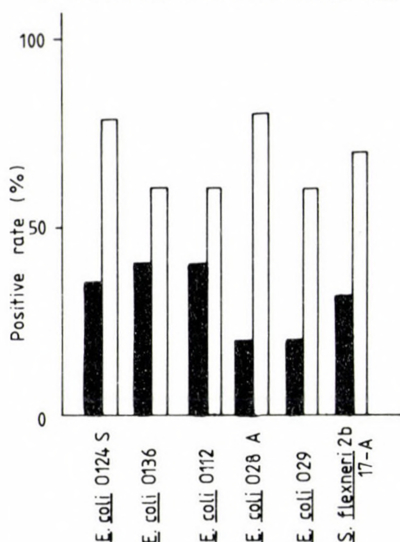


Fig. 1. Positive rate for the intestinal loop assay in untreated mice (solid columns) and mice treated with cyclophosphamide 3 days previously (open columns)

mice was 115.7 mg/cm after inoculation of *E. coli* O124 S, and 114.5 mg/cm after inoculation of *E. coli* O28 A. However, *E. coli* O124 R and O28 N were not capable of causing an inflammation in the intestinal loops in treated and untreated mice, same as observed with the noninvasive *S. flexneri* 17 N.

Upon enumeration of viable cells at 18 h after inoculation of the organisms into the loops of treated mice, every strain, except for strains of *E. coli* O124 R and O28 N, showed a significantly higher number of viable cells in loop homogenates and fluid. On the other hand, no *E. coli* cells were detected in control loops inoculated with B. H. I. Broth only. For example, it was found that $1 \times 10^9$ organisms/ml of *E. coli* O124 S increased to $7 \times 10^9$ organisms/ml. However, $2 \times 10^9$ organisms/ml of *E. coli* O124 R decreased to $1 \times 10^7$ organisms/ml.

## Discussion

Pathogenic mechanisms of intestinal infections are classified into four groups; enterotoxigenic, enteropathogenic, enteroinvasive and enterohaemorrhagic. *E. coli* O124, O135, O112, O28 A and O29, used in this study, were known to be capable of invading the intestinal tissues. These strains injured the epidermal cells locally as *S. flexneri* did [5]. The mouse ligated intestinal loop assay has been generally used for the detection of heat-labile enterotoxin. In this assay, inflammatory changes of ligated intestinal loops were evaluated 6–8 h after the operation. *E. coli* O6, producing enterotoxin, caused swelling in all intestinal loops, while *E. coli* O124 S

and R did not [3]. *E. coli* O6 was also positive in the assay of enterotoxin with cultured cells used for the detection of heat-labile toxin. And it was also positive in the suckling mouse assay used for the detection of heat-stable toxin. However, *E. coli* O124 S and R were also negative in two assays [3]. Recently, Fasano et al. [6] reported that enteroinvasive *E. coli* produced low levels of Vero cell cytotoxins. However, *E. coli* strains used in this study produced no Vero cell cytotoxins (Table I). It seems that these strains were unable to produce enterotoxin or produced extremely low values.

Concerning the difference in the latent period between enterotoxigenic and invasive strains, incubation time was prolonged to 18 h after the operation. *E. coli* O124 S, which did not exert inflammation 6–8 h after the operation, caused macroscopically recognizable inflammation with swelling and oedema after 18 h in about one third of the loops tested. To increase the positive rate of this assay, the animals were pretreated with cyclophosphamide, which is an inhibitor of neutrophils. Increasing numbers of viable cells were seen in these loops, and numerous bacterial cells were found in the epithelial cell fraction. *E. coli* O124 S, O136, O112, O28 A and O29 caused a keratoconjunctivitis in the Serény test. And they were also able to invade HeLa cells indicating an association of invasion of the strain with positive loop test (Table I).

To use mice instead of rabbits was superior not only in economy, equipment and availability, but also in practice. It should be noted that the mouse ligated intestinal loop assay could be used for the detection of enteroinvasive strains.

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# PHOSPHOLIPID CONTENT OF UNTREATED AND INSULIN TREATED CELLS OF *NEUROSPORA CRASSA* (WALL-LESS STRAIN)

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Phosphatidylcholin (51.2%) and phosphatidyl-ethanolamine (38.3%) are the most abundant phospholipids in the wall-less strain of *Neurospora crassa*. Insulin treatment exerted no change on the amount of phosphoinositides, although some previous results proved the existence of specific insulin receptors and specific effect of insulin on glucose metabolism in these cells. Long (20 h) treatment with insulin also failed to cause biologically significant changes.

The constituents and changes of phospholipids have become conspicuous when the importance of phosphatidylinositol cycle in signal transduction has been discovered. After binding to their membrane receptors many hormones, neurotransmitters trigger the cleavage of phosphatidyl 4,5-bis-phosphate ($PIP_2$) by phospolipase C, giving rise to diacylglycerol (DG), and inositol 1,4,5-trisphosphate ($IP_3$). $IP_3$ increases the intracellular $Ca^{++}$ content, while DG remaining in the membrane activates the protein kinase C [1, 2]. In the case of insulin, the binding to the subunit of the receptor causes the autophosphorylation of the subunit [3]. Some authors have found that due to insulin effect a phosphatidylinositol glycan of the membrane is hydrolized to DG and inositol phosphate glycan [4].

Signal transduction research on eukaryotic microbes has evolutionary importance as the mechanism appears entirely different in pro- and eukaryotic cells [5]. It has been suggested that signal transduction system occurring in higher organisms first developed in fungi during evolution [5]. A specific insulin receptor with tyrosine kinase activity was shown in a wall-less strain of *Neurospora crassa* [6–8].

In our study the phospholipid content of *N. crassa* is presented and the effect of insulin, especially on phosphoinositides, is discussed.

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## Materials and methods

*Culture.* Cultures of the wall-less strain of *N. crassa* (FGSC 4761) were grown in synthetic medium for 40 h. In each experiment one of the cultures was treated with insulin ($10^{-6}$M; Actrapid MC, Novo, Copenhage, Denmark) the other was the control. After 20 h treatment the cells were washed and cultured further for 20 h in insulin-free medium [7, 8]. Then the cells were centrifuged and labelled with $^{32}$P-Na-ortophosphate (60 μCi/ml protein, Izinta, Budapest). After 3 h the cells were washed (control and insulin treated groups alike) and were grouped, and treated further as follows: (i) the lipids were extracted immediately (0); (ii) the lipids were extracted after 30 s; (iii) the lipids were extracted after 30 s of insulin treatment ($10^{-6}$M). The experiments were repeated thrice, and each of them was performed in triplicates.

*Lipid extraction.* Two ml of ice-cold 10% trichloroacetic acid were added to 2 ml sample. After storage for 20 min at 4 °C the samples were centrifuged and 2 ml of chloroform:methanol:HCl (100:200:1) was added to the pellet. After 15 min 0.66 ml of chloroform and 0.66 ml water were given. Following centrifugation the upper phase was discarded. The lower organic phase was dried under $N_2$, resuspended in chloroform:methanol (6:1) and spotted on oxalate impregnated Silica Gel plates [9].

*Chromatography.* The separation of phospholipids was made by two steps one dimensional thin-layer chromatography [10]. The radiolabelled phospholipids were visualized by autoradiography. Medifort RP diagnostic film (Forte, Vác, Hungary) was used for 36 h at −18 °C. The phospholipids on the plates were visualized by iodine vapour. Phosphoinositide standards were used: PIP (L-phosphatidylinositol-4-phosphate), and PI (myo-inositol-4-phosphate) (Calbiochem, Luzern, Switzerland). The identified spots were scrapped into vials, 5 ml Turner cocktail was added and counted (Beckmann LS 9000). Results for each lipid are expressed as a percentage of total radioactivity. Significance was calculated by Student's "t" test.

## Results and discussion

Six phospholipids were separated from the wall-less strain of *N. crassa*: $PIP_2$ = phosphatidylinositol-4,5-bisphosphate; PIP = phosphatidylinositol-4-monophosphate; PI = phosphatidylinositol; PC = phosphatidyl-α-choline; PE = phosphatidyl-ethanol-amine; PS = phosphatidyl-serine. Our results are given in Table I.

Several hormones, neurotransmitters and growth factors stimulate the cleavage of $PIP_2$. The production of $IP_3$ [1] and DG, and the importance of these second messengers have been shown. But there are some contradictions in the results on the insulin effect. The tyrosine kinase activity of the receptor is basic in the response [3], but some other data show that another way can be involved also in the cell activation. In some cases the insulin triggered the production of second messengers from a membrane glycolipid, the phosphatidylinositol-glycan [11, 12]. Accordingly, the change in the amount of phosphoinositides due to insulin treatment is possible at least in certain cells. Of course, most of the experiments have been performed mainly on higher vertebrate cells, but some studies have proved that the phosphoinositides or/and metabolic products of them are important in the regulatory processes of

Protozoa e.g. in *Paramecium* [9]. In *Tetrahymena pyriformis* [9] insulin increased the amount of $PIP_2$ after insulin pretreatment (imprinting).

**Table I**

*Phospholipid content of control and insulin treated cells of N. crassa (%)*

| | Control group (without pretreatment) | | | Insulin pretreated (0 h) | | |
|---|---|---|---|---|---|---|
| | C 0 s | C 30 s | I 30 s | C 0 s | C 30 s | I 30 s |
| $PIP_2$ | 2.2 | 2.7 | 2.8 | 3.9** | 3.1 | 3.7 |
| PIP | 2.0 | 2.1 | 1.6 | 1.8 | 1.3 | 1.3 |
| PS | 4.1 | 3.0 | 2.9 | 3.9 | 3.1 | 2.9 |
| PC | 51.2 | 52.2 | 52.1 | 50.5 | 51.7 | 51.8 |
| PI | 1.8 | 1.2 | 1.4 | 1.1 | 1.2 | 1.3 |
| PE | 38.3 | 39.2 | 39.6 | 38.5 | 39.8 | 39.1 |

** Significant to 0 (Without pretreatment). The other data are not significant
C = time of extraction
I = time of extraction after insulin treatment

Not too much attention was paid to the regulation in eukaryotic microbes, although the role of second messengers in physiological processes in different species was reported [13–16]. In *Saccharomyces* and *Dictyostelium* the presence of phosphatidylinositol cycle has been shown [5–7]. The study of signal transduction in eukaryotic microbes may have a great importance as there are only sparse similarities in signal transduction systems in eu- and prokaryotes, although biochemically the eu- and prokaryotes are rather similar [5]. For example, receptor regulated phosphoinositide turnover haven't been found in bacteria, but it is characteristic of eukaryotes: fungi, plants and animal cells [5].

The specific effects of insulin, binding to its receptor, and the tyrosine kinase activity of the receptor was reported on the cells of a wall-less strain of *N. crassa* [6–8]. The evidence of some second messengers, like cAMP, $Ca^{++}$, $IP_3$ in different physiological processes is also known in this species [14, 16, 17, 18, 19]. On the other hand, there are only few data about the phospholipid content and the turnover of phosphoinositides in these cells [4].

**Table II**

*Comparison of phospholipid content (%) in different organisms*

| | *E. coli** | *N. crassa*** | *Tetrahymena**** | Mammalian liver* |
|---|---|---|---|---|
| PE | 70 | 38.3 | 48.0 | 7.0 |
| PS | trace | 4.1 | ? | 4.0 |
| PC | 0 | 51.2 | 13.8 | 24.0 |

* Alberts et al. [20]; ** Present paper; *** Kovács and Csaba [9]

Our data show that in this mutant strain of *N. crassa* the most abundant phospholipids are PC (51.2%), and PE (38.3%). The amount of phosphoinositides ($PIP_2$, PIP, PI) is 6% of total phospholipids. It differs from the phospholipid content of *Escherichia coli* [20] and eukaryotic *Tetrahymena pyriformis* [9] and mammalian liver cell (Table II) as well. While the most abundant phospholipid in *E. coli* is PE, like in *Tetrahymena*, in mammalian liver it is PC. The phospholipid content of *N. crassa* is more similar to mammalian liver than to the phylogenetically closer unicellular *Tetrahymena*. From our results it seems that insulin treatment cause no significant changes in the phospholipid content (control, not pretreated group), accordingly, in *N. crassa* the phosphoinositol cycle has no role in transduction of insulin effect. The long term (20 h) treatment increased the amount of $PIP_2$ (insulin pretreated group) although the short (30") insulin treatment had no effect on it. Several studies showed that the pretreatment with a hormone (imprinting) changes the binding capacity and though the degree of hormone effect in a subsequent treatment [21–23]. Such effect was shown in *Tetrahymena* even on the level of phospholipids, the quantity of $PIP_2$ decreased in pretreated cells, and without pretreatment there were no changes in it [9]. In *Neurospora* we could not show such effect, suggesting that although the long pretreatment increased the amount of $PIP_2$, this changes have no role in insulin transduction.

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# THE COURSE OF LCMV INFECTION IN EUTHYMIC AND ATHYMIC MICE PRETREATED WITH IMMUNOMODULATORY AGENTS

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Balb/c (euthymic) and nu/nu (athymic) mice were treated intraperitoneally with TP-4 (a synthetic tetrapeptid, thymopoietin sequence analog) or with Mannozym (1% zymosan suspension), and were infected intracerebrally with LCM virus. Both of the agents contributed to the development of fatal choriomeningitis, consequently stimulated the cellular immune response in euthymic mice, but the athymic mice either treated or not, survived the infection, consequently the agents had no effect on the course of LCM virus infection. Both agents exerted a thymus-dependent cellular immune response stimulating effect. That is, an immunostimulatory effect can be realized only in the presence of the thymus or the T-dependent lymphoid system.

It is known that the fatal lymphocytic choriomeningitis followed by intracerebral (i. cer.) LCM virus (LCMV) infection is the consequence of the cytotoxic reaction of LCMV antigen-specific T lymphocytes to cells expressing viral antigens on the leptomeninx [1–3]. The course of LCMV infection thus greatly depends on the cellular immune responsiveness of the animal. Acute lymhocytic choriomeningitis develops in adult mice with intact, developed immune system and the animals die on the 6–8th day after virus infection. In the absence of the thymus-dependent lymphoid system, as in genetically athymic nude mice [4], in mice thymectomized in newborn age [5–7], in newborn mice with undeveloped immune system [8] lymphocytic choriomeningitis fails to develop and the mice surviving the infection become virus carriers. Manifestation of LCMV infection in the form of fatal meningitis could be altered in mice both with normal and undeveloped immune system by immunomodulatory treatments. Immunostimulatory effect [9–14] accelerate and increase, immunosuppressive effects [15–19] hinder and decrease the time and rate of the appearance of the fatal lymphocytic choriomeningitis.

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In the present experiment it was examined whether pretreatment with two immunomodulatory agents, with Mannozym or with TP-4, can influence the course of LCMV infection in genetically athymic, nude or in euthymic mice.

## Materials and methods

*Experimental animals.* Six-week-old, SPF Balb/c nu/nu (nude, athymic), conventional Balb/c (euthymic) and CFLP mice of both sexes (LATI, Gödöllő, Hungary) were used.

*TP-4 synthetic product* (Gedeon Richter Pharmaceutical Product's Factory, Budapest). It is a tetrapeptid analog of the natural thymopoietin sequence (Arg-Lys-Asp-Val) [20].

*Mannozym preparation* (Institute of Serobacterial Production and Research Human, Budapest). Composition: 1 mg *Saccharomyces cerevisiae* polysaccharide per ml, suspended in saline (0.1% zymosan suspension), without preservative. The water-insoluble active substance (glucomannan) is built up mainly of glucose and mannose molecules [21, 22].

*LCMV infection.* The strain WE maintained in our laboratory by serial intracerebral (i. cer.) passages in mice was used. Brain suspensions and virus dilutions were prepared with PBS. Virus titration was performed by i. cer. inoculation of 6-week-old conventional Balb/c mice.

*Recovery of LCMV.* Young adult CFLP mice were inoculated i. cer. with 1:10 dilutions of brain suspensions prepared from one half of the brains of mice surviving LCMV infection and sacrificed on the 21st day of the experiment. Presence of LCMV was confirmed by the typical neurological symptoms and deaths of mice.

*Histology.* Half of the brains of mice surviving LCMV infection and sacrificed on the 21st day of the experiment, were fixed in formalin and embedded in paraffin. The sections were stained with haematoxylin-eosin.

## Experiments and results

The treatment of mice with TP-4 was carried out by intraperitoneal injection in 2 mg/kg doses, 7 times during two weeks. Treatment with M in 1 ml volume injected intraperitoneally was carried out 4 days and one day before LCMV infection. One ml dose of M is equal to 40 mg/kg of zymosan in case of 25 g body weight. Thirty mice were treated with TP-4, another 30 with M. One day after the last TP-4, or second M treatment two-third of the animals in each group were infected intracerebrally with 100 $LD_{50}$ previously titrated LCMV. The remaining one third of the animals were inoculated i. cer. with virus free normal mouse brain suspension. The groups, the treatments, and the numbers of mice are shown in Table I.

In virus infected groups the development of neurological symptoms (tremor, spasm) characteristic of lymphocytic choriomeningitis were registered daily. No deaths were recorded in the groups of mice not infected with LCMV. The experiment was terminated on the 21st day after LCMV infection.

**Table I**

*Mouse groups and treatments*

| Mice | Groups | Treatments of mice i. p. | Treatments of mice i. cer | Number of mice |
|---|---|---|---|---|
| Balb/c nu/nu nude | M-LCM | M | LCM virus | 20 |
| | (TP-4)-LCM | TP-4 | LCM virus | 20 |
| | LCM | – | LCM virus | 20 |
| | M | M | X | 10 |
| | TP-4 | TP-4 | X | 10 |
| | C | – | X | 10 |
| Balb/c (normal) | M-LCM | M | LCM virus | 20 |
| | (TP-4)-LCM | TP-4 | LCM virus | 20 |
| | LCM | – | LCM virus | 20 |
| | M | M | X | 10 |
| | TP-4 | TP-4 | X | 10 |
| | C | – | X | 10 |

X = normal mouse brain suspension

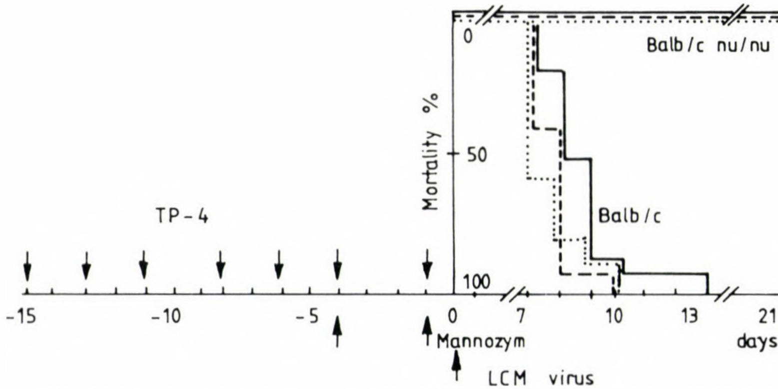


*Fig. 1.* Rate and time of deaths after LCMV infection. Groups: LCM ------; M-LCM - - - - -; (TP-4)-LCM · · · · · ·

Rate and time curve of death in LCMV infected groups during the experiment are presented in Fig. 1. Hundred per cent of nude mice survived the LCMV infection in each group (LCM, M-LCM, (TP-4)-LCM), neither disease (tremor or spasm) nor death occurred among them. The reisolation of the LCMV from the brain of

survivors at the 21st day of the experiment was successful, whereas the lymphocytic infiltration of the leptomeninx could not be demonstrated histologically. Thus the survivors have been asymptomatic virus carriers.

All of the euthymic mice died showing characteristic signs of lymphocytic choriomeningitis (tremor, spasm) both in the groups of untreated and pretreated animals (Fig. 1). However, the time and the daily rate of death differed in the groups treated with M or TP-4 and in the untreated one. Among the treated mice the lymphocytic choriomeningitis developed earlier, the treated mice died on 7 – 10th day instead of on the 7 – 14th day registered in the untreated group. The mortality rate was higher in treated groups on the first day of death (40 and 60%), than that in the untreated group (20%). This more than 10% difference could be evaluated as the stimulation of the cellular immune response.

## Discussion

In agreement with literary data [4] the athymic nude mice survived the LCMV infection without clinical sign of choriomeningitis and became virus carriers. In the absence of T-lymphocytes the lymphocytic infiltration of the meninx, and the fatal lymphocytic choriomeningitis failed to develop.

The cellular immune response stimulating effect of TP-4 or M was demonstrated earlier [23 – 26]. In the present experiment both of the examined agents, the M and the TP-4 stimulated the cellular immune response only in euthymic mice, while such effect could not be demonstrated in nude mice. Our results are in agreement with literary data that the TP-4 could diminish the immunosuppressive effect of cyclophosphamid, but this effect did not occur after thymectomy [25]. Thus, TP-4 is effective in animals with impaired T-dependent lymphoid system too, but only in the presence of the thymus.

Based on our earlier results, the effect of M could be evaluated similarly, since the M pretreatment stimulated the cellular immune response to LCMV infection both in suckling mice with undeveloped immune system and in aged mice being in physiological thymus involution [27, 28].

The present results reinforced our earlier observations that the preparations under study may be suitable for stimulation of insufficient immune system, but in the case of the total absence of the thymus or T-dependent immune system the treatment with these agents will be unsuccessful.

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# ANTHRAQUINONE PIGMENTS PRODUCTION BY BROWN MUTANT *TRICHODERMA VIRIDE* M-108 UNDER VARIOUS NUTRITION CONDITIONS

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In this work the influence of 12 carbon and 9 nitrogen sources on pigment production of brown mutant *Trichoderma viride* M-108 was tested. There were two concentrations of nutrient sources used and the tests were carried out in three light regimes. The pigmentation was good only on media containing sodium nitrate, sodium nitrite, sucrose, starch and glycerol and there was no pigment production on media with adenine, ammonium chloride and ammonium sulphate. In mixtures of pigments, individual compounds were determined by using thin-layer chromatography and UV spectra of colour fractions.

In 1978, 26 morphological and colour mutants of *Trichoderma viride* were isolated [1]. The mutants could be divided into three groups and this division was used as a basis for their study [2, 3]. One of these mutants (M-108) produced a lot of brown conidia and released purple-red pigment on the surface of the colony and into the medium. When the pigment was separated, two components responsible for the colour of brown conidia were isolated and identified by spectroscopy as 1,3,6,8-tetrahydroxyanthraquinone and 1-acetyl-2,4,5,7-tetrahydroxy-9,10-anthracenedione [4, 5]. Both compounds are known substances but were reported as metabolites of the fungus *T. viride* only by the authors mentioned. There were only reports about anthraquinones produced from *Trichoderma polysporum* as the antagonistic compounds in contact with the basidiomycete fungus *Fomes annosus* [6].

In the present work the results of comparative studies of various nutritive conditions on anthraquinone pigment production are reported. The release of pigments and their components were studied.

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## Materials and methods

*Strain. T. viride* M-108 mutant with brown conidia (CCM T-742) [3]. The strain was maintained on Czapek – Dox agar (CzDA).

*Media.* As basic cultivation medium CzDA was used [1]. Other types of media contained another carbon or nitrogen source in different concentrations (see Table I). Media with maltose, *D*-fructose, *D*-galactose, *D*-glucose, starch, sucrose, sodium nitrate, sodium nitrite, ammonium nitrate, urea and yeast extract as nitrogen source were also prepared as liquid media (without agar). Cultivation in liquid media was used for obtaining anthraquinone pigments.

*Cultivation conditions.* Mutant was evaluated after 21-day growth at three light regimes: A – alternate day and night light by cultivation on the laboratory desk; B – cultivation in dark with a 10-min exposure to day light at regular 24 h intervals; C – permanent cultivation in the dark. The temperature was 23 – 25 °C.

*Preparation of extracts, thin-layer chromatography (TLC), production and isolation of anthraquinones* were described by Betina et al. [5].

## Results

The production of conidia and then their pigmentation began when mutant M-108 was cultured on media with various carbon and nitrogen nutrients after 3 or more days. The pigments appeared on the surface of colonies as drops on media with normal carbon concentration in 6 to 14 days, on media with half carbon concentration in 5 to 7 days and on media without yeast extract but with various carbon sources in 8 to 15 days. When sodium nitrite was used as nitrogen source, pigment drops were on the surface of colonies after 5 days, when sodium nitrate, glycine and yeast extract were used as nitrogen sources drops appeared in 10 to 12 days and dependence on nitrogen concentration was very low. Only when glycine concentration was decreased to half, no pigment drops appeared on the surface of colonies. The same situation occurred when ammonium chloride, urea, adenine and ammonium sulphate were used in both concentrations. The pigment drops disappeared from the surface after 3 – 5 days. In the media, they usually appeared after their production on the surface of colonies.

In the pigment production and their following release into the media there were expressive differences between regime A (day – night) and B (10 min photoinduction) where the pigmentation was very intensive and regime C (cultivation in dark) where colonies produced pigments only in slight amounts or did not produce them at all (Table II). The complete inhibition of dark-red pigment production in all three regimes was obtained on media with normal concentration of *L*-arabinose without yeast extract, with half glycine concentration, and on media with normal or half concentration of ammonium chloride, adenine and ammonium sulphate. Very weak pigmentation was also found on other media containing L-arabinose and all types of media with cellulose, ammonium nitrate, urea and yeast extract as

**Table I**

*Carbon and nitrogen sources and their amounts in individual types of media*

| C-source | Medium | Amount of C-source (g/l) | Amount of yeast extract (g/l) |
|---|---|---|---|
| sucrose | $X_C$ | 30.0 | 5.0 |
| | $X_{C'}$ | 15.0 | 5.0 |
| | $X_b$ | 30.0 | 0.0 |
| Maltose, fructose, D-galac- | $X_C$ | 31.528 | 5.0 |
| tose, D-glucose, starch, | $X_{C'}$ | 15.764 | 5.0 |
| L-arabinose | $X_b$ | 31.528 | 0.0 |
| Glycerol | $X_C$ | 32.2 | 5.0 |
| | $X_{C'}$ | 16.1 | 5.0 |
| | $X_b$ | 32.2 | 0.0 |
| Lactose | $X_C$ | 30.0 | 5.0 |
| Cellulose, L-sorbose, mannose | $X_C$ | 31.528 | 5.0 |

| N-source | Medium | Amount of N source (g/l) |
|---|---|---|
| Sodium nitrate (basic CzDA) | $X_N$ | 3.0 |
| | $X_{N'}$ | 1.5 |
| Sodium nitrite | $X_N$ | 2.43 |
| | $X_{N'}$ | 1.215 |
| Ammonium chloride | $X_N$ | 1.88 |
| | $X_{N'}$ | 0.94 |
| Ammonium nitrate | $X_N$ | 1.4 |
| | $X_{N'}$ | 0.7 |
| Urea | $X_N$ | 1.0 |
| | $X_{N'}$ | 0.5 |
| Glycine | $X_N$ | 2.65 |
| | $X_{N'}$ | 1.325 |
| Adenine | $X_N$ | 0.02 |
| | $X_{N'}$ | 0.01 |
| Ammonium sulphate | $X_N$ | 1.93 |
| | $X_{N'}$ | 0.965 |
| Yeast extract[a] | $X_N$ | 11.43 |
| | $X_{N'}$ | 5.715 |

Carbon concentration was in all cases 12.6 g C/l; or 6.3 g C/l nitrogen concentration was in all cases 0.494 g N/l or 0.247 g N/l

[a] The nitrogen amount in yeast extract was determined by the Lowry method

**Table II**

*Review of pigment production and intensity of media colour due to pigments produced by mutant T. viride M-108 on individual types of media*

| Medium | Conc. of C | Type of cultivation<br>Regime A<br>Pigments | Colour intensity of medium | Regime B<br>Pigments | Colour intensity of medium | Regime C<br>Pigments | Colour intensity of medium |
|---|---|---|---|---|---|---|---|
| Source of C | $X_C$ | C | + + + + | C, D | + + + | C | + + |
| Sucrose | $X_b$ | C, D | + + + + | C | + + + | C | + |
| | $X_{C'}$ | C, D | + + | C, D | + | C | + |
| | $X_C$ | C, D | + + + + | C, D | + + + + | 0 | 0 |
| Maltose | $X_b$ | C, D | + + + | C, D | + + | C | + |
| | $X_{C'}$ | C, D | + + + | C | + + + | 0 | 0 |
| | $X_C$ | C, D | + + + | C, D | + + + | C, D | + |
| Fructose | $X_b$ | B, C, D | + + + | B, C, D | + | C, D | + |
| | $X_{C'}$ | C, D | + + + | C, D | + + + | C | + |
| | $X_C$ | C | + + + | C, D | + + + | C, D | + + + |
| D-Galactose | $X_b$ | C | + + | C, D | + | C | + |
| | $X_{C'}$ | C, D | + + + + | C | + + + | C | + |
| | $X_C$ | C, D | + + + | C, D | + + + | C, D | + |
| D-Glucose | $X_b$ | C | + + + | C | + + | C | + |
| | $X_{C'}$ | C | + + + | C | + + + | C | + |
| | $X_C$ | C, D | + + + + | C, D | + + + + | C, D | + + |
| Starch | $X_b$ | C, D | + + + | C, D | + + + | C | + + |
| | $X_{C'}$ | C | + + | C | + + | C | + |
| | $X_C$ | C, D | + | C | + | 0 | 0 |
| Cellulose | $X_b$ | C, D | + | C, D | + | C | + |
| | $X_{C'}$ | C, D | + | C | + | 0 | 0 |
| | $X_C$ | C | + + + + | C | + + + | C | + |
| Glycerol | $X_b$ | C, D | + + + | C, D | + + | 0 | 0 |
| | $X_{C'}$ | C | + + + + | C | + + | C | + |
| L-Arabinose | $X_C$ | B, C, D | + | B, C, D | + | 0 | 0 |
| | $X_{C'}$ | B, C, D | + | B, C, D | + | 0 | 0 |
| L-Sorbose | $X_C$ | C | + + + | C | + + | C, D | + + |
| Lactose | $X_C$ | B, C, D | + + + | x | x | x | x |

Continuation of Table II

| Source of N | | | | | | | |
|---|---|---|---|---|---|---|---|
| sodium | $X_N$ | C, D | + + + + | C, D | + + | C, D | + + |
| nitrate | $X_{N'}$ | C, D | + + + + | C, D | + + + + | C | + |
| sodium | $X_N$ | C, D | + + + + | B, C, D | + + + + | B, C, D | + |
| nitrite | $X_{N'}$ | C, D | + + + + | C, D | + + + + | C | + |
| ammonium | $X_N$ | 0 | 0 | 0 | 0 | 0 | 0 |
| chloride | $X_{N'}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| ammonium | $X_N$ | C, D | + | B, C, D | + | C, D | + |
| nitrate | $X_{N'}$ | C, D | + | C, D | + | 0 | 0 |
| urea | $X_N$ | C, D | + | C, D | + | B, C, D | + |
| | $X_{N'}$ | B, C, D | + | B, C, D | + | 0 | 0 |
| glycine | $X_N$ | C | + | C | + | C, D | + |
| | $X_{N'}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| ammonium | $X_N$ | 0 | 0 | 0 | 0 | 0 | 0 |
| sulphate | $X_{N'}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| adenine | $X_N$ | 0 | 0 | 0 | 0 | 0 | 0 |
| | $X_{N'}$ | 0 | 0 | 0 | 0 | 0 | 0 |
| yeast | $X_N$ | C, D | + | C, D | + | C, D | + |
| extract | $X_{N'}$ | C, D | + | C, D | + | 0 | 0 |

$X_C$ – medium with normal carbon concentration and with 0.5% yeast extract
$X_{C'}$ – medium with half carbon concentration and with 0.5% yeast extract
$X_b$ – medium with half carbon concentration without yeast extract
$X_N$ – medium with normal nitrogen concentration
$X_{N'}$ – medium with half nitrogen concentration
+ + + + – purplered coloured media
+ + + – darkred coloured media
+ + – red coloured media
+ – orangered coloured media
0 – pigments did not penetrate into the media or colonies did not produce red pigments
x – media were not tested

nitrogen source. The decrease of carbon concentration to half and the absence of yeast extract in these experiments influenced but slightly the production of pigments and their release into the media (Table II). The effect of nitrogen sources on pigmentation of brown mutant M-108 was very expressive. Good pigmentation resulted only on basic CzDA medium with sodium nitrate and on medium with sodium nitrite. A different pigmentation was on the medium with yeast extract used as sole nitrogen source where pigment drops appeared on the fungus colony but they did not penetrate into the medium (Table II).

In the mixture of pigments we tried to determine individual compounds by using thin-layer chromatography (TLC) (Table II). From chromatographs (Silufol foils, Kavalier, CSFR) the $R_F$ factors of substances obtained from TLC were enumerated. The compound C was compared with the standard and it was identified as 1-acetyl-2,4,5,7-tetrahydroxyanthraquinone ($R_F{}^4$ = 1). For compounds B (1,3,6,8-tetrahydroxyanthraquinone, $R_F{}^4$ = 1.53 – 1.59) and D (unknown structure, $R_F{}^4$ = 0.36 – 0.39) $R_F{}^4$ factors were enumerated. Which of these three substances produced mutant on individual types of media in three various cultivated regimes is shown in Table II. Irrespective of cultivation conditions the pigment's part C is always present. Part B appeared only during cultivation on several types of media. Its amount was very small and it may be assumed that it is produced also on the rest of the media but its amounts are so small that we cannot prove them by TLC. The pigments coloured the media in various shades of red. They were divided into 5 groups on the basis of colour intensity of media (Table II).

From silica gel column (100 – 160 $\mu M$, Lachema, Brno, CSFR) in three colour fractions the UV spectrum was measured. From the third fraction the UV spectrum with absorption maxima at 225, 263, 295 and 317 nm were obtained. The spectrum was the same as was the spectrum for standard compound C and in this fraction 1-acetyl-2,4,5,7-tetrahydroxyanthraquinone was detected. In the second fraction the absorption maxima 210, 253 and 290 nm were similar to absorption maxima of compound B (1,3,6,8-tetrahydroxyanthraquinone). Other fractions contained probably mixtures of compounds, which could not be used for the detection of individual pigment compounds.

## Discussion

The chemical structures and properties of pigments isolated from mutant M-108 were described in two papers by Betina et al. [4, 5]. When we enumerated $R_F{}^4$ factors for compounds B ($R_F{}^4$ = 1.53 – 1.59) and D ($R_F{}^4$ = 0.36 – 0.39) from pigments isolated from brown mutant M-108 none of them were identical with those described by Betina et al. [5]. There were also differences to spectra measured by these authors. For compound C the absorption maximum at 255 nm was absent which could have been due to the spoiling of the sample. In the second fraction the absorption maxima (210, 253 and 290 nm) remind absorption maxima of compound B (226, 250 and 275 nm). The mixtures of compounds are probable in the other fractions. They may contain next anthraquinone pigments e.g. pachybasin, chrysophanol and emodin. Some anthraquinones from *T. polysporum* were described as the antagonistic compounds produced by this fungus [6]. No reports about the influence of various nutritive sources on the production and structure of anthraquinone pigments have been published.

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# REVERSION OF UV LIGHT MORPHOLOGICAL AND COLOUR MUTANTS OF *TRICHODERMA VIRIDE*

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By using UV light 13 years ago from *Trichoderma viride* 8–7 Pers. ex S. F. Gray 82 colour and morphological mutants were prepared. The reversion of these mutants was high and in 1985 only 26 stable mutants (i.e. 32%) could be used for genetic studies. In 1991, from these 26 mutants we had only 6 stable types (i.e. 7.3% from the original state). These mutants were permanently transferred on fresh CzDA medium every 3–4 months and checked for morphology, growth, conidiation, heterokaryons and diploids production. The group of mentioned mutants was enlarged with mutants which were after 3–4 transfers on fresh medium, preserved dried to the present time. The largest differences were observed in microscopic appearance of colonies. Differences between growth rates, intensity of conidiation were comparable with those made 13 years ago.

In 1977 82 colour mutants from originally green pigmented strain of *Trichoderma viride* 8–7 were isolated [1]. Mutants were obtained by using UV light as mutagenic factor. They were of white, light yellow, yellow, lemon yellow, yellow-brown, light brown and brown colour. Besides the colour of conidia the growth rate was evidently reduced in some mutants (6 mutants) and conidiation was absent. From the resulting mutants 20 were nonconidiated, i.e. 24.4% of the total number of mutants. UV light activates numerous biosynthetic and metabolic processes [2] and also induces destructive reactions causing growth delay or killing of cells [3, 4]. As a mutagenic factor, it has the disadvantage that there is a high possibility of reversions. When the mentioned mutants were fully characterized in 1980 and 1985 [5, 6] it resulted in producing, in the first case 67, and in the second case only 26 stable forms (i.e. 32%). This shows that reversion frequency to parent strain is very high. From 26 stable mutants only 9 (i.e. 34.6%) were nonconidiating.

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## Materials and methods

*Strains. T. viride* 8–7 Pers. ex S. F. Gray is deposited at the Checho-Slovak Collection of Microorganisms, Brno, as CCM F-534. From this strain morphological and colour mutants were isolated [1], which were used in this work. Mutants were divided into two groups under their preservation conditions. Mutants from the first group were transferred every 3–4 months on the fresh CzDA medium during 13 years. Mutants from the second group were transferred on fresh CzDA medium only 3–4 times and then were preserved dried. Mutants from the first group were denoted M 3, M 32, M 76, M 103, M 105, M 108 and from the second group M 3*, M 5*, M 6*, M 14*, M 32*, M 34*, M 48*, M 59*, M 76*, M 100*, M 102*, M 105*, M 108*.

*Media.* Czapek-Dox medium (CzDA) with 0.5% yeast extract and 3% sucrose supplemented with 2% agar was used. For testing of conidia isolated from heterokaryons, the CzDA was supplemented with 0.24% sodium deoxycholate (CzDAD).

*Morphology of mutants, conidiation intensity and rate of mutant growth* were examined as previously described [6].

*Preparation of heterokaryons by anastomosis.* Four agar blocks from 4 mutants were placed on a plate with CzDA. At the point of contact the growing colonies formed hyphal connections in the form of anastomoses.

*Isolation of diploids.* Suspensions of conidia were prepared in distilled water from heterokaryons obtained by anastomosis. These suspensions were filtered through G 1 filters and then diluted to ensure the growth of 30–40 colonies on plates with CzDAD. Inoculated plates were incubated in the laboratory using natural light at 20–24 °C for 7–10 days. Since the conidia are mononucleate, green conidia would have to be diploids or their segregants.

## Results and discussion

*Mutant morphology.* In 1991, from the morphological and colour mutants isolated in 1977 (and published for the first time in 1978 [1]) only 6 stable mutants could be used to study the changes which occur during long time cultivation. This means that the frequency of reversion in our case was very high, being 92.7% of those tested in 1978, and 77.0% of those tested in 1985. The collection of mutants was enlarged with 13 mutants preserved dried. Mutants were as described before [6] divided into three groups on the basis of macroscopic appearance, colour of colonies and on the microscopic study of conidiation structures.

I. Colour mutants – they differ from the parental strain only in the colour of conidia. Their conidiation structures are not damaged. According to the colour of conidia they can be divided into three subgroups: (a) milky white (M 3, M 32, M 76, M 105, M 14*, M 32*, M 76*, M 105*); (b) yellow (M 103, M 3*, M 100*); (c) brown (M 108, M 108*).

Compared with the state described by Fargašová et al. [6] only mutant M 3 changed its morphological appearance from yellow to white and mutant M 103 changed from non-conidiating to white conidiating when they were permanently transferred.

II. Mutants with disturbed conidiation – their conidiation structures are damaged morphologically or functionally to different degrees. They were divided into the following subgroups:

(a) Mutants with morphological damage to conidiation structures – degeneration to a different degree. The conidia do not become separated from all phialides. They include mutants M 5*, M 6*, M 59*.

(b) Mutants with decreased intensity of conidiation – these have a normal structure and branching of conidiation structures but not all phialides give rise to conidia. These include mutants M 34*, M 48*, M 102*.

When conidiation structures of all mutants from this group were examined under a light microscope, the differences between them and the parent strain *T. viride* or colour mutants from group I were not so expressive as were described by Fargašová et al. [6]. In all cases the conidiation structures were properly formed and differences were only in their size, thickness and density. Some differences were observed also in appearance of hyphae. During microscopic observations made 13 years ago we observed that some mutants had hyphae with thicknesses and branching different from those of the parent strain. Before, vacuolized hyphae appeared only in mutant M 5. Now they were found in mutants M 6* and M 100* in a considerable amount, and in mutants M 3, M 32, M 76 and M 34* vacuoles were in marginally older parts hyphae. In mutants M 5*, M 105 and M 108 hyphae were thin as before and were twisted into structures resembling a ball. Hyphae of other mutants now resembled the parent strain. Formerly, there were often anastomosis between hyphae of mutant M 100, and mutant M 6 had hyphae like chains.

In group III described by Fargašová et al. [6] as non-conidiating mutants – the mutants have never been found to form conidial structures with conidia, we have now only mutant M 103*. Because its colour is now yellow and has complete conidiation, it must be arranged in group I. Non-conidiating mutant growth in all was very miserable, forming only small colonies with thin transparent mycelium and irregular edges and were the first which were incapable to growth. In 1985 there were only 9 non-conidiating mutants from 26 ones.

Also the appearance of some colonies was different as we described previously [6]. Mutants M 5*, M 6* and M 34* were described that form very thin transparent mycelium but now they formed more compact mycelium which is not as thick as the parent strain mycelium. Appearance of mutant M 59* and M 48* was the same as that previously. Mutant M 59* produced colonies with very thin mycelium exactly as mutant M 48* but it had also irregular border.

*Conidiation intensity.* Conidiation intensity reflected marked differences between the mutants from group I, divided in two parts (I – mutants permanently transferred on the fresh CzDA medium and I* – mutants preserved dried) and group II (II* – only mutants preserved dried). The parent strain *T. viride* CCM

F-534 had a conidiation intensity of $2.3 \times 10^7$ conidia per agar block (d = 14 mm). Mutants of group I permanently transferred on fresh CzDA medium had an intensity in the range of $8.7 \times 10^6$ to $1.8 \times 10^7$ and mutants of group I* preserved dried had very similar values of conidia number as group I (the values were between $3.8 \times 10^6$ and $2.2 \times 10^7$). There were only small differences between the parent strain and mutants of groups I and I* and these results correspond with results from year 1985. Mutants of group II* exhibited about 100-fold lower conidiation intensity as compared with the parent strain. The intensity of conidiation was in this group of mutants 10-fold higher then was described before [6]. It means that we can expect after few transfers their reversion to a wild strain connected with the production of green conidia. Conidiation intensity in *T. viride* mutants is shown in Table I.

**Table I**

*Conidiation intensity in T. viride mutants*

| Mutants permanently transferred | | Mutants preserved dried | | | |
|---|---|---|---|---|---|
| Group I | | Group I* | | Group II* | |
| Mutant | Conidiation intensity [a] | Mutant | Conidiation intensity [a] | Mutant | Conidiation intensity [a] |
| M 3 | $1.6 \times 10^7$ | M 3* | $1.5 \times 10^7$ | M 59* | $3.4 \times 10^5$ |
| M 32 | $1.5 \times 10^7$ | M 32* | $8.8 \times 10^6$ | M 48* | $5.8 \times 10^5$ |
| M 76 | $1.4 \times 10^7$ | M 76* | $1.2 \times 10^7$ | M 102* | $7.2 \times 10^5$ |
| M 105 | $1.2 \times 10^7$ | M 105* | $4.6 \times 10^6$ | | |
| M 108 | $1.8 \times 10^7$ | M 108* | $2.2 \times 10^6$ | | |
| M 103 | $8.7 \times 10^6$ | M 100* | $9.1 \times 10^6$ | | |
| | | M 14* | $3.8 \times 10^6$ | | |
| Parent strain | $2.3 \times 10^7$ | | | | |

[a] After 20 days number of conidia per agar block (d = 14 mm)

*Mutant growth rate.* Between our groups of mutants were differences not only in morphology of conidiation structures and conidiation intensity but also in growth rate of colonies. The average growth rate of mutants from group I slowed down after 72 h to about 22% of that of the parent strain. Thirteen years ago their growth rates were very similar to that of the parent strain [6]. Mutants of group I* grew at a rate very similar to that of the parent strain, indicating that this type of preservation did not influence the growth rates of mutants. Mutants of group II* were preserved only dried and their growth rates slowed down after 72 h about 38% as compared to the

parent strain. In 1985 their growth rates were, after 72 h of growth about 40% lower than that of the parent strain. Their growth rates were not influenced during preservation either. The growth rates of our mutant groups are shown in Fig. 1.

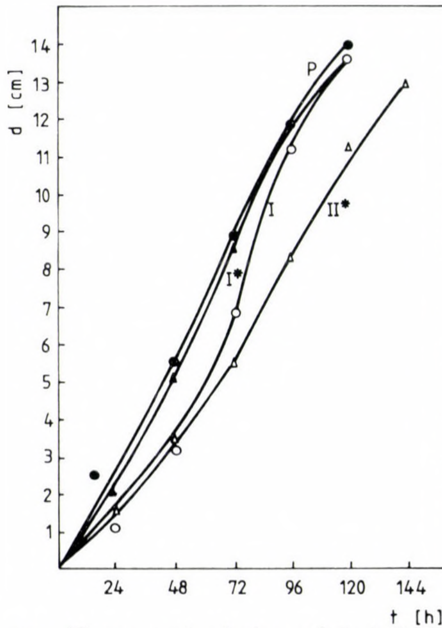


*Fig. 1.* Mean growth rates (diameter of colonies, cm) in individual groups of *T. viride* mutants. P = parent strain; I = group of colour mutants permanently transferred; I* = group of colour mutants preserved dried; II* = group of mutants with disturbed conidiation preserved dried

*Heterokaryosis of colour mutants permanently transferred.* Anastomosis was used to crossbreed 4 mutants forming white conidia, 1 mutant with yellow conidia and 1 with brown conidia. Complementation was considered to correspond to the appearance of green conidia, i.e. restoration of pigmentation of the parent strain. As described before [6], mutants with the same colour were not mutually complementary (Table II). Mutants with conidia of the same colour are probably constantly allelic and confirm correctness of our previous division to divide them into three complementation groups by their colour: white, yellow and brown.

**Table II**

*Complementation between colour mutants of T. viride permanently transferred on fresh medium*

| Mutant [a] | M 32 (w) | M 76 (w) | M 103 (y) | M 105 (w) | M 108 (b) |
|---|---|---|---|---|---|
| M 3 (w) | – | – | + | – | + |
| M 32 (w) | | – | + | – | + |
| M 76 (w) | | | + | – | + |
| M 103 (y) | | | | + | + |
| M 105 (w) | | | | | + |

[a]w – white conidia; y – yellow conidia; b – brown conidia

**Table III**

*Complementation between mutants of T. viride preserved dried*

| | | Group I* | | | | | | Group II* | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mutant [a] | M 14* | M 32* | M 76* | M 100* | M 105* | M 108* | M 48* | M 59* | M 102* (c) |
| Group I* | M 3* (y) | + | + | + | – | + | + | – | – | – |
| | M 14* (w) | | – | – | + | – | + | + | – | – |
| | M 32* (w) | | | – | + | – | + | – | + | – |
| | M 76* (w) | | | | + | – | + | – | – | – |
| | M 100* (y) | | | | | + | + | – | – | – |
| | M 105* (w) | | | | | | + | – | – | – |
| | M 108* (b) | | | | | | | – | + | – |
| Group II* | M 48* (lb) | | | | | | | | + | – |
| | M 59* (lb) | | | | | | | | | – |

[a]w – white conidia; y – yellow conidia; b – brown conidia; lb – light brown conidia; c – colourless conidia

*Heterokaryosis of mutants preserved dried.* Anastomosis was used to mutually crossbreed 10 mutants preserved for nearly 13 years as dried. Three of these mutants were previously described as mutants with disturbed conidiation (M 48*, M 59*, M 102*). They had either deformed phialides (M 59) or incomplete ones from which conidia often failed to separate (M 48, M 102) [6]. Now mutant M 59* had only shorter and thicker phialides than the parent strain and mutants M 48* and M 102* had normal fructification structures. However, the amount of conidia was lower by about $10^2$-times compared with the parent strain. Crossbreeding of 7 colour mutants from group I* in all combination gave the same results as crossbreeding between mutants permanently transferred (Table III). In this group we found only one

mutant, M 3* interesting. It was permanently transferred white but during preservation as dried its colour was yellow as 13 years ago. Now it was complemented with all 4 white and 1 brown mutant but not with the yellow mutant of group I*. Mutant M 102* (colourless) gave no heterokaryons with other mutants of groups I* and II* (see Table III). This does not imply its incompatibility with all others but may indicate, in addition to disturbance of conidiation structures, also disturbances in genes responsible for the contact and transfer of genetic material from the hypha of one mutant into the hypha of another one. Mutants with disturbed conidiation (M 48*, M 59*) formed heterokaryons after crossbreeding between themselves. This result agrees with results introduced in previous work [6]. After crossbreeding between mutants with disturbed conidiation and colour mutants both preserved as dried, heterokaryons were formed only in case when mutant M 108* was crossbred with mutant M 59* and mutant M 14* with mutant M 48*. Previously no crossbreeding between colour mutants and mutants with disturbed conidiation was made and so no comparisons could be made.

*Isolation of diploid.* Heterikaryons M 3 × M 108, M 76 × M 108, M 105 × M 108, M 3* × M 108*, M 100* × M 108*, M 32* × M 100*, M 32* × M 108*, M 76* × M 108*, M 105* × M 3*, M 76* × M 3*, M 3 × M 103, M 105 × M 103, M 32 × M 103, M 76 × M 103, M 103 × M 108, M 14* × M 100*, M 105* × M 100*, M 76* × M 100*, M 14* × M 3*, M 14* × M 108* were used to isolate and spread conidia. The pigmentation of the resulting colonies was then studied. On parallel plates (30 – 40 colonies per plate) no green colonies appeared. This indicates that, as in previous work [6], in the relatively small conidial populations under study (cca 300 – 400 colonies) no fusion of nuclei took place after anastomosis. On the plates only colonies with the morphology of crossbreeding mutants appeared. Their proportion was different from case to case but they can be divided into 4 groups with proportions approximately 1:3 (M 3 × M 108, M 76 × M 108, M 105* × M 3*, M 76 × M 103), 1:2 (M 105 × M 108, M 3* × M 108*, M 100* × M 108*), 1:1 (M 32* × M 100*, M 32* × M 108*, M 76* × M 108*, M 14* × M 100*, M 76* × M 100*, M 14* × M 3*) and 1:0.5 (M 76* × M 3*, M 105 × M 103, M 3 × M 103, M 32 × M 103, M 103 × M 108, M 105* × M 100*, M 14* × M 108*).

From our mutants the most stable were the coloured ones. One of them (M 108) was also used for isolation and identification of two anthraquinone pigments which have so far not been obtained from other strains of *T. viride* [7]. The most unstable were non-conidiating mutants which grew slowly and formed only small colonies with thin mycelium. Now we have no mutants with disturbed conidiation which were permanently transferred. Mutants from this group are all from cultures preserved dried. The ability of their reversion is very high.

It is known that the damages caused by UV light can be repaired by exposition to light (photoreactivation) or by incubation in the dark (dark repair). Also the third mechanism of reparation was described. In this case reparation takes place after DNA replication. In our case probably the second process took place. The possibility of repairing UV damages is high and living organisms have not only one way to protect themselves. How high is the ability to correct mistakes after exposure to UV light we can see also in our case with mutants of *T. viride*.

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# OCHRATOXIGENICITY OF *ASPERGILLUS OCHRACEUS* GROUP AND *PENICILLIUM VERRUCOSUM* VAR. *CYCLOPIUM* STRAINS ON VARIOUS MEDIA

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Production of ochratoxin A (OA) by strains of *Aspergillus ochraceus* group (*A. ochraceus* – 21, *A. sclerotiorum* – 1, *A. sulphureus* – 1) and *Penicillium verrucosum* var. *cyclopium* strains – 11, on various media was investigated. Thirteen percent of *A. ochraceus* strains and 18% of *P. verrucosum* var. *cyclopium* formed OA growing on sterile crushed wheat for 14 days at 25 to 27 °C (preliminary experiments). Toxin concentrations were 5.0 to 7.0 μg/kg. Five strains OA-positive on crushed wheat and four OA-negative strains were cultivated on various liquid nutritional media (YES, RM, OAT, CG and AFP). All the strains tested, including OA-negative ones, produced OA on certain liquid media. Concentrations of OA were low again (trace to 16.0 μg/l). The largest number of mould strains examined produced OA on YES (55.5%) and RM media (44.0%), but the highest concentration (16.0 μg/l) was formed on YES and CG media.

Ochratoxin-producing moulds are widespread contaminants of various foods and feeds. Varasky et al. [1] analyzed 155 of chicken feed samples and found that 19% of samples were contaminated with *Aspergillus ochraceus*. About 10% of these strains were ochratoxigenic. Cuero et al. [2] found toxigenic strains in 45% among *A. ochraceus* strains isolated from corn samples. Škrinjar [3] tested *Penicillium verrucosum* var. *cyclopium* strains, isolated from Edam cheese, on OA production and found that only 3% of strains were ochratoxigenic. Cvetnić and Pepeljnjak [4] studied the ability of OA production by *A. ochraceus* strains isolated from stored grains and dried meat collected in the period 1980–1987 from individual households in the nephropathic and non-nephropathic areas in Yugoslavia. They described that fungal strains from nephropathic areas produced OA in 37% of and those from non-nephropathic area in 35%. The toxin concentrations were from 0.07 to 240 mg/l.

The purpose of this study was to examine the ability of OA production by strains of *A. ochraceus* group and *P. verrucosum* var. *cyclopium*, isolated from beans.

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## Materials and methods

*Cultures and inoculum preparation.* The ability of OA production by 23 strains of *A. ochraceus* group (*A. ochraceus* Wilhelm – 21, *A. sclerotiorum* Huber – 1, *A. sulphureus* (Fres.) Thom and Church – 1) and 11 *P. verrucosum* Dierckx var. *cyclopium* (Westling) Samson, Stolk and Hadlok strains on various media was investigated. All of the strains tested were isolated from beans during the year 1990 in Vojvodina, Yugoslavia. The cultures were grown on Sabouraud maltose agar (SMA) slants for 7 days at 25 °C before use. The conidia were harvested from the SMA slants with 5 ml of sterilized distilled water and diluted to 99 ml with phosphate buffer containing 0.5% Tween 80. The final conidial suspension was adjusted to contain approximately $10^6$ conidia/ml.

*Growth of media.* In preliminary experiments all the strains were cultivated on sterile crushed wheat (25 g) for 14 days at 25 to 27 °C. Sterile medium was inoculated with 5 ml of inoculum. After 24 h 5 ml of sterile distilled water was added to the medium.

In further experiments the fungal strains that were OA-positive on crushed wheat [5] and four OA-negative were cultivated on various nutritional media as follows: YES (yeast extract, 2%; sucrose, 15%; distilled water to one litre), RM (rice meal, 7.5%; distilled water to one litre), OAT (oat meal, 3%; distilled water to one litre), CG (corn grits, 7.5%; distilled water to one litre) and AFP (yeast extract, 2%; bacteriological peptone, 1%; lactose 0.5%; $K_2HPO_4$, 0.1%; $MgSO_4{\cdot}7H_2O$, 0.05%; distilled water to one litre). The media (50 ml) were inoculated with 5 ml of inoculum and then incubated for 14 days at 25 to 27 °C. All experiments were performed in triplicates.

*OA analysis.* The method described by Balzer et al. [5] was used for determining of OA as follows: 25 g/ml/ of sample was extracted with 90 ml of acetonitrile and 10 ml of tap water, agitated with a mixer (3000 rpm) for 15 min and then filtered; 50 ml of filtrate was defatted with 2 × 25 ml of n-hexane. Detection of OA was performed by thin-layer chromatography (TLC). Concentrations of OA were determined visually. Pure OA from *A. ochraceus* was supplied by Fluka Biochemika 7411, Switzerland.

## Results and discussion

Since there is an opinion that cereals are the best substrate for mycotoxin synthesis [6], in preliminary experiments. *A. ochraceus* group and *P. verrucosum* var. *cyclopium* strains were cultivated on sterile crushed wheat for 14 days. It was found out that only three strains of *A. ochraceus* group (13%) (*A. ochraceus* 90-P2, *A. sclerotiorum* 12-II, *A. sulphureus* 9-NM) produced OA on wheat (Table I). Concentrations of the toxin were relatively low (5.0 and 7.0 μg/kg). Approximately the same results obtained cultivating *P. verrucosum* var. *cyclopium* strains on crushed wheat. One can notice from Table II that two *P. verrucosum* var. *cyclopium* strains, 90P-MS5 and 90P-MS6, out of the 11 investigated, had the ability to produce OA.

In further experiments, five mould strains that were toxin-positive in preliminary investigations (*A. ochraceus* 90-P2, *A. sclerotiorum* 12-II, *A. sulphureus* 9-NM, *P. verrucosum* var. *cyclopium* 90P-MS5 and 90P-MS6) and four toxin-negative strains (*A. ochraceus* 90-P1 and 90-P4, *P. verrucosum* var. *cyclopium* 90P-MS4 and 90P-MS7) were tested for OA production growing on YES, RM, OAT, CG and AFP

media. Results are given in Table III. It was shown that all the strains tested, including toxin-negative ones on wheat, produced OA on some media although sometimes at concentrations hardly detectable (trace). *Aspergillus* and *Penicillium* strains that produced OA on wheat formed this toxin on some media again. Toxin concentrations were low (from trace to 16.0 μg/l). It was noticed that YES and RM were the best media for toxin production. The largest number of fungal strains investigated produced OA on YES and RM, but the highest concentration (16.0 μg/l) was formed on YES and CG media.

**Table I**

*Production of ochratoxin A by moulds of A. ochraceus group on sterile crushed wheat*

| Mould species | Strain number | Concentration of ochratoxin A $\mu g \cdot kg^{-1}$ |
|---|---|---|
| *A. ochraceus* | 90-P1 | – [a] |
| *A. ochraceus* | 90-P2 | 5.0 |
| *A. ochraceus* | 90-P3 | – |
| *A. ochraceus* | 90-P4 | – |
| *A. ochraceus* | 90-P5 | – |
| *A. ochraceus* | 90-P6 | – |
| *A. ochraceus* | 90-P7 | – |
| *A. ochraceus* | 90-P8 | – |
| *A. ochraceus* | 90-P9 | – |
| *A. ochraceus* | 90-P10 | – |
| *A. ochraceus* | 90-P11 | – |
| *A. ochraceus* | 90-P12 | – |
| *A. ochraceus* | 90-P13 | – |
| *A. ochraceus* | 90-P14 | – |
| *A. ochraceus* | 90-P15 | – |
| *A. ochraceus* | 90-P16 | – |
| *A. ochraceus* | 90-P17 | – |
| *A. ochraceus* | 91-PD4 | – |
| *A. ochraceus* | 91-PD4A | – |
| *A. ochraceus* | 91-PD2 | – |
| *A. ochraceus* | 91-PD2A | – |
| *A. sclerotiorum* | 12-II | 7.0 |
| *A. sulphureus* | 9-NM | 5.0 |

[a] Not detected

**Table II**

*Production of ochratoxin A by P. verrucosum var. cyclopium strains on sterile crushed wheat*

| Strain number | Concentration of ochratoxin A $\mu g \cdot kg^{-1}$ |
|---|---|
| 90P-MS3 | –[a] |
| 90P-MS4 | – |
| 90P-MS5 | 5.0 |
| 90P-MS6 | 5.0 |
| 90P-MS7 | – |
| 90P-MS8 | – |
| 90P-MS9 | – |
| 90P-MS10 | – |
| 90P-MS11 | – |
| 90P-MS12 | – |
| 90P-MS/II | – |

[a] Not detected

**Table III**

*Production of ochratoxin A by strains of A. ochraceus group and P. verrucosum var. cyclopium on various media*

| Moulds species | Strain number | Concentrations of ochratoxin A $\mu g \cdot l^{-1}$ | | | | |
|---|---|---|---|---|---|---|
| | | YES | RM | OAT | CG | AFP |
| *A. ochraceus* | 90-P1 | –[a] | trace | – | trace | – |
| *A. ochraceus* | 90-P2 | – | trace | – | – | – |
| *A. ochraceus* | 90-P4 | 5.5 | – | – | – | – |
| *A. sclerotiorum* | 12-II | 16.0 | – | – | – | – |
| *A. sulphureus* | 9-NM | 8.5 | 5.2 | – | – | – |
| *P. verrucosum* var. *cyclopium* | 90P-MS4 | 8.0 | – | trace | – | – |
| *P. verrucosum* var. *cyclopium* | 90P-MS5 | – | – | – | – | – |
| *P. verrucosum* var. *cyclopium* | 90P-MS6 | – | – | – | 16.0 | – |
| *P. verrucosum* var. *cyclopium* | 90P-MS7 | 8.0 | trace | – | – | – |

[a] Not detected

YES = yeast extract + sucrose;
RM = rice meal;
OAT = oat meal;
CG = corn grits;
AFP = yeast extract + peptone + lactone + K + Mg;
SMA = Sabouraud-maltose agar

Frisvad [7] is of the opinion that the best media for toxin production depend on the mould species and sometimes even on the strains. His experience has shown that a series of media might be necessary to ensure that biosynthetic capabilities are detected. For *Penicillium* and *Aspergillus* toxins he suggested CYA, YES and OAT media. In our experiments it was established that about 55% of mould strains tested produced OA on YES and 11% on OAT medium.

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# ANTIBODIES AGAINST INVASION PLASMID CODED ANTIGENS OF SHIGELLAE IN HUMAN COLOSTRUM AND MILK

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Colostral and milk samples of Swedish, Vietnamese and Costa Rican mothers living under various socioeconomic conditions were tested for the presence of shigella invasion plasmid coded antigen (Ipa) specific antibodies. IgA antibodies of this specificity were found in significantly higher titres in samples of Vietnamese (600 ± 338) than in samples of Swedish or high income Costa Rican mothers (190 ± 224 and 290 ± 241, respectively; $p < 0.05$). Specific IgA titres in the low income group of Costa Rican mothers (470 ± 338) did not differ significantly from the values obtained in Vietnam. While no Ipa specific IgM could be detected in any of the samples tested, specific IgG was found in 90% of the Vietnamese colostrum. These data indicate that antibodies which could be responsible for the dysentery-preventing effect of breast feeding are indeed present in human colostrum and milk in areas where shigellosis occurs with relatively high incidence.

Breast feeding is generally thought to provide a passive protection against various types of infectious diseases [1, 2]. Beyond its beneficial effect on the

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nutritional status of the babies, several antimicrobial factors have been described in human colostrum and milk. These include specific immunoglobulins – mostly secretory IgA (sIgA) – T lymphocytes, leukocytes active in ADCC reaction, macrophages, PMNs, and non-immune factors such as lactoferrin, transferrin, lactoperoxidase, and lysozyme [3–7].

Dysentery is one of the most important enteric infections in developing countries. *Shigella* bacteria infect and multiply within the colonic epithelial cells leading to the ulceration of the intestinal mucosa (for a recent review see [8]). Two main groups of antigens on the surface of shigellae have been shown to be important for the virulent phenotype. One of them is the type specific lipopolysaccharide (LPS) antigen with an essential but still precisely not defined role in the pathogenesis [9]. There is also a set of protein antigens coded by the invasion plasmid of this pathogen, therefore often referred to as invasion plasmid coded antigens (Ipa). They are shared by all virulent *Shigella* strains and seem to play a role in the invasion of the host epithelium [8, 10].

Natural infection or vaccination elicit a strong antibody response against both antigen complexes in the serum and also on the intestinal mucosal surface [11, 12]. IgA antibodies against the shigella LPS [13, 14] and Ipa [14, 15] have been described in human colostrum and milk, too. Several investigations showed that there was a lower incidence of dysentery among breast fed infants [16–18], and breast milk also had a significant severity-mitigating effect even in malnoutrished children [18, 19]. It is reasonable to assume that one of the factors, by which colostrum and breast milk contributes to the passive protection against dysentery of breast fed babies, is antibacterial antibodies specific to various surface antigens. Previously we have shown that LPS specific antibody titres in colostrum samples of mothers from various geographical areas, or from families of different socioeconomic levels of the same country show a strong correlation with the possible previous exposure to shigellosis (Achi et al. submitted). Here we present our data comparing the colostrum and milk Ipa-antibody titres in Swedish, Vietnamese and Costa Rican mothers living under various socioeconomic conditions.

## Materials and methods

*Study populations.* Colostrum and milk samples were collected from Vietnamese, Swedish and Costa Rican mothers. In Vietnam, 38 mothers living in the Hanoi area under low income conditions were involved in the study. Colostrum was obtained from day 1 to day 5 post partum and milk samples from days 30 and 90 after delivery. Colostrum samples (n = 8) in Sweden were collected on the first day after delivery at the Department of Obstetrics and Gynaecology of the Huddinge Hospital, Huddinge. For Swedish mothers no special attention was paid to determine their socioeconomic status. Costa Rican mothers living in the metropolitan area of San José were recruited from low, middle and high socioeconomic status. The 34 mothers from the low socioeconomic conditions lived in overcrowded slums

or marginal areas of the capital and had very low incomes. The 23 women contacted at private clinics, with high income and excellent living conditions constituted the high socioeconomic group, while 37 mothers having incomes and living conditions in--between were considered to represent an intermediate socioeconomic level. Details about the social requirements for these categories were given elsewhere (Achi et al. submitted). Samples from Costa Rican mothers were taken at days 1, 4, 7 and 30 postpartum.

Mothers were questioned about symptoms of enteric infections in the three months prepartum and only those with a negative answer were included in the study.

*Colostrum and breast milk samples.* Samples were centrifuged at 10 000 g for 10 min to remove lipids and cells. The supernatants were collected, aliquoted and were immediately frozen. From Vietnam and Costa Rica frozen samples were transported to Sweden for further analysis.

*Antigen preparation.* For the Ipa-ELISA bacterial water extracts known to be rich in Ipa-s were used as antigens according to Oaks et al. [20]. In order to avoid the influence of colostral and milk anti-LPS antibodies on the results, extracts were made from a rough *Escherichia coli* K-12 strain (SP10) harbouring the invasion plasmid (pWR110) of a *S. flexneri* 5 isolate, M90T [21] and also from its plasmid less parent strain J53. To coat EIA plates (Dynatech), water extracts were used at a protein concentration of 2 μg/well as determined by the method of Lowry [22].

*EIA procedure.* The EIA was performed according to Oaks [20]. Samples were diluted 1:1000 in PBS containing 1% fetal calf serum. Alkaline phosphatase – conjugated rabbit anti-human IgA in dilution 1:3000; anti-human IgG in 1:4000; and anti-human IgM in 1:2000 dilution (Dakopatts) were used as second antibodies. Reactions were developed using p-nitrophenylphosphate substrate, and the optical densities (OD) were read after 100 min at 405 nm of a Titertek Multiskan photometer (Flow Laboratories). To increase the specificity of the assay all the samples were tested against extracts made from the plasmid harbouring (SP10) and plasmid negative (J53) strains. For any further analysis corrected OD values – calculated by substrating OD (J53) from OD(SP10) were used. The results were expressed as relative titres, e.g. corrected ODs multiplied by the dilution of the samples. Vietnamese and Costa Rican samples were considered to be positive when titres exceeded the mean +2 SD value of those from Swedish mothers.

*Statistical methods.* The Mann – Whitney test was used to analyse the data.

## Results

As shown in Fig. 1, colostrum samples from Vietnam exhibited the highest Ipa specific IgA titres with an average of 600 ± 338. This was significantly higher ($p < 0.05$) than the corresponding values of Swedish and Costa Rican mothers of high income class (190 ± 224 and 290 ± 241, respectively). The same comparison between Vietnamese mothers and those from Costa Rica at low (470 ± 484) or intermediate (400 ± 394) socioeconomic status did not provide a statistically significant difference ($p > 0.05$).

Concerning the mean value, the Vietnamese colostrum samples showed rather high Ipa specific IgG titres, although the variation among the individual samples was extensive (420 ± 544). Nevertheless, this mean titre was significantly higher ($p < 0.01$) than that found in other study groups varying from 35 ± 37 for low income Costa Rican mothers to 15 ± 14 for the Swedish mothers (Fig. 1.).

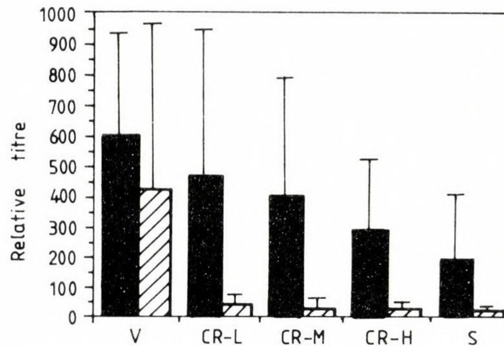


*Fig. 1.* Invasion plasmid antigen specific IgA and IgG titres in human colostrum. Solid columns, IgA; shaded column, IgG. V: Vietnamese mothers; CR-L, CR-M CR-H: Costa Rican mothers from low, intermediate and high socioeconomic status; S: Swedish mothers

Using a cut off value defined as the mean ±2 SD of the corresponding Swedish values, 40% of the Vietnamese samples were found to be positive for Ipa specific IgA, followed by the Costa Rican low, intermediate and high income groups with percentages of 29, 26 and 5, respectively. A similar sequence of order was seen for IgG with 90, 26, 14 and 10% (Fig. 2). It should be noted however, that even the positive samples from Costa Rica showed very low IgG titres close to, or often within the range of error of the EIA technique. No IgM responses was detected against Ipa (titres < 25) in any of the samples tested.

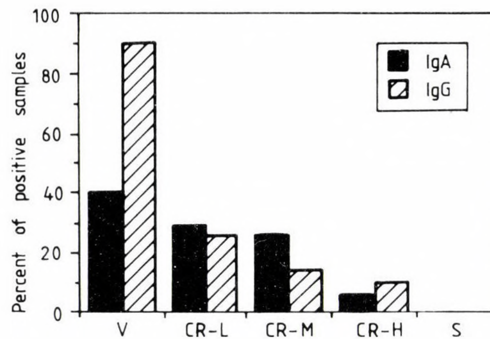


*Fig. 2.* Frequency of colostrum samples positive for invasion plasmid antigen specific IgA (solid columns) and IgG (shaded columns). V: Vietnamese mothers; CR-L, CR-M, CR-H: Costa Rican mothers from low, intermediate and high socioeconomic status; S: Swedish mothers

The change of Ipa specific antibody titres during the first three months of lactation was also tested. Milk samples from those Vietnamese mothers and Costa Rican mothers of low and intermediate socioeconomic status were tested for IgA whose colostrum sample were positive by the above criteria. For Ipa specific IgG,

milk samples only from Vietnamese mothers with positive colostrum-values were analysed. The results are summarized in Fig. 3.

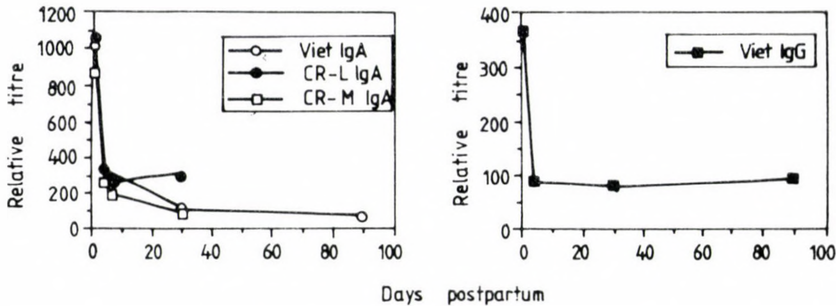


*Fig. 3.* Changes of invasion plasmid antigen specific IgA and IgG titres during lactation. Viet: Vietnamese mothers; CR-L, CR-M: Costa Rican mothers from low and intermediate socioeconomic status

For IgA, the titres considerably dropped already by the 4th day postpartum and stabilized by day 30 at a low – in case of low income Costa Rican mothers at a slightly higher – but constant level. Concerning IpA specific IgG, the decrease of titres were even more dramatic. Day 4 samples already showed the low value which was maintained up to the end of the 90 days study period.

## Discussion

Several epidemiological studies have shown that breast feeding protects infants against enteric infections. Two kinds of observations suggest that human milk might have a beneficial effect in protecting children against dysentery. First, shigellosis was shown to be less frequent and less severe among breast fed, than among the control babies [16–18]. Furthermore, similarly to the data of Mata et al. [17] obtained in Guatemala, we found in Vietnam that the highest incidence rate of shigellosis was recorded among children of the age when weaning usually takes place [23]. This correlated well with the appearance of shigella LPS specific antibodies. On the other hand, children under 1 year were less frequently suffering from dysentery, though usually they did not have shigella specific antibodies (e.g. probably were not protected by active immunization due to previous infections) [23].

The mechanisms by which the human milk protects brest fed babies against enteric infections are still not clear. Non-immune factors such as lactoferrin, lysozyme or the influence of milk diet on the intestinal flora could provide a broad range, non-specific protection [3, 4, 6]. In some cases specific antibodies have been

implied to interfere with interactions between bacterial virulence factors and host cells [24–26].

The presence of a smooth LPS antigen on the surface of shigellae is necessary for the full virulence of the bacterium. However, it is not known what role this complex bacterial polysaccharide plays in the pathomechanism of dysentery [9]. Some observations indicate that anti dysentery immunity is serotype (e.g. LPS) specific [27], but to explore whether it is mediated by specific immunoglobulins needs further investigations.

Recently, Cleary et al. [15] speculated that antibodies against the shigella invasion plasmid coded antigens may contribute to the passive immunity provided by breast feeding. Some of these proteins (Ipa B and C) are known to be involved in the invasion of epithelial cells as well as in the rapid escape of bacteria from the phagocytic vacuole within the host cell [8, 10]. With Ipa B specific monoclonal antibodies Mills et al. [28] were able to block the plaque formation (a complex assay measuring the invasion, intracellular multiplication, intra- and intercellular spread of bacteria [29]). It is reasonable to assume that an interference with these steps of the pathogenesis by specific secretory antibodies would result in an effective protection against the infective microorganism.

Our results presented here show that IgA antibodies specific to these antigens are frequently found in colostrum of mothers living under low socioeconomic conditions (e.g. the group of Vietnamese or the low income group of Costa Rican mothers), while samples from mothers living under better conditions exhibited no or low IgA responses (e.g. Swedish or the high income Costa Rican mothers). These results together with our previous observations using purified LPS antigens (Achi et al. submitted) suggest that mothers living under less favourable circumstances were more exposed to shigella infections than their counterparts in Sweden or in wealthier communities in Costa Rica.

While the anti-Ipa IgA response of Vietnamese and low income Costa Rican mothers were very similar, high Ipa specific IgA titres were seen only in Vietnamese mothers and only in the first few days postpartum. The origin of the colostral IgG, whether it was produced locally or was the result of passive diffusion from the serum, was not investigated during this study. However, one can speculate that low income Costa Rican mothers might have experienced only one or a few shigella infections in the past (which could be in accordance with the relatively low incidence of shigellosis in Costa Rica [30, 31]) resulting in a long lasting memory in the enteromammary sIgA system. On the other hand, in Vietnam, where shigellosis is endemic, mothers could have been repeatedly exposed to this pathogen or the last exposure could be more recent inducing a detectable Ipa specific IgG response in the colostrum. It is noteworthy that recently Oberhelman et al. [32] could show a strong Ipa specific IgG response on the intestinal mucosal surface of Peruvian patients.

Comparing the present data to our previous observations about antibody titres against the O antigen of various enteropathogens of the same colostral samples (Achi et al. submitted), it seems that there is no obvious correlation between the magnitude of anti-LPS and anti-Ipa responses in the colostrum of the same individual (data not shown). Similar observations were made recently testing serum and faecal extract samples of volunteers vaccinated with an *aro* D *S. flexneri* vaccine strain (An Li, Pál et al. submitted) and also when analysing the serum immune response of dysenteric Vietnamese and Swedish patients (Phung Dac Cam, Pál et al. submitted). Whether this is due to individual differences in responsiveness to various antigens of the same pathogen, or to differences in anamnestic histories concerning previous shigella infections needs further investigations.

Currently, experiments are in progress in our laboratory to determine by what mechanism(s) Ipa- and LPS-specific colostral antibodies could be effective in protecting against dysentery.

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# ESTABLISHMENT OF AN MT4 CELL LINE PERSISTENTLY PRODUCING INFECTIVE HIV-1 PARTICLES

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Human T cell Lymphotropic Virus-I (HTLV-I) carrying human T cell line MT4 is highly sensitive to Human Immunodeficiency Virus-1 (HIV-1). After HIV-1 infection cell clusters characteristic of intact MT4 rapidly disintegrate, syncytia appear and the cells die. Surviving MT4 cells were subcultured following HIV-1 infection of high multiplicity. We succeeded to establish an MT4 cell line continuously producing infective HIV (MT4/HIV-1). The original and the HIV-1 infected MT4 cells were morphologically similar. The MT4/HIV-1 cells proved to be nearly 100% positive in indirect immunofluorescence assay using the serum of an HIV-1 antibody positive individual. OKT4 surface antigen could not be demonstrated on MT4/HIV-1 cells. On electron microscopic pictures typical and atypical virus particles could be seen near the surface of the cell membrane. The persistently produced virus particles were infective for H9 and MT4 cells. The antigenic structure of the virus produced by MT4 cells was similar to that produced by H9 cells.

HIV-1, the causative agent of AIDS [1, 2], infects T4 cells [3] among them MT2 and MT4 cells [4]. The human T cell line MT4 described by Miyoshi et al. [5] originated from cord blood lymphocytes cocultured with leukemic cells of a patient with adult T cell leukemia. These cells carry HTLV-I provirus in their genomes and are highly sensitive to HIV-1 infection. HIV-1 infected MT4 cells undergo drastic and rapid cytopathic effects; the cells growing in clusters disintegrate and soon die [6]. Based on these properties virus titration by $TCID_{50}$ assay, a plaque assay and an assay based on HIV-1 induced prevention of reclustering of MT4 cells were developed [4, 7].

As MT4 cells are excellent indicators in HIV-1 investigations, we have made efforts to establish an MT4 cell line continuously producing the virus.

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## Materials and methods

*Cells.* H9 and virus producing H9/HTLV-IIIB human T lymphocytes were kindly provided by R. C. Gallo. MT4 cell line was obtained through the courtesy of D. Nosik (Moscow) with the permission of N. Yamamoto. The cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 IU/ml of penicillin and 100 μg/ml of streptomycin. The cells were subcultured twice weekly.

*Virus.* Clarified cell free supernatants of H9/HTLV-IIIB were used as virus source. After determining their reverse transcriptase (RT) activity, serial dilutions were made and 1.0 ml aliquots of these dilutions were added to the wells of a 24 well plate (Linbro), each well containing $10^5$ MT4 cells in 1.0 ml. The cytopathic effect was followed by light microscope and by regular measuring of RT activity of the supernatant.

*Immunofluorescence test.* Indirect immunofluorescence was carried out as described previously [8]. Briefly, cell smears were fixed with cold acetone for 10 min. The cells were then incubated with an HIV-1 antibody positive human serum, finally incubated with FITC conjugated anti-human IgG.

*RT assay.* We used a microassay for measuring RT activity described previously [9]. Poly (rA): oligo $(dT)_{12-18}$ as template primer was immobilized on Whatman DE 81 filter paper squares. $^3H$ TTP and virus-RT buffer mixture were dropped on the squares. They were incubated for 2 h, washed, dried and cpm was measured in a scintillation cocktail using a Packard 1600CA liquid scintillation analyzer.

*Fluorescence activated cell sorting (FACS).* The cell surface receptors (presence of T4 receptor) of control and HIV-1 infected cells were investigated by FACS analysis, using a FACSTAR (Becton Dickinson) analyzer. (We thank FACS investigation to R. Mihalik, National Institute of Haematology and Blood Transfusion, Budapest).

*Electron microscopic investigations.* Cell suspensions were centrifuged at 1400 rpm for 10 min and the cells obtained were fixed with 2.5% glutaraldehyde, postfixed with osmium tetroxide and embedded in araldite and investigated in a JEM 100 C type electronmicroscope. (We thank the electron microscopical investigations to Márta Csik and A. Pintér, "B. Johan" National Institute of Hygiene, Budapest.)

*HIV infectivity titration.* The method described by V. A. Johnson was used for measuring $TCID_{50}$/ml [10]. Sixty wells (6 horizontal × 10 vertical rows of a 96-well flat bottomed microtiter plate (Greiner) were used; 133 μl RPMI complete medium were pipetted to the wells of the first vertical row, and 150 μl to the second to tenth vertical rows; 67 μl aliquots of undiluted virus fluid were added into the wells of the first vertical row (1:3 dilution of the virus suspension). After mixing, 50 μl fluid from each of the six wells in the first vertical row was transferred to the wells of the next rows so as to make serial fourfold dilutions. From the 10th row 50 μl fluid was discarded or transferred to a second plate. Finally 50 μl MT4 cell suspension containing $0.4 \times 10^6$ cells/ml were pipetted into each of the wells. The cultures were halved twice a week by discarding 100 μl cell suspension and adding 100 μl fresh medium. CPE was investigated of the 12th day by light microscopy.

*Western blot analysis.* For western blotting, virus was concentrated from the clarified culture fluids of H9/HTLV-IIIB and MT4/HIV cells by ultracentrifugation (30 000 rpm on a Beckman L7–55 ultracentrifuge, using a SW40 rotor for 2 h at 4 °C). The virus pellet was mixed with sample buffer, boiled, then electrophoretized on 5–10% gradient gel according to the method of Laemmli [11]. The polypeptides in the gel were blotted on to nitrocellulose sheets. Strips were cut out from the sheets, and incubated with the sera of HIV-1 antibody positive individuals. After thorough washing and incubation with HRP conjugated goat anti-human IgG, the reaction was visualized by addition of diaminobenzidine.

## Results

The results of a comparative study of HIV-1 infection of H9 and MT4 cells are shown in Fig. 1. This type of experiment was repeated several times with similar results. RT activity of supernatants of HIV-1 infected MT4 cells rapidly increase following infection, showing that the cells produced virus in a great quantity. The peak is followed by a rapid decrease of RT activity, indicating that in consequence of the cytopathic effect of the virus, the cells are not able to produce viruses any more.

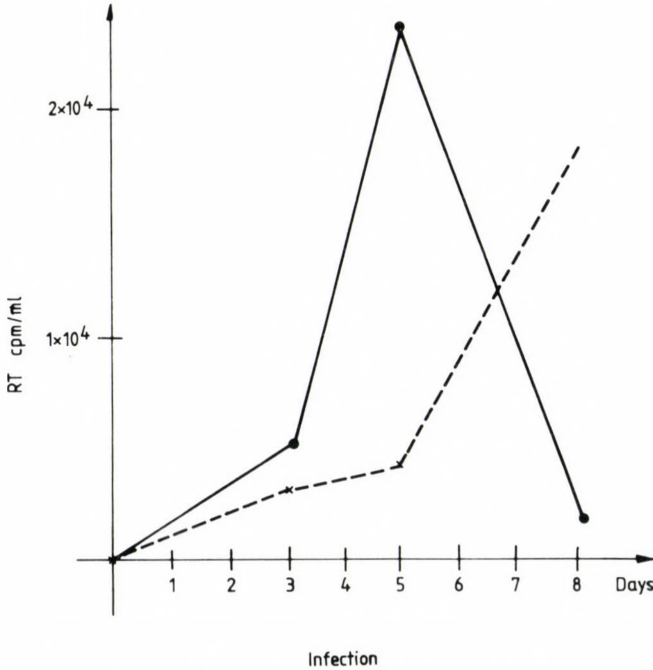



*Fig. 1.* Reverse transcriptase activity of supernatants of MT4 ( ----- ) and H9 cells ( - - - - ), after HIV-1 infection

In correlation with the viral dosis used for infection, MT4 clusters consisted of almost only dead cells by the 10–20th day after infection. Despite of the poor condition of the cultures we continued to feed them by regular changing of the culture medium. In the wells containing MT4 cells infected with high concentrations of the virus, living cells appeared by the 28–30th day of culturing. These surviving cells were subcultured. They are now for more than 10 months in culture.

By light microscopy no difference could be observed in the morphology of control and the surviving HIV-1 infected MT4 cells. Both are growing in large

clusters. Growth characteristics of the control and HIV infected MT4 cells proved to be also similar.

More than 90% of the original MT4 cells were positive for OKT4 surface antigen. This antigen could not be demonstrated on the surface of the surviving HIV-1 infected MT4 cells by FACS analysis (Fig. 2).

Surviving HIV-1 infected MT4 cells proved to be nearly 100% positive in indirect immunofluorescence test using a HIV-1 antibody positive human serum. The mixture of control and infected MT4 cells are excellent indicator cells in immunofluorescence confirmation investigations (Fig. 3).

RT activity of the supernatants of MT4/HIV-1 cell line was measured regularly in order to compare it with that of H9/HTLV-IIIB supernatant. The RT values given in Table I show that the cells are producing the virus continuously.

**Table I**

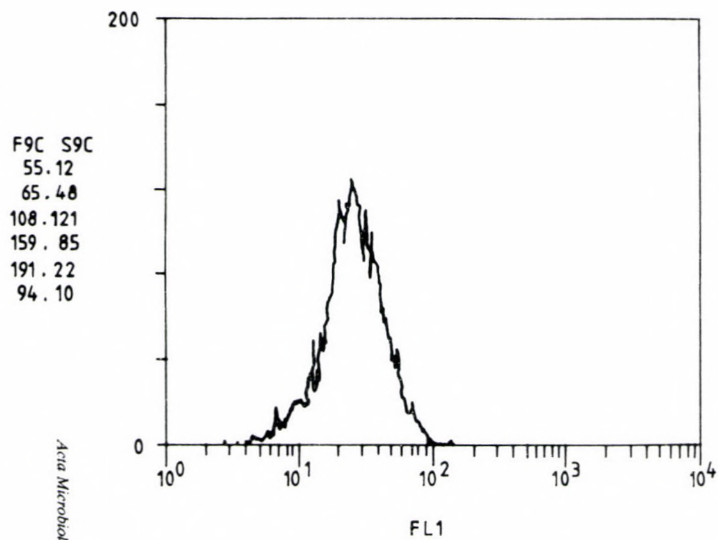
*RT activities (cpm/ml) of virus producer and control cell cultures measured at different intervals*

| Cell line | RT values measured on | | | | |
|---|---|---|---|---|---|
| | 04.01.1991. | 04.02.1991. | 27.03.1991. | 10.04.1991. | 20.05.1991. |
| H9/HTLV-III/B | 11454 | 31971 | ND* | 38917 | 41013 |
| MT4/HIV-1 | 35824 | 26443 | 61457 | 24425 | 29646 |
| MT4 | 468 | 492 | 256 | 287 | 246 |

*Not done

The virus produced by MT4/HIV-1 proved to be infective for both H9 and MT4 cells. The titre of the supernatant of these cells was $10^{3.2}$ $TCID_{50}$/ml on MT4 cells. This is, however, considerably lower than the value ($10^{5.9}$) obtained with virus produced by H9/HTLV-IIIB cells.

By electron microscopic investigation, large number of virions can be seen near the cell surface of MT4/HIV-1 cells (Fig. 4). The virions are morphologically different. There are typical HIV-1 virions and so called doughnut shaped particles present as well.



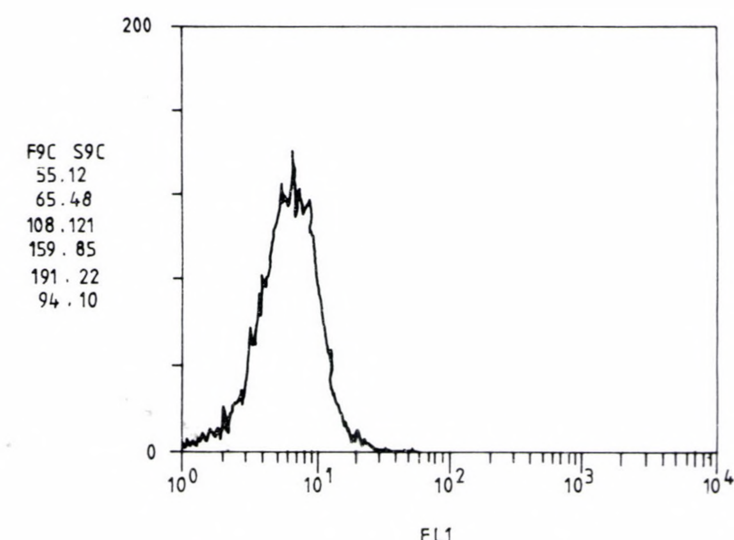




*Fig. 2.* FACS analysis of MT4 and MT4/HIV-1 cells. (A) More than 90% of MT4 cells carry OKT4 antigen on their surface. (B) HIV-1 producer MT4 cells carry no OKT4 antigens

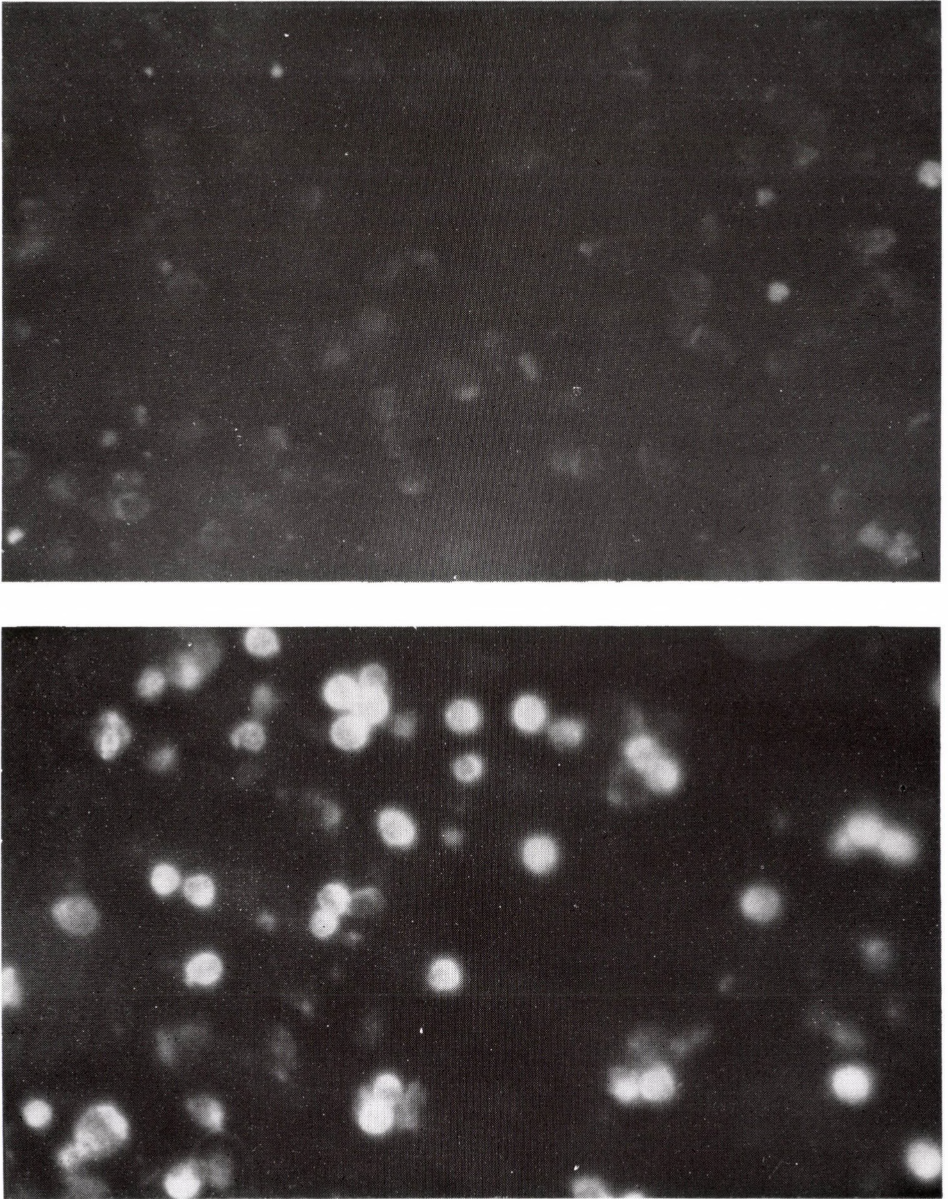


*Fig. 3.* Immunofluorescence assay using MT4/HIV-1 cells. MT4 (A) and MT4/HIV-1 (B) cells were incubated with the serum of a HIV-1 antibody positive individual. No specific fluorescence can be seen on MT4 cells (A), but a marked fluorescence can be seen on the virus producer cells (B)

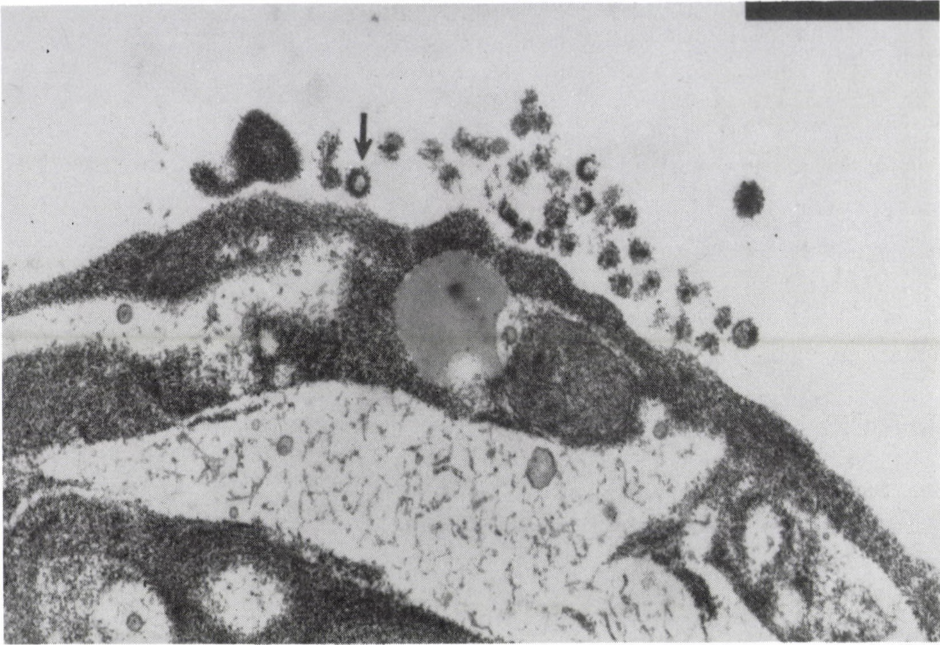


*Fig. 4.* Electron microscopic picture (magnification: 48 000x) of the MT4/HIV-1 cell line. Virus particles, among them doughnut shaped ones (arrow) near to the membrane of the cell

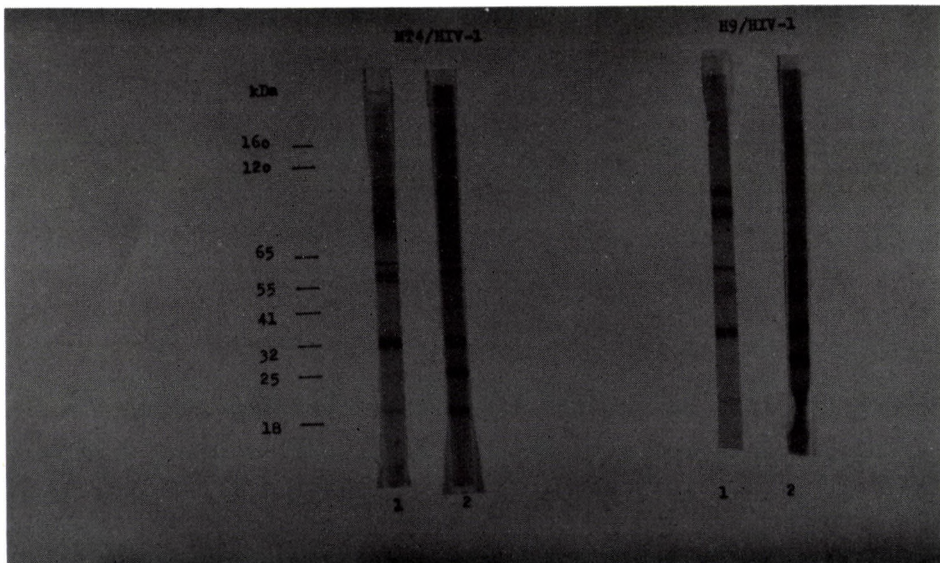



*Fig. 5.* Western blot analysis of virus antigens produced by MT4/HIV-1 and H9/HIV-1 cells. Virus antigens were separated and visualized as described in Materials and methods

Western blot analysis of the virus produced by MT4 cells proves the presence of the virus polypeptides characteristic to HIV-1. Figure 5 shows the typical bands by the use of two different positive human sera.

## Discussion

HTLV-I transformed cell lines are highly susceptible to HIV-1 infection and the affected cells are killed in a short time [6].

The mechanism of the cytotoxic effect of the AIDS virus in HTLV-I carrying cells is in connection with the inhibition of DNA synthesis, and the pronounced cytolysis inhibits subculturing of the infected cells [12].

Subsequently, Ikuta et al. [13] succeeded to get persistently HIV-1 producing MT4 cell lines named M4HB1, M4LB1 and a cloned line named M4HB2. Though the supernatants of these cells proved to be infective to MT4 cells, their $TCID_{50}$/ml value was lower than that of Molt4/HTLV-IIIB cells. The infectivity of the supernatant of the cloned cell line was undetectable. This latter line produced so called doughnut shaped, defective virus particles without normal core structure. Two possible reasons were proposed why a few MT4 cells survive after drastic cytolysis by HIV-1 infection.

(i) A small population of MT4 is different from the major population. This minor population may be defective in enzymes involved in the maturation of HIV-1 env proteins.

(ii) The HIV-1 used for infection contained defective viruses as well. The intact HIV kills MT4 cells, whereas surviving cells produced defective particles. This latter possibility does not answer why infective particles are present in persistently infected uncloned cells.

Goto et al. [14] isolated three more clones from persistently HIV-1 infected MT4 cells producing large numbers of infectious and noninfectious HIV-1 particles.

The virus particles produced by MT4/HIV-1 established in our laboratory are different in morphology. Among them there are typical and doughnut shaped ones as well. Comparing the RT and $TCID_{50}$/ml values of MT4/HIV-1 and H9/HTLV-IIIB supernatants there is some contradiction: while the RT values are similar in the two cell lines, there is a difference in the infectivity of them. The infectivity of the MT4/HIV-1 is much lower. Though the virus particles produced by our infected MT4/HIV-1 cells are different in morphology, this cell line seems to be stable for a long time and proved to be very useful for a confirmatory fluorescence assay. Experiments are going on to separate MT4/HIV-1 cells producing typical and doughnut shaped viruses, respectively.

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# EFFECT OF INHIBITORS OF HELA CELL STRUCTURES AND FUNCTIONS ON *ESHERICHIA COLI* HB101 (PRI203) ENTRY PROCESS

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We investigated the effect of some eucaryotic cytoplasmic structure and function inhibitors on the entry into HeLa cells of the *Escherichia coli* HB101 K12 strain, harbouring the recombinant plasmid pRI203, in which is cloned a 3.2 Kb chromosomal fragment of *Yersinia pseudotuberculosis*. Substances impairing microfilament structures and functions (cytochalasin B and trifluoroperazine) significantly reduced invasion ability whereas microtubule organization inhibitors (colchicine and vinblastine) were ineffective. Data obtained with a lipophilic weak base (methylamine), which raises the pH of intracellular vesicles, demonstrated that, in entry pathway of *E. coli* HB101 (pRI203), endosome acidification is not required. Host cell energy has been shown to contribute to bacterial internalization since the presence of oxidative phosphorylation and glycolysis inhibitors (sodium azide, 2-dinitrophenol and 2-deoxy-*D*-glucose) during the invasion process, affected bacterial entry.

Within the family, *Enterobacteriaceae*, invasive *Yersinia* species "in vivo" pass through the intestinal epithelial layer and replicate in the underlying reticuloendothelial tissues including the lamina propria and lymph follicles [1–4]. These bacteria "in vitro" are able to enter non-phagocytic cultured animal cells and the molecular mechanisms involved require the cooperation between bacteria and host cells [3, 4]. Since epithelial cells are not normally able to take up microorganisms, invasive *Yersinia* spp. must encode factors that facilitate intracellular penetration. Whereas a 70 Kb plasmid is required for virulence but is not essential for entry [5], invasive bacteria appear to have different independent internalization

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pathways, the most efficient being mediated by an outer membrane protein called "invasin" able to recognize mammalian cell receptors and whose determinant is located on chromosome [6]. The invasin-encoding gene *(inv)* has been cloned from *Yersinia pseudotuberculosis* in the plasmid pRI203 which allows normally non-invasive *Escherichia coli* K12 to invade cultured epithelial monolayers [7]. It has been hypothesized that invasin binding to its receptors must transmit an intracellular signal responsible for initiating the internalization of bacteria; consequently, bacterial entry into the host cells takes place through an endocytic process or more likely by a mechanism of induced phagocytosis [4, 8].

Whereas enteric yersiniae have been shown still invasive in the presence of bacterial RNA and protein synthesis inhibitors [6], the role of host cell physiological conditions and structures in the initiation of the yersinia entry has not yet been well defined. Eucaryotic cells appear to participate to bacterial entry by providing for invasin, receptors of the integrin superfamily, belonging to cell adhesion transmembrane glycoproteins [8, 9–11]. Integrins are heterodimeric proteins acting as receptors for different molecules at the extracellular side of the membrane while their cytoplasmic domains are coupled with cytoskeletal activity [12, 13]. Moreover, membrane glycoproteins and glycolipids containing N-acetyl glucosamine and N-acetyl neuraminic acid have been also demonstrated to act as receptorial binding sites for attachment and internalization of invasin-expressing bacteria [14].

Although the nature of host receptors and their binding affinity play a significant role in the early phases of internalization, it is necessary to further clarify the metabolism involvement of non professional phagocytes in the steps of the entry process subsequent to bacterial adhesion. For this purpose, several inhibitors of eucaryotic cytoplasmic structures and functions were tested for their potential effect on the internalization in HeLa cells of the *E. coli* HB101 (pRI203) strain [7].

## Materials and methods

*Organisms and media. E. coli* HB101 (pRI203) was kindly provided by Dr. Falkow (Stanford Medical School, California 94305, USA); pRI203 plasmid carries a chromosomal DNA fragment of *Y. pseudotuberculosis* that converts the *E. coli* strain HB101 into a microorganism capable of invading cultured animal cells [7].

*Host cells.* HeLa $S_3$ cells (epithelioid carcinoma of human cervix) were grown as monolayers at 37 °C on 24-well tissue culture clusters (Costar) by loading $1.5 \times 10^5$ cells/ml in Eagle's minimal essential medium (MEM, Gibco Laboratories) plus 1.2 g/l $NaHCO_3$, 2 m*M* glutamine, 100 U/ml penicillin, 0.2 mg/ml streptomycin and 10% heat inactivated foetal calf serum in 5% $CO_2$.

*Invasion assays.* Invasiveness was assayed in HeLa cells as described by Conte et al. [15]. Briefly, HeLa cell monolayers ($3 \times 10^5$ cells) were infected with bacteria ($3 \times 10^7$ c.f.u.) grown on Trypticase Soy Broth (TSB-BBL) for 1 h at 37 °C. After this incubation period, the monolayers were washed and fresh medium containing 50 μg/ml gentamicin was added to eliminate extracellular bacteria. After 2 h incubation at 37 °C, HeLa cells were treated with trypsin-EDTA (mixture of 0.05% trypsin (1:250) and

0.02% EDTA) for 5 min at 37 °C, lysed with 0.5% natriumdeoxycholate for 5 min at 37 °C, then diluted in PBS, plated on TSA and incubated overnight at 37 °C. The total number of viable internalized bacteria was determined by colony forming unit (c.f.u.) counts.

*Chemicals*. Stock solutions of colchicine, trifluoroperazine, vinblastine, cytochalasin B, methylamine dinitrophenol, sodium azide and 2-deoxy-D-glucose, purchased from Sigma Chemical Co., were made up in phosphate-buffered saline (PBS) pH 7.4 (with the exception of cytochalasin B which was preliminary dissolved in dimethyl sulphoxide) as follows: 5 m*M* colchicine, 5 m*M* trifluoroperazine, 1 m*M* vinblastine, 0.5 mM cytochalasin B, 100 mM methylamine, 10 m*M* dinitrophenol, 40 m*M* sodium azide and 2.5 M 2-deoxy-*D*-glucose.

*Inhibitor assays on bacterial entry*. Different trials were carried out: the cells were preincubated for 25 min at 37 °C with the inhibitors before bacterial infection and/or the cells were incubated with the inhibitors during the infection step for 1 h at 37 °C; in other experiments the drugs were added to monolayers for 1 h at 37 °C after the infection.

Controls were performed to exclude a direct bactericidal effect of cellular inhibitors used. For this purpose bacteria were incubated with inhibitors for 1 h at 37 °C. At the concentrations used in the invasion assays none of the above mentioned drugs caused significant modifications of the number of bacteria as compared with drug-free controls. Preliminary investigations were also carried out to assess the influence of each drug on HeLa cell morphology, viability (monitored by neutral red uptake) and growth (measured by counting the cell number). The highest non-cytotoxic and non-bactericidal concentration of each drug which did not affect any parameter considered was 2 μ*M* colchicine, 5 μ*M* vinblastine, 10 μ*M* cytochalasin B, 10 μ*M* trifluoroperazine, 5 m*M* methylamine, 10 m*M* sodium azide, 2 m*M* dinitrophenol and 50 m*M* 2-deoxy-*D*-glucose.

## Results

The effect on *E. coli* HB101 (pRI203) invasion ability of drugs acting on cytoskeleton structures (colchicine, vinblastine, cytochalasin B), on calmodulin (trifluoroperazine), on low pH compartments (methylamine), on oxidative phosphorylation (sodium azide) and on glycolysis (2-deoxy-*D*-glucose) was studied in the experimental HeLa cell model.

To determine the effect of the drugs on bacterial invasion HeLa cells ($3 \times 10^5$) were exposed to non-cytotoxic and non-bactericidal concentrations of each chemical for 25 min at 37 °C prior the infection and during the entire period of contact (1 h at 37 °C) with bacterial inoculum ($3 \times 10^7$ c.f.u.). Then drugs were removed and gentamicin containing medium was added in order to eliminate non-internalized bacteria. Results are reported in Tables I and II. Substances impairing microfilament structures and functions (cytochalasin B, trifluoroperazine) significantly reduced the number of bacteria recovered from the cell lysate by comparison with chemical-free control counts (by up to 2.2 $\log_{10}$ c.f.u./ml and 1.9 $\log_{10}$ c.f.u./ml of 4.8 $\log_{10}$ c.f.u./ml) whereas microtubule organization disrupting agents (colchicine and vinblastine) were found to be ineffective (Table I). The addition of methylamine which raises the pH of intracellular low pH compartments did not produce a significant reduction of bacterial invasion (Table II). On the contrary, inhibitors of

oxidative phosphorylation (sodium azide and dinitrophenol) and of glycolysis (2-deoxy-*D*-glucose) were found to cause a strong dose-dependent inhibition of bacterial entry (by up to 3.4, 2.8 and 1.5 $\log_{10}$ c.f.u./ml, respectively of 4.8 $\log_{10}$ c.f.u./ml) (Table II).

**Table I**

*Effect of inhibitors of cytoskeleton structures and functions on HeLa cell invasion by E. coli HB101 (pRI203)*

| Chemical | Concentration (μM) | Recovery of bacteria from HeLa cell lysate ($\log_{10}$ c.f.u./ml*) |
|---|---|---|
| None | – | 4.8 |
| Colchicine | 2 | 4.6 |
| | 0.4 | 4.9 |
| Vinblastine | 5 | 4.6 |
| | 1 | 4.7 |
| Cytochalasin B | 10 | 2.2 |
| | 5 | 3.6 |
| | 1 | 4.1 |
| Trifluoroperazine | 10 | 1.9 |
| | 2 | 4.2 |

Non-bactericidal and non-cytotoxic concentrations of the different compounds were added to HeLa cells before (25 min, 37 °C) and during the infection period with bacteria (1 h, 37 °C)

* Mean standard deviation < ± 0.20

Successively, in order to investigate the *E. coli* HB101 (pRI203) invasion step affected by the inhibitors, the action of each effective drug was checked by incubating the compounds with HeLa cells (a) only prior the infection (for 25 min at 37 °C); (b) during the infection period with bacteria (for 1 h at 37 °C); or (c) after the removal of bacterial inoculum (for 1 h at 37 °C). Results reported in Table III clearly demonstrated that metabolic inhibitors did not induce relevant modifications of the number of bacteria recovered from the cell lysate when added prior the infection (a) or after the penetration step (c). Only dinitrophenol was also active if preincubated with HeLa cells before the addition of bacteria. All the inhibitors were able to reduce the invasion efficiency when added during the infection period (b).

**Table II**

*Effect of HeLa cell inhibitors acting on low pH compartments and metabolism on invasion by E. coli HB101 (pRI203)*

| Chemical | Concentration (mM) | Recovery of bacteria from HeLa cell lysate ($\log_{10}$ c.f.u./ml*) |
|---|---|---|
| None | – | 4.8 |
| Methylamine | 5 | 4.4 |
| | 1 | 4.8 |
| Sodium azide | 10 | 3.4 |
| | 2 | 4.4 |
| Dinitrophenol | 2 | 2.8 |
| | 0.4 | 4.5 |
| 2-Deoxyglucose | 50 | 1.5 |
| | 20 | 2.9 |
| | 4 | 4.5 |

Non-bactericidal and non-cytotoxic concentrations of the different compounds were added to HeLa cells before (25 min, 37 °C) and during the infection period with bacteria (1 h, 37 °C).

* Mean standard deviation < ± 0.20

**Table III**

*Effect of cytochalasin B (10μM), trifluoroperazine (10 μM), sodium azide (10 mM), dinitrophenol (2 mM) and 2-deoxy-D-glucose (50 mM) on E. coli HB101 (pRI203) entry process in HeLa cells*

| Chemical | Recovery of bacteria from HeLa cell lysate ($\log_{10}$ c.f.u./ml*) | | |
|---|---|---|---|
| | (a) | (b) | (c) |
| None | 4.9 | 4.9 | 4.9 |
| Cytochalasin B | 4.7 | 2.5 | 4.8 |
| Trifluoroperazine | 4.0 | 3.6 | 4.5 |
| Sodium azide | 4.7 | 4.1 | 4.8 |
| Dinitrophenol | 2.9 | 2.8 | 4.8 |
| 2-Deoxy-D-glucose | 3.9 | 2.2 | 4.7 |

(a) Inhibitors were added to HeLa cells before infection (25 min, 37 °C); (b) inhibitors were added to HeLa cells during the infection step (60 min, 37 °C); (c) inhibitors were added to HeLa cells after infection step (60 min, 37 °C)

* Mean standard deviation < ± 0.20

## Discussion

The early interactions of invasive bacterial species with epithelial cells are complex. In the experimental model represented by *E. coli* HB101 (pRI203) carrying the *Y. pseudotuberculosis* chromosomal *inv* gene [6, 7, 9] and HeLa cells, host cells have been shown to contribute to bacterial adherence and invasion by providing specific glycoprotein receptors [9, 14]. Besides to the invasin expression, the efficiency of bacterial entry is influenced by environmental conditions such as $O_2$ tension, temperature [13] presence of nutrilites (unpublished results) and of electric charged macromolecules [15]. Further information has been obtained by the present research for the comprehension of the invasion process by studying the participation of the non professional phagocyte metabolism in this phenomenon.

For this purpose different HeLa cell functions were separately evaluated. One of these is represented by cell motility; since all motility associated events need the participation of cytoskeleton structures and calmodulin, their role in bacterial penetration of HeLa cells was investigated by treating the cells with colchicine, vinblastine, cytochalasin B and trifluoroperazine.

These compounds respectively disrupt microtubule free units [16], induce reorganization of microtubule structures in living cells [17], inhibit microfilament formation [18] and interact with microfilament contractile apparatus by acting on calmodulin [19]. Results obtained clearly demonstrated that microfilament but not microtubule inhibitors prevent bacterial invasion. Similar data were obtained for *Yersinia enterocolitica* [3], *Salmonella cholerae-suis* [3] and *Shigella flexneri* [20] internalization process.

The requirement of intracellular low pH vesicles in the internalization process has been studied with a weak base, methylamine. This lysosomotropic agent, enters vesicles readily and upon protonation causes marked vacuolar swelling, accumulates in intracellular compartments, preventing endosome acidification [21]. Results obtained after treatment with methylamine support that endosome acidification is not required for bacterial invasion. This finding is in agreement with previous reports which demonstrated that *Y. enterocolitica* [3] and *S. flexneri* [22] invasion process was unaffected by the addition of different lysosomotropic agents.

As to the participation of the host cell metabolism, results here obtained with both oxidative phosphorylation (dinitrophenol and sodium azide) and glycolysis (2-deoxy-*D*-glucose) inhibitors suggest that *E. coli* HB101 (pRI203) penetration is a cell energy and cell metabolism dependent process. Different behaviour has been reported for *Salmonella typhi-murium* which invasion was only slightly reduced in the presence of dinitrophenol and iodioacetic acid whereas *S. flexneri* invasion was reduced by 99% [23].

The active participation of HeLa cells is required for *E. coli* HB101 (pRI203) entry, differently from that observed for bacterial cells, since invasive yersiniae in "in vitro" experiments have been shown to adhere to epithelial cells on ice or to invade in the presence of bacterial RNA and protein synthesis inhibitors [6] in contrast to invasive salmonellae which must be viable and must synthetize RNA and proteins [23].

Taken together the data presented here provide evidence for the role of cytoskeleton components, sensitive to cytochalasin B and calmodulin action, and of host cell efficient metabolism in the invasion process of HeLa cells by *E. coli* HB101 (pRI203). In conclusion, bacteria may function as ligands by binding to receptors of non-professional phagocyte surface but invasion process is achieved by host cell mechanisms subsequent to bacterial adhesion.

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# CHLOROQUINE INHIBITS THE INSULIN BINDING AND THE IMPRINTING OF NUCLEAR ENVELOPE IN *TETRAHYMENA*

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Chloroquine possessing lysosomotrop effects inhibits the development of insulin imprinting both on the plasma membrane and the nuclear envelope. Simultaneously chloroquine can inhibit the insulin binding in the nucleus itself but not in the plasma membrane. There is a dose dependent increase of binding of labelled insulin to the nuclear envelope. The control related binding of nuclei pretreated with insulin or chloroquine is similar and have a direct ratio, independently of the applied concentrations.

The unicellular *Tetrahymena* contains hormones characteristic to the higher levels of evolution [1–3], and has the capacity to bind these hormones with receptors. The hormonal imprinting is formed at the first encounter with the hormone. As the result of hormonal imprinting the receptor-like structures which were present previously turn into stronger and more specific; the binding capacity and responsiveness are changed [4–6]. The *Tetrahymena* will transfer these alterations to progeny generations and the effect of the first encounter with the hormone is present after hundreds of generations, too [7]. A protozoon lives in a constantly altering environment. The recognitive character of the plasma membrane has a main importance in the aspects of survival and producing offspring generations. The presence of receptor-like structures is obvious while the plasma membrane has the contact to the environment. But previous experiments showed that the nuclear envelope of *Tetrahymena* contains receptors [11, 12] like the nuclear envelope of mammalian cells [8–10]. They are able to bind hormone and following the first encounter with the hormone the imprinting takes place.

The polypeptide hormones following the interaction with the receptor in the plasma membrane are internalized to the cell. In the cell the hormones are separated from the receptors and their degradation occurs. The products of degradation could play a role in development of imprinting in the case of plasma membrane and nuclear envelope as well. In our previous experiments [13], investigating the

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lysosomotrop bacitracin [14] an inhibitor of degradation of insulin, we found that it inhibits the development of imprinting in the plasma membrane. In our present experiments we are going to test the effects of chloroquine a molecule possessing lysosomotrop and acidotrop character; how can this molecule influence the insulin binding and the insulin imprinting of the plasma membrane and the nuclear envelope.

## Materials and methods

*Cultures of Tetrahymena. Tetrahymena pyriformis* GL cells cultured in 0.1% yeast extract containing 1% Bacto tryptone medium (Difco, Michigan, USA) were used.

*Treatments.* Treatments were carried out on freshly transferred 24 h cultures of cells in tubes of 10 ml volume. (i) Insulin Semilente MC (Novo, Copenhage, Denmark), 10−6 *M*. (ii) Chloroquine (Sigma, USA) 2.5 n*M*. (iii) Insulin + Chloroquine 10−6 *M* and 2 n*M*. (iv) The control group had no treatment at all. The time of treatment was 1 h, then the cells were transmitted into 150 ml flasks containing fresh medium. After 24 h the nuclei were isolated.

*Isolation of nucleus.* Cells were centrifuged, washed in 10 m*M* NaCl, then homogenized in the homogenization buffer (0.04 w/v Na-deoxycholate, 0.04 v/v NP 40, 6 n*M* $CaCl_2$, 5n*M* NaCl, 1.5 m*M* $Na_2HPO_4$, 250 μg/ml spermidine tetrahydrochloride, pH = 7.6) [15]. In this environment the disintegration of cells was 90% without damage of nucleus. The cells and the buffer containing mixture had a 10 min rest then they were centrifuged, suspended again and treated in the manual Potter homogenizator five times. These were followed by centrifugation at 3000 g and washing (6 m*M* $CaCl_2$ 0.28 *M* raffinose). After this the nuclei were set on "sucrose pillow" (2.14 mol/l sucrose) by VAC 602 ultracentrifuge at 15 000 g for 30 min. The nuclei were suspended in the washing buffer (0.25 *M* sucrose, 10 m*M* MgCl, 10 m*M* Na-acetate pH = 7.6). All the steps of isolation were carried out at 0 °C.

*Binding-assay.* The isolated nuclei were incubated with 0.025, 0.05, 0.1, 0.2 mg/ml FITC insulin (FITC: protein ratio 0.42, concentration of protein 0.2 mg/ml) for 1 h. Then the nuclei were washed with PBS and were dried onto surface of slides.

The intensity of fluorescence was measured by a Zeiss Fluoval cytofluorimeter. A Hewlett-Packard HP41 CX calculator connected to the cytofluorimeter supplied the numeric data simultaneously serving the values of significance of inter group variation. In each group three replica assays were performed. Intensity of fluorescence was measured in 20 nuclei in each group, this way the individual points of the figures were representing the average values of 60 nuclei.

*Experiments on the plasma membrane.* Cell cultured and treated in the same way described above were incubated with 0.2 mg/ml FITC-insulin (ratios see above) for 1 h. Then they were washed with PBS and dried onto slides. In the four replica assays of experiment the measurement was similar to the above mentioned.

## Results and discussion

The increase of FITC-insulin binding of nuclei of control cells was concentration dependent. The result was similar in the case of insulin pretreated (imprinted) cells, but here at the lowest FITC-insulin concentration the nuclei have already a higher insulin binding than the control nuclei. This advantage increased by

the increasing of the concentration of labelled insulin. The labelled insulin binding in the nuclei of chloroquine treated cells also increased in a concentration dependent way as well as in the insulin + chloroquine pretreated group. The chloroquine itself could inhibit the insulin binding related to the control; the insulin binding of the chloroquine + insulin pretreated group remains on the control level (Fig. 1). If we relate our data to the prevailing control level (100%; Fig. 2) it is clear that the binding value is around the peak even in the lowest labelled insulin concentration and the second lowest concentration (0.05 mg/ml) reaches the maximal level which never changes any more. This means that the increase of the labelled insulin concentration results an increased insulin binding on the nuclear envelope (in controls, too). This binding value is stationary and characteristic to the controls and pretreated cells alike.

The above findings show clearly that the pretreatment with insulin (imprinting) can increase the hormon binding of the offspring generations (after 24 h about 7–8 new generations) by the specification of receptors, but the insulin treatment together with chloroquine does not allow the formation of this increase of binding. Itself the chloroquine strongly inhibits the insulin binding (24 h later) and it is presumable that the previous effect and the provoked increase by insulin together can produce a level which is identical to the control value. We can not determine whether chloroquine could inhibit the binding of insulin to the imprinted – higher binding capacity – receptors or only there is an addition of the negative chloroquine and the positive insulin effects.

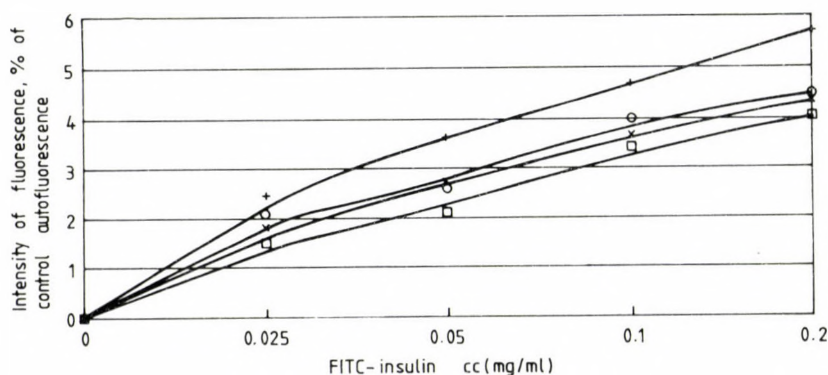


*Fig. 1.* Dose-dependent binding of FITC-insulin in nuclei of control and pretreated *Tetrahymena*; o control; × insulin + chloroquine; + insulin; □ chloroquine

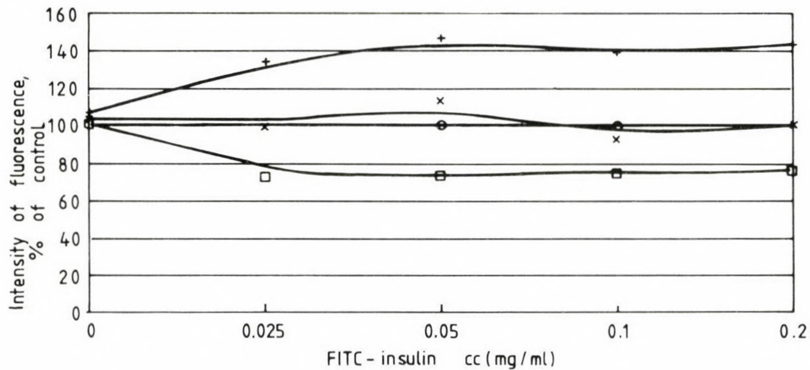


*Fig. 2.* Dose-dependent binding of FITC-insulin to nucleus; the prevailing control value is 100%. The insulin or chloroquine pretreated groups related to the control and chloroquine + insulin groups differ significantly in each concentrations applied ($p < 0.01$). o control; × insulin + chloroquine; + insulin; □ chloroquine

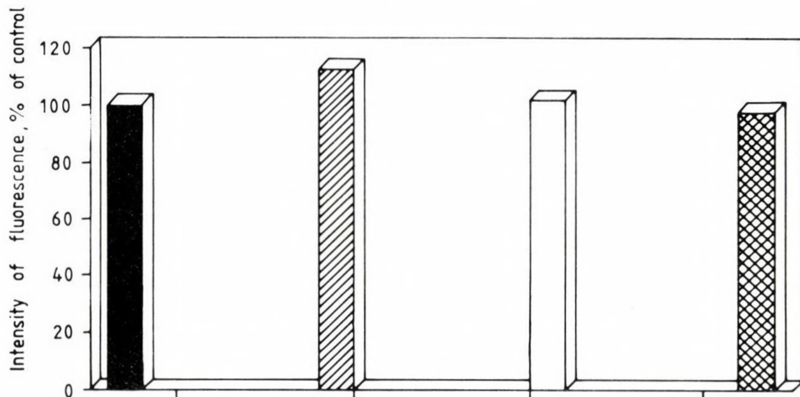


*Fig. 3.* Binding of FITC-insulin (0.2 mg/ml) to plasma membrane of *Tetrahymena*. The insulin pretreated group differs significantly ($p < 0.01$). Solid column, control; shaded column, insulin; open column, chloroquine; hatched column, chloroquine + insulin

In the case of plasma membrane alone the chloroquine could not inhibit the binding of insulin (Fig. 3). The significantly increased binding of labelled insulin evoked by insulin-imprinting is not detectable when chloroquine is also present during the imprinting. This makes possible – especially in the case of plasma membrane – that there is not an addition of positive and negative influences (here is no sign of the negative effect) but chloroquine can inhibit the development of imprinting. According to these results it is presumable that a similar mechanism works in the case of nuclear envelope.

Chloroquine as a lysosomotrop substance influencing the acidity of cell compartments [16], inhibits the degradation of proteins [17]. In the case of insulin the amount of intact hormone doubled while the activity of degradation falls to the half [18, 19]. It seems that chloroquine can drive the hormone to a retroendocytotic route from the processes of degradation [19]. This way there is no possibility of dissociation of internalized receptor and hormone complex [20–22] and the internalized receptor and the hormone are accumulated intracellularly or move toward the surface of the cell. Meanwhile chloroquine does not inhibit the enzyme degradating insulin [17], and fragments of insulin – that may have a role in the process of imprinting according to our experiments – are not formed.

The above-mentioned facts could explain the reason for the lack of imprinting in the plasma membrane and nuclear envelope, both membranes having a similar character in this aspect. There is no evidence for the inhibition of insulin binding on the nuclear envelope by chloroquine, when the plasma membrane exerts no such effect following the same treatment. It is presumable that only the intracellular chloroquine can induce these effects on the nuclear envelope directly, while the excess of chloroquine was washed out from the environment of the cell.

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# EVALUATION OF INDIRECT ENZYME LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF COXSACKIEVIRUSES IN CLINICAL SAMPLES AND ITS COMPARISON WITH DOT-IMMUNOBINDING ASSAY

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Indirect enzyme linked immunosorbent assay (ELISA) was applied for the direct detection of coxsackieviruses in clinical samples viz. rectal swabs (RS) and throat swabs (TS) collected from patients admitted to various nursing homes and local hospitals. Results indicate the presence of different CVA types in 65 (62.5%) out of 104 RS, and 18 (52.9%) out of 34 TS samples. Dot-immunobinding assay was also standardized for the identification of CVA types employing 52 RS samples and the results compared with indirect ELISA. Dot-immunobinding detected more CVA types in a relatively larger number of specimens than indirect ELISA.

Coxsackieviruses group A (CVA) and B (CVB) have been recognized as potential aetiological agents causing a wide spectrum of clinical syndromes, thus assuming immense clinical importance. Aseptic meningitis is one of the major clinical affliction among children caused by multiple CVA and CVB types (sometimes in conjunction with Echo viruses). Outbreaks and sporadic cases incriminating either a single or mixed CVA and CVB types, have been frequently reported, e.g. respiratory tract infections and diarrhoea, specially among children [1, 2], acute haemorrhagic conjunctivitis involving CVA-24 [3], cardiovascular diseases [4], diabetes mellitus [5], pericarditis and myocarditis among adults [6–8].

Occurrence of antigenic variants in CVB and extensive cross reactivity among CVA groups render serological diagnosis difficult [9]. Consequently, in order to establish infections attributable to coxsackieviruses, it is pertinent to isolate the virus and identify them by a sensitive, specific and simple detection system. Among the conventional methods for the identification of enteroviruses including coxsackieviruses, the versatility of neutralization test (Nt) remains a specific, confirmatory test system [10, 11]. However, the inherent disadvantages and

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intricacies associated with conventional techniques, e.g. complement fixation test (CFT), haemagglutination inhibition (HAI) and fluorescent antibody test (FAT) in addition to Nt, have minimized their application [12–14].

With the introduction of ELISA systems this technique is being increasingly exploited for the identification of CVA [15] and CVB [16] types by applying sandwich ELISA. However, indirect-ELISA has also been employed successfully for the detection of certain enteroviruses and foot and mouth disease (FMD) viruses [17, 18]. Besides, nitrocellulose based dot-immunobinding assay (dot-ELISA) has also been applied, though to a limited extent for the detection of enteroviruses [19] including polioviruses [20].

This paper describes the efficacy of indirect-ELISA for the detection of coxsackieviruses in clinical specimens and compares its compatibility with dot-immunobinding assay standardized for coxsackieviruses group A.

## Materials and methods

*Clinical samples.* One hundred and four rectal swab (RS) and 34 throat swab (TS) samples were collected from patients of different age groups, manifesting diarrhoeal and respiratory distress, admitted in various nursing homes and local hospital of the city. Samples were collected in Hanks' balanced salt solution and brought to laboratory on ice.

*Antisera.* Lim Benyesh Melnick (LBM) enteroviral horse antisera pools, O, P, J, L, A and C (National Institute of Health, USA) and monospecific equine antisera (American Type Culture Collection, USA) were used for the detection.

*Preparation of clinical samples.* After initial centrifugation at 2000 rpm, clinical samples (TS and RS) were treated with 1000 units/ml of penicillin, 1000 μg/ml of streptomycin and 200 mg/ml of nystatin. These were allowed to interact for about an hour at room temperature and then subjected to sterility test. The samples were then centrifuged at 1500 rpm for 20 min and the supernatants collected was further centrifuged at 5000 rpm for 20 min and later checked for sterility. Supernatants thus collected were stored at −20 °C until used.

*ELISA system.* Indirect ELISA system applied in the present work was based broadly on the method described by us earlier [21, 22]. In short, polystyrene plates (Dynatech, USA) were coated with RS and TS samples, prepared above using carbonate, bicarbonate buffer (pH 9.6) and incubated at 37 °C for an hour followed by overnight incubation at 4 °C. Plates were then washed thrice with phosphate buffer saline containing 0.05% Tween 20 (PBST-pH 7.4), which was eventually followed after each major step. Uncoated sites were blocked with 1% bovine serum albumin (Sigma, USA) and incubated for an hour at 37 °C. In case of RS samples monospecific sera to CVA types 18, 20, 21, 22 and 24 generally encountered in diarrhoea [9] were applied (2 Ab units/100 μl) in the next step. However, in TS samples LBM pools containing antisera to CVA types, 2 to 6, 8, 10, 21 and CVB4 and 5, normally existing in throat infection [9] were initially detected. Having detected appropriate LBM pools, corresponding monospecific sera were then added (2Ab units/100 μl) in a second set of experiment. Since both LBM and monospecific sera were raised in equine, in the subsequent step, rabbit anti-equine sera (1:3000) raised in the laboratory was used. Alkaline phosphatase conjugated goat anti rabbit immunoglobulin G (Sigma, USA) was added in 1:1000 dilution followed by the addition of p-nitrophenylphosphate, substrate (Sigma) diluted (1 mg/ml) in diethanolamine buffer (pH 9.8). Yellow reaction product

developed on incubation at room temperature for nearly 30 min. The reaction was stopped with 20 μl/well of 3 N NaOH. Optical densities were measured at 410 nm absorbance by Mini-ELISA Reader (Dynatech, USA).

*Dot-ELISA*. The test was standardized for the identification of CVA types in 52 RS samples after modification of the method described by Bode et al. [23]. Briefly, nitrocellulose paper (NCP) was cut in small sizes with a view to accomodate 4 to 6 samples having plastic support on one side. RS samples (5 μl/dot) were immobilized on NCP with tris-HCl buffer (pH 7.4) and incubated for an hour at 37 °C. However, PBST also served well the purpose of coating the samples onto NCP. Thereafter NCP was washed thrice by shaking in PBST (pH 7.4) solution. This washing step was followed after each step. NCP was then dipped in 3% BSA diluted in PBST with 0.05% Tween-20 for blocking the untreated sites and incubated for an hour. Monospecific monkey CVA antisera (CMC, India) were diluted to contain 4 Ab units/100 μl and NCP put in the antibody solution. It was then placed in a solution containing horse radish peroxidase conjugated goat antihuman IgG (1:800) using PBST as diluent and incubated at 37 °C for 45 min. Diaminobenzidine (Sigma) diluted 1 mg/ml, used as an insoluble substrate, was diluted in phosphate citrate buffer (pH 5.0) plus 0.015% hydrogen peroxide. After 5 to 10 min discernible brown spot is perceptible on washing with plain water, which is indicative of positive samples.

## Results

Indirect ELISA was applied to identify the incidence of CVA types in clinical samples viz. 104 RS and 34 TS samples as represented in Table I. Sixty five (62.5%) out of 104 RS samples were detected positive for CVA types which existed either as alone viral constituent, 26 (40%) or in a combination of two CVA types, 25 (38.4% or as a mixture of three types, 14/21.5%).

Table II shows the incidence of CVA and CVB types in 34 throat swab samples, of which 18 (52.9%) were detected as positive. CVA 5 predominated other CVA types being present in 8 TS samples existing in combination with other types, on the contrary CVB 5 and A 10 were present as a lone viral entity in 3 and 1 TS samples, respectively, without any association with other types.

Fifty two RS samples were subjected to dot-ELISA system and the results obtained were compared to indirect ELISA as shown in Table III – a to d. Out of 52 RS samples 35 (67.3%) were detected as positive for the presence of CVA types by dot-ELISA. Comparison with indirect ELISA system indicates that both systems identified 27 of 35 (77.1%) dot-ELISA positive RS samples (Table III – a to c). Similar CVA types were identified by both methods in 12 (44.4%) of 27 specimens are presented in Table III – a. Table III – b depicts the identification of partially similar types identified in 13 (48.1%) of 27 samples by dot and indirect ELISA. Amongst these one CVA types was similarly detected in 12 of 13 samples by both the procedures viz. A18 (2 samples), A20 (4), A21 (2) A22 (1), A24 (3). Only in one sample two types (A18, A21) were identically detected.

**Table I**

*Detection of different types of coxsackieviruses in rectal swab (RS) samples by indirect ELISA system*

| CVA types detected | 18 | 20 | 21 | 22 | 24 |
|---|---|---|---|---|---|
| Single viral type | 7 | 7 | 5 | 2 | 5 |
| Dual combinations | CVA 20(7)<br>CVA 22(5)<br>CVA 24(3)<br>CVA 21(4) | CVA 21(4) | CVA 22(2) | CVA 18(5)*<br>CVA 21(2)* | CVA 18(3)* |
| Triple combinations | CVA 20+21(4)<br>CVA 20+24(2)<br>CVA 22+24(4) | CVA 22+24(2) | CVA 20+24(1)<br>CVA 22+24(1) | CVA 18+24(4)*<br>CVA 20+24(2)*<br>CVA 21+24(1)* | CVA 18+20(2)*<br>CVA 18+22(4)* |

Total number of samples, 65. CVA types in combination with those shown in line number 1 have been put in parentheses. Combinations marked with asterisks under CVA 22 and CVA 24 columns are repetitions

**Table II**

*Detection of coxsackieviruses group A and B in throat swab (TS) samples*

| Virus types | Frequency of occurrence of CVA and CVB types in TS samples | | |
|---|---|---|---|
| | As lone viral constituent | Combination of two types | Mixture of three types |
| CVB 4 | 1 | – | – |
| CVB 5 | 3 | – | – |
| CVA 10 | 1 | – | – |
| CVA 5 | – | 4: A2(1), A10(1)<br>A7(2) | 4: A2, A4(2)<br>A7, A10(1)<br>A2, B4(1) |
| CVA 2 | – | 2: A6(1), A8(1) | 1: A3, A8(1) |
| CVA 3 | – | 1: A8(1) | 1: A2, A10(1) |

Number of samples showing the presence of different CVA types, in association with types indicated in column 1, have been put in parentheses

**Table III**

*Coxsackieviruses A types identified by indirect (I) and dot (D) ELISA*

| Specimen | A-18 | | A-20 | | A-21 | | A-22 | | A-24 | | No. of types | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nos | I | D | I | D | I | D | I | D | I | D | I | D |
| a 11 | + | + | + | + | – | – | – | – | – | – | 2 | 2 |
| 16 | + | + | + | + | – | – | – | – | – | – | 2 | 2 |
| 19 | + | + | – | – | – | – | – | – | – | – | 1 | 1 |
| 36 | – | – | + | + | – | – | – | – | – | – | 1 | 1 |
| 68 | + | + | – | – | + | + | – | – | – | – | 2 | 2 |
| 69 | + | + | + | + | + | + | + | + | – | – | 4 | 4 |
| 73 | + | + | + | + | + | + | + | + | – | – | 4 | 4 |
| 77 | – | – | – | – | + | + | – | – | – | – | 1 | 1 |
| 83 | – | – | – | – | – | – | – | – | + | + | 1 | 1 |
| 87 | – | – | – | – | + | + | + | + | + | + | 3 | 3 |
| 90 | – | – | + | + | – | – | – | – | – | – | 1 | 1 |
| 95 | + | + | – | – | – | – | – | – | – | – | 1 | 1 |
| b 14 | + | + | – | + | + | + | – | + | – | – | 2 | 4 |
| 17 | + | + | – | – | – | + | – | + | – | – | 1 | 3 |
| 21 | – | + | – | – | + | – | + | – | + | + | 3 | 2 |
| 24 | + | + | – | – | + | – | + | – | – | – | 3 | 1 |
| 28 | + | – | – | – | + | + | + | – | – | + | 3 | 2 |
| 30 | + | – | – | – | – | – | + | + | – | – | 2 | 1 |
| 56 | + | – | + | + | – | + | – | + | – | – | 2 | 3 |
| 65 | + | – | + | + | – | – | – | – | – | + | 2 | 2 |
| 71 | – | – | – | – | – | + | – | – | + | + | 1 | 2 |
| 74 | – | – | + | + | – | – | – | + | – | + | 1 | 3 |
| 75 | – | + | – | – | – | + | – | + | + | + | 1 | 4 |
| 78 | – | + | – | + | + | + | – | – | – | + | 1 | 4 |
| 94 | – | + | + | + | – | – | + | – | + | – | 3 | 2 |
| c 34 | – | – | + | – | – | – | – | + | – | – | 1 | 1 |
| 58 | – | – | + | – | – | + | – | + | – | – | 1 | 2 |
| d 15 | – | – | – | – | – | + | – | – | – | – | 0 | 1 |
| 28 | – | – | – | – | – | – | – | + | – | + | 0 | 2 |
| 45 | – | – | – | – | – | + | – | – | – | + | 0 | 2 |
| 53 | – | – | – | + | – | – | – | – | – | – | 0 | 1 |
| 57 | – | – | – | + | – | – | – | – | – | + | 0 | 2 |
| 76 | – | – | – | – | – | + | – | – | – | – | 0 | 1 |
| 80 | – | + | – | – | – | + | – | – | – | – | 0 | 2 |
| 96 | – | + | – | – | – | – | – | – | – | – | 0 | 1 |

I = Indirect ELISA; D = Dot ELISA
a = Similar types of CVA identified by both ELISA systems b = Partially similar CVA types
c = Different CVA types d = CVA types identified by dot – but not by indirect – ELISA

Table III – c indicates that only in 2 (7.4%) of 27 samples, different types were identified by the two ELISA systems. Table III – d presents result on 8 specimens found positive by dot but negative by indirect ELISA.

## Discussion

The application of conventional serologic techniques, e.g. Nt, HAI, CFT and FAT for the detection of enteroviral types, have proved either intricate or suffer from unquestionable limitations [9, 24]. Though Nt, still stands as an undisputed and perfect detection tool, nevertheless the use of intersecting enteroviral pools and the maintenance of cells or laboratory animals render this test as time consuming and relatively cumbersome. With the introduction of solid phase immunoassay, which is an extremely versatile, sensitive and specific system the inherent problems of conventional systems have been alleviated. Yolken and Torsch [15], Katze and Crowell [16] have applied sandwich ELISA for the identification of CVA and CVB types, respectively. Indirect ELISA was also exploited to identify foot and mouth disease (FMD) viruses even at a low concentration [17, 18] and coxsackieviruses group A from mouse torso antigens [21]. It was elucidated by Hermann et al. [25] on the basis of experimental evidence that ELISA performed with adsorbed antigen or adsorbed antibody was approximately equally sensitive and specific for the identification of certain types of enteroviruses. In a previous study, with a view to compare the potential of indirect ELISA vis-à-vis sandwich ELISA, for the identification of ECHO viruses in sewage samples, we found both ELISA systems relatively comparable, with latter having a slight edge over indirect ELISA [22]. It has, however, been indicated that removal of extraneous cellular antigen is imperative for the success of indirect ELISA based enteroviral identification [25]. An additional advantage in indirect ELISA is the use of only one specific antibody instead of two specific antibodies raised in two different animals, consequently reducing the time for the test. Since the indirect ELISA is now gaining grounds, we screened 104 RS and 34 TS samples employing this simple test system. Results reveal that 26 (40%) contain a single viral constituent of CVA types closely followed by the combination of two viruses in 25 (38.4%) out of 65 positive RS samples. Preponderance of A18 was evident being present in 37 samples existing either alone or coexisting with other CVA types, more discernibly with A20. Incidence of CVA22 was generally encountered in conjunction with other types than as a lone constituent.

In this study an attempt has been made to detect coxsackieviruses directly from the processed clinical specimens. The isolation of coxsackieviruses is indeed a complex process requiring human and monkey kidney cell cultures in addition to day-old mice. By directly applying the partially purified antigens this complicity could be overcome. Yolken and Torsch [15] have reported the direct detection of CVA antigens in 8 of 11 stool samples without manifesting any cross reactivity with other related viruses. This apart, low concentration of FMD viruses [18] and respiratory syncytial virus [26] were successfully detected directly from the clinical samples.



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Dot-ELISA has also been exploited, though to a limited extent, for the detection of enteroviruses [19, 27]. In the present study we standardized dot-ELISA for the detection of CVA types and found 35 (67.3%) out of 52 RS samples as positive for CVA types. Results explicitly show that CVA types could be detected in 27 (51.92%) of 52 RS samples by both the tests. While 25 out of 27 RS samples positive by both the systems detected similar [12] or partially similar types [13], only two samples detected different CVA types. However, eight samples negative by indirect ELISA turned positive by dot-ELISA. It implies therefore that while compatibility exists between the two ELISA systems tested, dot-ELISA detects more CVA types and in a relatively higher number of samples. It has been elucidated that NC-based enzyme immuno assay (EIA) has an excellent capacity to immobilize virus antigen and that the sensitivity is enhanced 10- to 100-fold higher than the conventional EIA [19]. These workers have also reported the detection of the minimum concentration of 0.08 ng of CVB 5 when viral antigen was suspended in PBS as coating buffer. This observation substantiates the detection of relatively more number of CVA types by dot-ELISA as compared to indirect ELISA. However, indirect ELISA provided OD values of the viruses whereas observations are only subjective in dot-ELISA. Nevertheless the relative ease and rapidity with which it can be performed makes dot-ELISA an acceptable detection system.

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# LATEX AGGLUTINATION AND ADENOVIRUSES I. DETECTION OF POLYCLONAL ANTIBODIES BY LATEX AGGLUTINATION TEST

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A latex agglutination (LA) test was developed for the detection and measurement of polyclonal anti-adenovirus antibodies. Latex particles, coated with purified hexon, penton or fibre antigens displayed specific agglutination with 21 rabbit immune sera directed against the different antigens and the complete virus of 10 human and 2 animal adenovirus types. The sensitivity of LA was compared to that of passive haemagglutination, complement fixation (CF) and immunodiffusion reactions. In experiments with immune sera as well as with human serum samples the sensitivity of LA test proved to be in the same range as that of CF test. Because of its easy and rapid performance (slide agglutination within 4 min) LA method may substitute for CF reaction in serological adenovirus diagnosis.

Latex agglutination (LA) is a very useful, simple and rapid method, known since more than 30 years [1] and used in several different fields of immunology and microbiology [2]. With adenoviruses (AV), however, the data are very scarce, dealing mostly with commercial preparations for enteric AV-es [3–8]. Only Sanekata et al. [9] reported on successful experiments for the detection of AV h41. Data concerning antibody detection, purified AV antigens or monoclonal antibodies were not reported up till now. Therefore we intended to investigate these possibilities for the application of LA method in the AV research. This paper reports on experiments for the detection of polyclonal anti-AV antibodies with a LA test.

## Materials and methods

*Viruses, antigens.* AV types h1, h2, h3, h5, h6, h7, h8, h9, h10 and h12 were propagated in HEp-2 cells, h35 in "293" [10] cultures. Purified hexon, penton and fibre antigens were prepared by repeated anion exchange, hydrophobic interaction chromatography and gelfiltration, as reported [11–13].

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*Sera.* Immune sera directed against the different human AV-es and purified antigens were produced in rabbits, as described earlier [12]. Serum to bovine AV bos2 was kindly supplied by Gy. Berencsi. Serum to canine AV can1 was a commercial preparation. Selected human sera, having high antibody level to AV h1 hexon in passive haemagglutination test were studied in the experiments, too.

*Complement fixation (CF) tests* were performed in microtiter plates, as in previous studies [12].

*Passive haemagglutination (HA)* was carried out with tannic acid treated sheep erythrocytes sensitized with different purified AV antigens, as already described [14, 15].

*Immunodiffusion (ID)* with the different purified AV antigens was performed in 1% agar, as reported previously [12].

*Protein* concentration of the purified antigens was determined with the method of Lowry et al. [16].

*Latex agglutination (LA) test.* Latex suspensions were kindly supplied by K. Fanta and L. Bánkuti (Human Institute for Serobacteriological Production and Research, Budapest). Of 5 different preparations the best results were obtained with a sulphate latex, particle diameter 137 nm. A 1% suspension of latex in PBS was mixed with equal volume of purified AV antigen, containing 0.5 mg/ml protein in the same diluent. The mixture was shaken for 2 h at 37 °C, then centrifuged at 11 000 rpm for 2 min and the sedimented latex resuspended as a 1% suspension in PBS, containing 5 mg/ml bovine serum albumin (BSA). After another 2 h at 37 °C, centrifugation was repeated as before and the sediment suspended in PBS, containing 0.5 mg/ml BSA, as a 0.25% suspension. The coated latex was stored at 4 °C and its reactivity remained stable for at least 5 years. Test was performed on glass slides, mixing equal volumes of diluted serum and latex suspension. The slides were constantly moved to and fro and agglutination appeared within 4 min. PBS and normal rabbit serum were used as controls, as well as a BSA-coated latex, to exclude aspecific agglutination.

## Results

*Specificity of latex agglutination test.* The suspensions of latex particles coated with different purified AV antigens, i.e. hexons of ten human AV types (1, 2, 3, 5, 7, 8, 9, 10, 12 and 35), two types of penton (h1 and h5) and a fibre (h2) were tested for their specificity with polyclonal rabbit antisera directed against ten human (1, 2, 3, 5, 6, 7, 8, 9, 10 and 12) and two animal (bos2 and can1) viruses, as well as with six anti-hexon (human types 1, 5, 7, 8, 9, and 10), two anti-penton (h1 and h5) and three anti-fibre (h1, h5 and h8) sera. The results are summarized in Table I. The sera used in LA were tested with latex particles coated with BSA, too. Figure 1 shows, that a type 1 anti-hexon serum reacts with hexon-coated latex, but does not agglutinate the BSA-control particles. The hexon-coated latex did not show aggregate in normal rabbit serum or in PBS.

**Table I**

*Reactivity of rabbit immune sera in LA test to different adenovirus antigens*

| Rabbit immune serum directed against type | Antigen on latex particles: Hexon of types h1, 2, 3, 5, 7, 8, 12, 35 | Penton of types h1 | Penton of types h5 | Fibre of type h2 |
|---|---|---|---|---|
| h1 virus | + | + | – | – |
| h2 virus | + | – | – | + |
| h5 virus | + | – | + | – |
| h3, 6, 7, 8, 9, 10, 12 virus | + | – | – | – |
| bos 2 virus | + | – | – | – |
| can 1 virus | + | – | – | – |
| h1 hexon | + | – | – | – |
| penton | – | + | + | – |
| fibre | – | + | – | – |
| h5 hexon | + | – | – | – |
| penton | – | – | + | – |
| fibre | – | – | + | – |
| h7 hexon | + | – | – | – |
| h8 hexon | + | – | – | – |
| fibre | – | – | – | – |
| h9 hexon | + | – | – | – |
| h10 hexon | + | – | – | – |

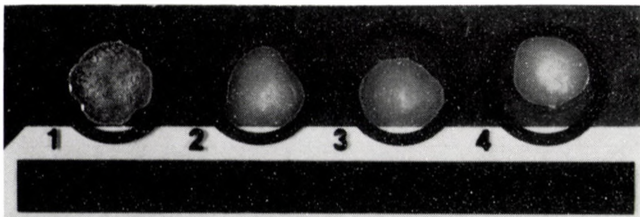


*Fig. 1.* Latex agglutination test. 1 – AV h1 hexon-coated latex particles in 1:20 h1 anti-hexon rabbit immune serum; 2 – AV h1 hexon-coated latex particles in PBS; 3 – AV h1 hexon-coated latex particles in 1:10 normal rabbit serum; 4 – BSA-coated latex particles in 1:20 h1 anti-hexon rabbit immune serum

*Sensitivity of latex agglutination test.* The LA titres of 18 human serum samples were tested with eight hexon (1, 2, 3, 5, 7, 8, 12 and 35), two penton (1 and 5) and a fibre (type 2) antigen. The reaction was performed in serial twofold dilutions with a 1:10 initial dilution, the highest titres were 1:320. The distribution of the titres are shown in Table II.

Ten of these sera were also tested with other serological methods, i.e. passive HA, ID and CF tests. The CF titres were between < 1:4 to 1:32. The results with

three different hexon types (h1, h3 and h35) obtained in the other three reactions are compared in Table III.

The comparison was made with 10 rabbit immune sera, too. All these sera precipitated the hexon of AV h1 in ID test. The titres obtained with the three other methods are summarized in Table IV.

**Table II**

*Distribution of LA titres of 18 human sera reacting with different adenovirus antigens*

| Number of sera having LA titres | Coating antigen on latex (types according to subgenera) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | | B | | | | | C | | | D |
| | 12 | 3 | 7 | 35 | 1 | 2 | 5 | 1 | 5 | 2 | 8 |
| | hexon | | hexon | | | hexon | | penton | | fibre | hexon |
| 1:80 to 1:320 | 3 | 7 | 3 | 7 | 9 | 3 | 8 | – | 1 | – | 2 |
| 1:10 to 1:40 | 13 | 9 | 11 | 8 | 9 | 12 | 10 | 8 | 13 | – | 12 |
| <1:10 | 2 | 2 | 4 | 3 | – | 3 | – | 10 | 4 | 18 | 4 |
| Mean titre 1: | 34 | 51 | 32 | 61 | 84 | 30 | 64 | 6 | 13 | | 31 |

**Table III**

*Comparison of reactivity of 10 human sera by passive haemagglutination (HA), latex agglutination (LA) and immunodiffusion (ID) methods*

| Serum | Reaction with hexon antigens of adenovirus type | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | h1 | | | h3 | | | h35 | | |
| | HA* | LA* | ID | HA | LA | ID | HA | LA | ID |
| 1 | 128 000 | 40 | + | 128 000 | 40 | + | 32 000 | 40 | + |
| 2 | 128 000 | 160 | + | 4 000 | 80 | + | 4 000 | 80 | – |
| 3 | 64 000 | 40 | + | 2 000 | 80 | – | 4 000 | 80 | + |
| 4 | 128 000 | 20 | – | 2 000 | 40 | – | 4 000 | 80 | + |
| 5 | 128 000 | 80 | + | 4 000 | 80 | – | 64 000 | 160 | + |
| 6 | 128 000 | 80 | + | 64 000 | 80 | + | 16 000 | 80 | + |
| 7 | 256 000 | 80 | + | 500 | 20 | + | 16 000 | 80 | + |
| 8 | 512 000 | 160 | – | < 250 | < 5 | – | 500 | 20 | – |
| 9 | 128 000 | 40 | + | 4 000 | 80 | – | 4 000 | 20 | + |
| 10 | 128 000 | 80 | – | 8 000 | 20 | + | 8 000 | 20 | + |

* Reciprocal titre

**Table IV**

*Antibody titres[1] of 10 rabbit immune sera with AV h1 hexon, tested by passive haemagglutination (HA), latex agglutination (LA) and complement fixation (CF) tests*

| Rabbit immune serum to type | HA | LA | CF |
|---|---|---|---|
| h3 virus | 1 600 | 80 | 16 |
| h6 virus | 1 600 | 80 | 64 |
| h8 virus | 1 600 | 80 | 32 |
| bos2 virus | 6 400 | 40 | n.t.[2] |
| can1 virus | 32 000 | 80 | n.t. |
| h1 hexon | 640 000 | 800 | 600 |
| h5 hexon | 16 000 | 400 | 200 |
| h8 hexon | 32 000 | 200 | 200 |
| h9 hexon | 16 000 | 200 | 200 |
| h10 hexon | 8 000 | 200 | 400 |

[1] Reciprocals
[2] Not tested

## Discussion

According to results presented in Table I and Fig. 1 the LA test with polyclonal sera is unequivocally specific with the different AV antigens. Using fibre or penton antigens for coating, type-specific or subgenus-specific antibodies can be detected. Using a hexon antigen for coating the latex particles, however, a genus-specific reagent can be obtained, which is reactive with antibodies to all, or almost all [17] mammalian AV-es. In this case the value of LA test is identical with that of CF test. As the coating procedure, as well as the test itself is very easy and rapid to perform, and the coated particles remain stable for at least 5 years, while the test can be made and evaluated in 4 min, in the possession of a single purified hexon preparation of the 47 human AV types [18], LA test may substitute for CF test in diagnostic work.

Comparing the susceptibility of LA test with other methods, the highest titres were obtained with HA (Tables III and IV). Because of the exceedingly high values, HA is very useful in research work [12–14, 19, 20], but not suitable for diagnostic purposes. LA titres of human sera (Table III) were somewhat, but not significantly higher than CF titres, while with immune sera (Table IV) the values were in the same range. Sensitivity of ID (Table III) remains unequivocally behind LA, especially considering that the test is performed with undiluted sera.

It can be concluded, that for the detection of anti-AV antibodies the LA test has similar value, but more favourable accomplishment as the CF test.

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# ANTIGENIC RELATIONSHIPS AMONG THE MEMBERS OF ADENOVIRUS SUBGENERA DETERMINED BY MONOCLONAL ANTIBODIES

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Epitope composition of human adenovirus (AV) serotype 13 (subgenus A), 19, 26 and 27 (subgenus D), as well as 41 (subgenus F) was studied with 23 selected monoclonal antibodies (MAbs) raised against different hexon types. With the help of three panels of MAbs great differences were shown in the epitope structure of subgenus D hexons. Comparing two AV13 strain hexons (a prototype strain and an intermediate strain), it was shown that the epitopes recognized by the MAbs were present in different combinations even on strains of the same serotype. Therefore the epitope composition of the subgenus D hexons seems to be heterogeneous.

Since the viruses and viral protein components are antigenically complex, the use of multireacting antisera limits the appropriate antigenic analysis to considerations of the total antigenicity of a complex viral protein and precludes the characterization of individual epitopes of a protein. The widely used monoclonal antibodies (MAbs) are powerful tools for antigenic analysis [1–4], therefore a map of all epitopes on the adenovirus (AV) hexon might finally be achieved with the MAbs.

During the years passed since the AV-es have been discovered, a great number of information is accumulated on the molecular structure and composition of the major capsid component, known as hexon. The capsomeric form of the complete hexon is a trimer [5]. A variety of antigenic determinants has been found on the hexon molecules of which the determinant common to mastadenoviruses is called genus specific antigen [6]. All the human and non-human mammalian AV-es belong to Mastadenovirus genus [7, 8]. The presence of type, subgenus and intersubgenus specific determinants has been demonstrated as well [9–11].

Our experiments proved the existence of intertype specific epitopes, too [12–15]. The specificity of these epitopes did not coincide with the current

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classification of AV-es into subgenera [8, 15]. It was thought that, with the study of additional purified hexon types, and by comparison of the reactivity patterns (RPs) of the newly characterized hexons with those of investigated earlier, further and more detailed informations may be obtained.

In this paper, we report on the antigenic analysis of 5 human AV hexons belonging to subgenera A, D and F, as well as on the differences of epitope structure of two AV13 hexons.

## Materials and methods

*Virus strains. Purification of hexon antigen.* The enteric AV serotype 41 was kindly supplied by J. E. Herrmann (Worcester, Mass, USA). It was propagated on AV5 DNA transformed human cell line "293" [16]. AV18 (subgenus A) was originally isolated from a patient [17], and than cultivated on HEp-2 cells. AV types 19, 26 and 27 (subgenus D) were prototype strains. For cultivation of these serotypes HEp-2 cells were used. The prototype strain of AV13 from H. G. Pereira was propagated on 293 cells [18].

For the purification of all hexon types, DEAE Sephadex A-50 (Pharmacia, Sweden) anion exchange chromatography was used [19]. After the repeated chromatographies, the protein concentration of the preparations was determined by Lowry's method [20].

*Monoclonal antibodies.* MAbs used in the present experiments originated from three different panels of MAbs. Eleven MAbs directed against crystallized AV1 hexon [12] and representing different RPs [14], 7 MAbs from the series of MAbs directed against AV35 hexon [21] with 4 different RPs, as well as 5 MAbs specific for bovine adenovirus (BAV) type 2 hexon showing 3 separated RPs [13] were used to study the antigenic structure of the hexons.

*Indirect ELISA experiments.* All ELISA experiments were carried out on 96-well polystyrene plates (Falcon Microtest II. T. C.). The purified hexon preparations were used in 40 $\mu$g/ml concentration for coating, and the antibodies of the ascitic fluids were studied in serial two-fold dilutions. To detect the MAbs bound to the epitopes of hexons anti-mouse immunoglobulins (goat) labelled with horse-radish peroxidase (HRPO) were used. Labelling was carried out in our laboratory by the method of Nakane and Kawaoi [22]. The enzyme substrate was o-phenylene diamine and the colour change was assessed at 492 nm in a Medicor-Medilab EPD-1 photometer. Not only the types of reactivity, but the titres of the specific antibodies were determined and expressed as:
$\log_2$(reciprocal dilution of the ascitic fluid $\times 10^{-2}$).

## Results

*Comparison of the epitopes of AV18 and AV12 (subgenus A).* With the help of the 23 selected MAbs, at least 5 differences could be detected between the epitopes of the hexons of AV12 and 18. Two of these epitopes recognized by the MAbs 2A6

**Table I**

*Indirect ELISA titres of 23 selected MAbs with hexons of AV12 and AV18 (subgenus A)*

| Specificity of the epitopes[1] | MAbs directed against AV1 | AV35 | BAV2 | Hexon types of subgenus A 12 | 18 |
|---|---|---|---|---|---|
| Genus specific epitope | 1A3 | | | 5[a] | 8 |
| | | 35H15 | | 12 | 10 |
| | | | IV.F3 | 13 | 12 |
| Intertype specific variations/overlapping epitopes[2] | H12 | | | 9 | 9 |
| | | 35H26 | | 9 | 8 |
| | 2A6 | | | 0.3 | 8 |
| | 2A1 | | | –[b] | 8 |
| | 2D6 | | | 0.3 | 10 |
| | 1A5 | | | 0.3 | – |
| | 2C1 | | | 0.3 | – |
| | 2B5 | | | – | – |
| | 2C6 | | | – | – |
| | 1B2 | | | – | – |
| | | 35H49 | | 8 | 8 |
| | | 35H43 | | 11 | 10 |
| | | | B11 | – | 6 |
| | | | CA12 | – | 13 |
| | | | A12 | – | 8 |
| | | 35H18 | | – | – |
| | | 35H62 | | – | – |
| Type specific epitopes | 5D2 | | | – | – |
| | | 35H51 | | – | – |
| | | | IV.F5 | – | – |

[1] Determination of the epitope specificity based on the evaluation of RPs with all the hexon types studied

[2] Intertype specific/overlapping epitopes were studied with competitive binding ELISA (24)

[a] Titres were expressed as $\log_2$ (reciprocal dilution $\times$ $10^{-2}$)

[b] No positive reaction in 1:100 dilution

**Table II**

*Indirect ELISA titres of 23 selected MAbs with hexons of subgenus D*

| Specificity of the epitopes[1] | MAbs directed against | | | Hexon types of subgenus D | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | AV1 | AV35 | BAV2 | 8 | 9 | 10 | 13* | 13** | 19 | 26 | 27 |
| Genus specific epitope | 1A3 | | | 11[a] | 10 | 11 | 11 | 11 | 11 | 12 | 12 |
| | | 35H15 | | 12 | 12 | 12 | 11 | 7 | 11 | 7 | 7 |
| | | | IV.F3 | 12 | 10 | 10 | 10 | 8 | 10 | 8 | 8 |
| Intertype specific variations/overlapping epitopes[2] | H12 | | | 11 | 12 | 11 | 11 | 10 | 7 | 7 | 5 |
| | | 35H26 | | 11 | 11 | 11 | 11 | 11 | 10 | 5 | 7 |
| | 2A6 | | | 11 | 11 | 10 | 8 | –[b] | 7 | 7 | – |
| | 2A1 | | | 9 | 9 | 8 | 8 | – | 7 | 9 | – |
| | 2D6 | | | 13 | 13 | 12 | 13 | – | 10 | 11 | – |
| | 1A5 | | | 11 | 11 | 9 | 7 | – | 10 | – | – |
| | 2C1 | | | 9 | 9 | 8 | 8 | – | 7 | – | – |
| | 2B5 | | | 11 | 12 | 9 | 8 | – | 7 | – | – |
| | 2C6 | | | 10 | 9 | 9 | 8 | – | 7 | – | – |
| | 1B2 | | | 11 | 11 | 10 | 7 | – | 7 | – | – |
| | | 35H49 | | 9 | 9 | 8 | 8 | 5 | 11 | – | – |
| | | 35H43 | | 11 | 10 | 10 | 10 | – | 10 | – | – |
| | | | B11 | 3 | 2 | 2 | 2 | 3 | 2 | 3 | 5 |
| | | | CA12 | – | – | – | – | 5 | 12 | 7 | 7 |
| | | | A12 | – | – | – | – | 6 | 8 | 7 | 7 |
| | | 35H18 | | – | – | – | – | – | – | – | – |
| | | 35H62 | | – | – | – | – | – | – | – | – |
| Type specific epitopes | 5D2 | | | – | – | – | – | – | – | – | – |
| | | 35H51 | | – | – | – | – | – | – | – | – |

1, 2 Explanation is shown in Table I

a Titres are expressed as $\log_2$ (reciprocal dilution $\times 10^{-2}$)

b No positive reaction in 1:100 dilution

* Intermediate strain

** Prototype strain

**Table III**

*Reactivity pattern of 23 selected MAbs with hexon of human and animal adenoviruses*

| Designation of the MAbs | Human adenoviruses according to subgenera | | | | | | | | | | | | | | | | | Animal adenoviruses | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | | B | | C | | | | D | | | | | | | E | F | Bovine | | | Ovine | Simian |
| | 12 | 18 | 7 | 35 | 1 | 2 | 5 | 6 | 8 | 9 | 10 | 13* | 19 | 26 | 27 | 4 | 41 | 1 | 2 | 3 | 3 | 16 |
| 1A3, 35H15, IV.F3 | +[a] | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| H12, 35H26 | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | -[b] | - | - | - | + |
| 2A6 | (+)[c] | + | + | (+) | + | + | + | + | + | + | + | - | + | + | + | + | + | nt | - | nt | nt | + |
| 2A1 | - | + | + | + | + | + | + | + | + | + | + | - | + | + | - | + | + | nt | - | nt | nt | + |
| 2D6 | (+) | + | + | - | + | + | + | + | + | + | + | - | + | + | - | + | + | nt | - | nt | nt | + |
| 1A5, 2C1 | (+) | - | + | - | + | + | + | + | + | + | + | - | + | - | - | + | - | nt | - | nt | nt | - |
| 2B5, 2C6, 1B2 | - | - | + | - | + | + | + | + | + | + | + | - | + | - | - | + | - | nt | - | nt | nt | - |
| 35H49 | + | + | + | + | - | - | - | - | + | + | + | + | + | - | - | + | + | nt | + | nt | nt | + |
| 35H43 | + | + | + | + | - | - | - | - | + | + | + | - | + | - | - | + | + | nt | - | nt | nt | + |
| B11 | - | + | - | - | + | + | + | + | + | + | + | + | + | + | + | + | + | - | + | nt | + | nt |
| CA12, A12 | - | + | - | - | + | + | + | + | - | - | - | + | + | + | + | + | + | - | + | nt | - | nt |
| 35H18, 35H62 | - | - | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - | nt | nt | nt | nt | - |
| 5D2 | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | nt | nt | nt | nt | - |
| 35H51 | - | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | nt | nt | nt | nt | - |
| IV.F5 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + | - | - | - |

a Titres expressed as $\log_2$ (reciprocal dilution X $10^{-2}$) are higher than 1 = 1:200

b Titres are lower than 0.3 = 1:100

c Titres are 0.3 = 1:100

nt = not tested

* Prototype strain

and 2D6 on the AV18 hexon have to be very similar to the epitopes of the other hexon types (Tables I and III) [12], but different from the epitope(s) present on AV12, because of the great difference between the titres. Four other MAbs (2A1, B11, CA12 and A12) reacted only with AV18 and there was no positive reaction with AV12. Comparing the combinations of positive reactions with all the hexon types involved in the experiments, these four MAbs recognize at least three different epitopes (Table III). Two MAbs (1A5 and 2C1) showed only minimal difference in reactions with AV12 and 18, and five MAbs (2B5, 2C6, 1B2, 35H18, 35H62) were negative with both hexon types of subgenus A. Three of these MAbs (2B5, 2C6, 1B2) were positive with a series of other hexons, while MAbs 35H18 and 35H62 recognize probably a subgenus specific epitope of subgenus B hexons [14] (Table III). MAbs reactive with the type specific epitopes of AV1, AV35 and BAV2 hexons (5D2, 35H51, IV.F5, respectively) gave no positive reaction in the experiments with subgenus A hexons.

*Differences in the epitope structure of subgenus D hexons.* Epitope composition of hexons of seven different AV serotypes, as well as two of two different AV13 strains (subgenus D) were investigated and compared in indirect ELISA experiments (Tables II and III). Two of the epitopes recognized by 5 different MAbs were present on all hexon types studied from subgenus D. One of them recognized by the MAbs 1A3, 35H15 and IV.F3 is probably the genus specific epitope of the hexon, the other one (positive with the MAbs H12 and 35H26) could be a common epitope of human and simian AV hexons (Table III). Based on the combinations of positive results with other MAbs (2A6, 2A1, 2D6, 1A5, 2C1, 2B5, 2C6, 1B2 and 35H43) the epitope structure of AV8, 9, 10, 19, as well as of the intermediate strain of AV13 seems to be much more similar to each other than to the epitope composition of the other hexon types of subgenus D (Table II). Three of these MAbs (2A6, 2A1 and 2D6) reacted with AV26, too, but gave no positive reaction with AV27 hexon. From the subgenus D all the hexon types, including the different AV13 strains reacted with the MAb B11, and MAb 35H49 recognized a common epitope of AV types 8, 9, 10, 19 and of both strains of AV13. Another epitope was present on the prototype strain of AV13, as well as on types 19, 26 and 27 of subgenus D (MAbs CA12 and A12). These latter epitopes, however, are present on other hexon types of subgenera A, C, E, F as well as on those of animal origin. The combinations of RPs are shown in Table III.

Comparison of the epitope structure of an intermediate strain and of a prototype strain of AV13 hexon have shown differences in the epitope map.

Completing these experiments with the study of AV41 of subgenus F (Table III), as well as with the results of the previous experiments, the number of the RPs (13 different types of cross-reactions) did not change, but the epitopes recognized by the MAbs were present in different combinations on the newly studied hexon types

(AV18, 19, 26, 27, 41 and the prototype strain of AV13) showing especially great variation on the hexons of subgenus D.

## Discussion

For the studies of the epitope composition of different hexon types, MAbs directed against three different hexons were investigated. Additional serotypes of subgenera A, D and F were used in indirect ELISA experiments to study their RPs and to compare with our previous experiments [13, 15, 21, 23, 24]. As Table III shows, the newly involved hexon types reacted with the MAbs raised against the genus specific epitope of the AV hexon (MAbs 1A3, 35H15, and IV.F3), and with various combinations of the other MAbs. Polyclonal AV antisera are known to vary in their numbers and patterns of cross-reactions [25–27] and the antigenic cross-reactions are much more frequent among serotypes of the same subgenus, but the intermediate strains show great serological diversity [28, 29].

The only subgenus, the members of which reacted uniformly, is the subgenus C. The epitope composition of AV12 and 18 (subgenus A), AV7 and 35 of subgenus B [14] as well as of the members of subgenus D show different variations (Tables I, II and III). In the subgenus D, however, there is close genetic relationship of all AV-es [28, 30] suggesting a close antigenic relationship, too. With horse antisera [31], major antigenic relationship was found among given hexon types, as types 8-9-10 of subgenus D. In our experiments, the results with the members of subgenus D show that the antigenic structure of the hexon types 8-9-10 is much more similar to each other, and to hexon types of subgenus C, and E, but significant differences could be found with other hexon types of subgenus D. The antigenic variation of the intermediate strain of AV13 was shown, too (Table II). The intermediate AV13 strain was proved to be identical with AV9 by restriction enzyme analysis and serum neutralization, while in haemagglutination inhibition test its behaviour resembles to type 13 [18]. Based on the results with MAbs, the hexon epitope structure of the intermediate strain seems to be similar to that of AV9 hexon. In the present study, we compared the results of indirect ELISA experiments, therefore the differences found in types of cross-reactions cannot be explained with the different assay systems [12, 14, 21, 32]. Because most of the epitopes recognized are actually discontinuous [33], the combination of positive and negative reactions could be resulted by these very small areas of the surface of the hexon molecules.

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# DIAZALD, A NEWLY RECOGNIZED ANTIMICROBIAL AGENT AND ITS SPECTROPHOTOMETRIC DETERMINATION

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Diazald, a chemical intermediate for the synthesis of biologically active compounds, was found to be a potent in vitro antimicrobial agent against yeasts, yeast-like and filamentous fungi as well as Gram-positive and Gram-negative bacterial strains. Its activity is not inhibited by either para-aminobenzoic acid (PABA) or the nitroso group-specific 2-aminothiazole-methoxyimino acetic acid (ATMAA). This suggests that the molecule as such is responsible for the antimicrobial activity. For its quick measurement a sensitive spectrophotometric method has been developed.

Diazald (N-methyl-N-nitroso-p-toluenesulfonamide (Fig. 1) is a known intermediate or precursor for the preparation of diazomethane (diazirane, azimethylene), a versatile and widely used reagent for, among others, the methylation of a large number of biologically active chemicals and also for epoxide formation, as well as ring expansion [1–5]. The study of its antimicrobial activity was initiated on two considerations.

Recently we developed a colour reaction for the determination of the antitumour nitrosourea drug products and drug substances [6]. Since some of these drugs, especially streptozotocin [7] is known to have antibacterial activity, we screened a series of non-urea nitroso compounds for antimicrobial activity. Among them diazald was found to possess inhibitory activity against *Bacillus subtilis* ATCC 6633 used as a screening strain.

Furthermore, since diazald has a modified sulfonamide structure, we followed up this activity by testing it against a number of bacterial and fungal strains. In fact, diazald turned out to be in vitro a broad-spectrum antimicrobial agent.

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In addition, like the antitumour nitrosoureas [6], diazald produces a cherry red colour reaction with ceftizoxime and also with its side chain, ATMAA. Consequently we undertook the study of the quantitative aspect of this colour reaction.

## Materials and methods

*Diazald* was purchased from Aldrich, p-aminobenzoic acid (PABA) and dimethylsulfoxide (DMSO) both from Sigma Chemical Company. The sample of 2-aminothiazole-*syn*-methoxyimino acetic acid (ATMAA) was a donation of Lonza.

*Bacterial and fungal strains.* Those listed in Tables I and II, are either standard (ATCC) or often used laboratory test strains, or recent clinical isolates.

*Agar media.* For bacteria, trypticase-soy agar or occasionally the semisynthetic peptone-glucose agar [8] and, for yeasts and fungi, the Sabouraud-glucose agar media were used to perform the disc susceptibility tests.

*Diazald disc preparation.* Diazald was dissolved in DMSO, then diluted with sterile deionized water. An aliquot of 20 μl was applied to sterile, antibiotic Sensi-discs of 6.35 mm diameter (BBL) to yield final disc concentrations of 200, 100 and 20 μg. After allowing to dry in a sterile hood, the disc were stored in brown containers in the refrigerator. All the control antibiotic susceptibility discs used were purchased from their corresponding commercial producers.

*Agar diffusion disc susceptibility assay.* The procedure was essentially the method described originally by Bauer et al. [9], adopted and modified by the National Committee for Clinical Laboratory Standards (NCCLS) [10]. The surfaces of the relevant agar media were seeded (flooded), to obtain even growths (not too light, not too heavy), with the appropriate dilutions of spores (*B. subtilis, T. mentagrophytes, M. gypseum, A. fumigatus*) or bacterial and yeast growth in the liquid media. The inoculated (seeded) agar plates were allowed to dry before the discs were placed on the surface. The plates seeded with bacteria were incubated at 37 °C overnight and those inoculated with yeast or fungi were incubated at 30 °C for 2 – 7 days, respectively. Then the diameters of the inhibition zones were measured and recorded. In all plates duplicate discs were used and the assay were repeated at least twice. The data given in the Tables represent the average values of the inhibition zone diameters.

*Disc approximation method.* To ascertain whether the activity of diazald can be antagonized by PABA or ATMAA, diazald discs and PABA- or ATMAA-saturated discs were placed in established proximity on the seeded agar surface. An eventual antagonistic effect is expressed by truncation of the otherwise round inhibition zones [11, 12].

*Instrumentation.* The quantitative colorimetric determination of diazald was performed similar to those described earlier [13, 14]. To three ml of dilutions (DMSO-water) of diazald was added 0.3 ml of solutions of ATMAA (0.25%) and acidified with one drop (about 0.05 ml) of cc. acetic acid. The red colour started to develop immediately and reached its maximum intensity after one hour at room temperature. At this point the absorbances (optimally at 500 nm) were measured, against a control blank, using a Bausch and Lomb Spectronic 70 spectrophotometer. The graph was constructed by plotting the log of absorbance (intensity of red colour) on the ordinate and the concentration of diazald on the abscissa (Fig. 2).

## Results and discussion

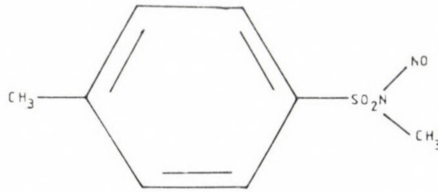


*Fig. 1.* Chemical structure of diazald

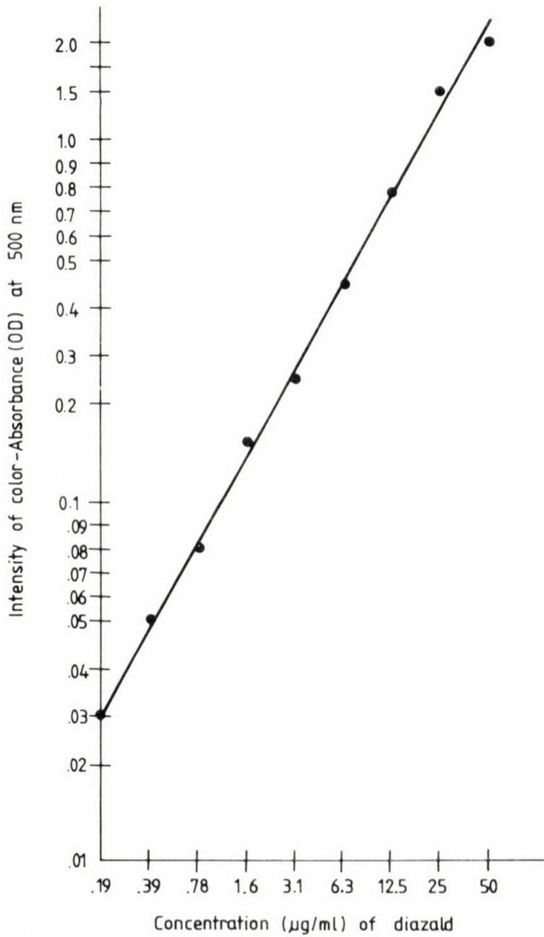


*Fig. 2.* Spectrophotometric curve of the coloured reaction product of diazald with ATMAA. Note the sensitivity of the reaction and the linearity of the curve

Data of Tables I and II clearly demonstrate that diazald is a broad-spectrum antibacterial agent. As Table I shows it has in vitro activity against a series of yeast-like organisms. Impressive is the activity against all strains of *Candida albicans* tested, also against *C. terreus* and *C. guillermondi*, as well as both strains of the yeast *C. neoformans*. The activity is somewhat less against *C. albidus* and surprisingly against *C. parapsilosis*. This would suggest certain selectivity in action against *Candida* species and also *S. cerevisiae*. Its activity against the dermatophyte strains (*T. mentagrophytes* and *M. gypseum*) is better at higher than at lower concentrations. Its potent activity against *A. fumigatus* would require the study of its activity against many strains of filamentous phytopathogenic and saprophytic fungi. This relates also to the inclusion of more dermatophyte strains in further studies. The relatively lower order of activity (smaller inhibition zones) of the control Fungizone (amphotericin B), griseofulvin and actidione in these experiments may be attributable to concentration and/or agar-diffusibility differences.

**Table I**

*Antifungal activity of diazald*

| Strains | Diameter of inhibition zones (mm) Diazald content of discs (μg) | | | |
|---|---|---|---|---|
| | 200 | 100 | 20 | Control discs |
| *Candida albicans* # 759 | 30 | 27 | 18 | 16(F) |
| *Candida albicans* (lab. strain) | 25 | 20 | 8 | 17(F) |
| *Candida albicans* (fresh isolate) | 21 | 13 | 12 | 14(F) |
| *Candida albidus* MAC 116 | 19 | 12 | T | 19(F) |
| *Candida guillermondi* | 29 | 17 | 16 | T(F) |
| *Candida parapsilosis* | 10 | T | – | 10(F) |
| *Candida terreus* | 33 | 22 | 20 | 11(F) |
| *Cryptococcus neoformans* # 1678 | 31 | 29 | 21 | 10(F) |
| *Cryptococcus neoformans* # 104 | 29 | 28 | 10 | 8(F) |
| *Saccharomyces cerevisiae* | 17 | T | T | 9(F) |
| *Trichophyton mentagrophytes* | 27 | 21 | T | 9(G) |
| *Microsporum gypseum* | 25 | 17 | T | 12(G) |
| *Aspergillus fumigatus* | 47 | 40 | 19 | 19(A) |

(F) – Fungizone; (G) – Griseofulvin; (A) = Actidione; T = Trace activity

**Table II**

*Antibacterial activity of diazald*

| Strains | Diameter of inhibition zones (mm) Diazald content of discs (μg) 200 | 100 | 20 | Control discs |
|---|---|---|---|---|
| *Staphylococcus aureus* ATCC 25923 | 18 | 10 | T | 45(C) |
| *Staphylococcus aureus Tour* (Mouse pathogen) | 35 | 30 | 15 | 45(C) |
| *Staphylococcus aureus* (Methic. resistant) | 25 | 17 | 7 | T(C) |
| *Bacillus subtilis* ATCC 6633 | 30 | 27 | 18 | 39(C) |
| *Escherichia coli* ATCC 25922 | 32 | 25 | 13 | 45(C) |
| *Morganella morganii* # 179 | 19 | 13 | 10 | 28(C) |
| *Pseudomonas aeruginosa* ATCC 15629 | 0 | 0 | 0 | 19(C) |

(C) = Ceftizoxime; T = Trace activity

Table II demonstrates that diazald possesses selective antibacterial activity. It appears to be less impressive than its antifungal activity. It has better activity against the highly mouse-pathogen *S. aureus* Tour strain than against the ATCC 25923 strain. The control ceftizoxime is superior to it. Interestingly it has activity against the methicillin resistant staphylococcus strain against which ceftizoxime, as expected, is not effective. Its activity against *E. coli* is demonstrable, but again ceftizoxime is much better. The same situation holds with *M. morganii*. It has no activity, even at high concentration, against *P. aeruginosa* which is, as known, only slightly inhibited by ceftizoxime.

In studying its mode of action, using the disc approximation test, it was found that, in contrary to the sulfonamides, it is not inhibited either by PABA or the nitroso-group reactive ATMAA. This finding leads to the conclusion that the entire molecule as such is responsible for the antimicrobial activities.

Diazald is the first modified sulfonamide molecule which has a definite antifungal activity. All the antibacterial sulfonamides contain an amino group directly attached to the benzene ring at the para position. Mafenide (Sulfamylon) is the only sulfonamide which contains a methylamino group (instead of amino) at the para position. This "small" modification made it effective, unlike the other sulfonamides, against anaerobic bacteria, especially against the gas gangrene-producing clostridia. This effect has therapeutic uniqueness and importance. Diazald is a further modified sulfonamide, with a p-methyl, as well as a methyl and a nitroso substituent at the sulfonamide group (Fig. 1). These molecular modifications produced the first sulfonamide molecule with impressive broad in vitro activity, including against fungi. Further studies are needed to establish its in vivo efficacy.

Another interesting aspect of diazald is that it is a chemical intermediate or precursor with noted antimicrobial activity. In this respect it is an addition to the list of the many famous drugs which originally were studied as chemical intermediates [15].

Our earlier recognition that the antitumour nitrosoureas developed a concentration-dependent colour reaction with ceftizoxime [6] prompted us to find out whether diazald, a non-urea-nitroso compound, could produce a similar colour-reaction with ATMAA, the side chain of ceftizoxime. This study led us to the development of a sensitive spectrophotometric method for the chemical detection and quick quantitative determination of diazald (Fig. 2).

The experimental data obtained with diazald reported in this paper represent a part of a broad ongoing project, originated by JVU aimed at the chemical, analytical, pharmacological and microbiological studies, and eventual practical applications of the aminothiazole-syn-oxyimino beta-lactam antibiotics and their molecular constituents [6, 13, 14, 16–24].

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# ADHERENCE OF RUMINAL *STREPTOCOCCUS BOVIS* AND *LACTOBACILLUS* STRAINS TO PRIMARY AND SECONDARY CULTURES OF RUMEN EPITHELIUM*

(A NOTE)

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Six strains of rumen *Lactobacillus* and four *Streptococcus bovis* strains isolated from rumen wall and fluid samples were examined for the adherence to cells of primary and secondary cultures of ruminal epithelium (REC) prepared from sheep and calf. *S. bovis* adhered to the keratinized REC. Ruminal lactobacilli did not adhere. The presence of rumen lactobacilli in mixture had no influence on the adherence of *S. bovis* strains. No difference was observed in the adherence of tested bacteria to epithelial cells of primary or secondary cultures, but adhesion was only detected on keratinized cells.

Ruminal streptococci and lactobacilli produce very large populations in rumen contents especially in young ruminants and belong to dominant microflora of these animals. Several authors have identified these bacteria also among the epimural microflora [1–3]. From our point of view they may serve as suitable recipients of foreign genetic materia for the improvement of the microbial gene pool of ruminants.

The long range aim of our investigation is to study the development of rumen microflora in young ruminants. The epimural rumen microflora seems to be the most suitable for this purpose because despite its low percentage (only about 1%) of total

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rumen microflora population [1] it is considerably stable and resistant to dietary changes.

In our first experiments we demonstrated the good adherence ability of some *S. bovis* strains [4] to cultured primary ruminal epithelial cells (REC). The purpose of this study was: (i) to compare the adherence ability of four such *S. bovis* to that of six *Lactobacillus* strains to REC; (ii) to compare adhesive properties of primary and secondary cultures of REC; (iii) to test if there is a difference between cultured REC of sheep and calf in adhesiveness to streptococci.

## Materials and methods

Four *Lactobacillus* strains (*Lactobacillus plantarum* 4, 13, 53; *Lactobacillus cellobiosus* CCM 4000) isolated earlier, two fresh isolates of *Lactobacillus* strains (*Lactobacillus plantarum* 1/5, 2/7) and four *Streptococcus bovis* strains (AO 24/85, 47/3, 4/1, 46/2) isolated earlier from ruminal wall and fluid samples of lambs and calves were used for the adherence experiments as described before [4]. *S. bovis* and lactobacilli were identified on the basis of cellular morphology, cultural characteristics and fermentation products.

The rumen lactobacilli were grown anaerobically at 37 °C in Rogosa broth and the *S. bovis* strains were grown aerobically at 37 °C in Nutrient broth No. 2 (Immuna, Šarišské Michal'any). All cell densities were adjusted to $10^8$ bacteria per ml before use in the adherence assay. The isolation of ruminal epithelial cells and preparation of rumen epithelial primoculture was performed from a sheep and a calf as described before [4]. The secondary culture of rumen epithelium was prepared from 30-days' ruminal epithelial primoculture by passaging of the cells after treatment with 0.25% trypsin and 0.02% EDTA solution for 5 min into next collagen-coated polystyrene flasks with MEM-H medium. These cells were then cultured for 12 days at 37 °C and at 100% humidity of air and subsequently used for experiment. The epithelial origin of the cells of primo- and secondary cultures was checked with anti-cytokeratin pan-monoclonal antibody (Boehringer Mannheim GmbH) by the method of Overbeck et al. [5].

Bacterial adherence tests were described before [4]. Bacterial counts were about $10^8$ bacteria per ml either at individual strains or at combination of both species *S. bovis* and lactobacilli.

## Results

The experiments were performed on rumen epithelial primary and secondary cultures after 12 days' culturing when all types of epithelial cells were developed, non-keratinized as well as keratinized cells.

Bacteria of all 4 *S. bovis* adhered only to differentiated epithelial cells in both primary and secondary cultures. The epithelial keratinization had also a considerable influence on bacterial adherence. Keratinized cells were inhabitated by bacteria much more than those non-keratinized. Strains of ruminal lactobacilli were not able to adhere to the cells of rumen epithelium. The presence of rumen lactobacilli in mixture did not have an influence on the adherence of streptococcal strains. All *S. bovis* strains adhered to ovine as well as to calf's epithelial cells in the irregardless to the animal species of isolation.

## Discussion

Studies of the bacterial populations of the rumen of milk-fed calves have shown [2] that species of *Micrococcus, Staphylococcus, Lactobacillus, Corynebacterium, Streptococcus, Flavobacterium* and some coliform bacteria predominate to produce very large populations in the ruminal contents of these animals. As these calves mature and progress towards the use of hay and forage feeds, this characteristic bacterial population appears to retreat to the rumen wall to become the rumen-epithelium-associated population [2]. In contrast, according to study of Mueller et al. [3], *Lactobacillus ruminis* had become the dominant microorganism of adherent epimural microflora at 4 weeks of lambs' age, representing 46.7% of the isolates. However, at 6 weeks of age and later, *L. ruminis* was not isolated from lambs. In the opinion of Rieu et al. [6], however, lactobacilli were absent from the rumen wall of 2–21 days old lambs.

In our results a great difference in the adherence between lactobacilli and *S. bovis* strains was detected. Although the *S. bovis* strains were isolated some time ago and have been subcultured repeatedly and subjected to various storage procedures they retained their adhesive properties. It seemed probable that they did not loose their glycocalyces or ligands responsible for their adherence. The species specificity of *S. bovis* strains isolated earlier (4; this paper) as well as of a fresh *S. bovis* isolate from rumen wall [7] was not observed in the adherence to rumen epithelial cells. It is interesting to note that ruminal bacteria adhered to epithelial cells of secondary culture as well as to epithelial primoculture and therefore the receptors of these ruminal cells showed the same properties also after passaging. Studies of Simpson et al. [8] suggest that fibronectin on the epithelial cell surface serves as a receptor for the attachment of streptococci. The presence of fibronectin on the cell surface both of primary and secondary cultures should now be detected by anti-fibronectin pan-monoclonal antibody.

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# ANNUAL MEETING OF THE HUNGARIAN SOCIETY FOR MICROBIOLOGY

SZÉKESFEHÉRVÁR, JULY 7 – 9, 1992

ABSTRACTS OF PAPERS AND POSTERS

# PLENARY SESSION

H. MILCH

## Molecular epidemiology of bacterial plasmids

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For determining phenotypic and genotypic characters in *Escherichia coli, Klebsiella, Enterobacter, Shigella sonnei, Salmonella typhi-murium, Pseudomonas aeruginosa, Staphylococcus aureus* and coagulase-negative staphylococci we elaborated and applied a complex typing method that allows the drawing of epidemiological and clinical conclusions. Examinations of *E. coli* O124 and *E. coli* O157 strains are good examples. Studying the regularity in the cause of changes in phage types, we demonstrated the role of R-plasmids. We examined the antibiotic resistance determinants, resistance-patterns, the frequency of transferable resistance and plasmid characterization in case of *E. coli* strains belonging to different serotypes associated with different diseases. The antibiotic resistant *E. coli* strains derived from outbreaks in new-born wards carried R-plasmid in 78%. Strains of animal origin were R-plasmid carriers in 55%. R-plasmid was demonstrated in the frequent *E. coli* serogroups in 48.8 – 100%. R-plasmids were demonstrated also among the strains resistant to one or two antibiotics. The antibiotic resistant *E. coli* strains of human origin are considered as R-plasmid carriers. For comparison I demonstrate the results of our relevant examinations of other enteric bacteria. *S. typhi* strains were antibiotic resistant in 0.3% and resistance was determined by R-plasmid in all of them. The resistance in the different enteric bacteria – though they differed in the frequency of resistance – was determined by R-plasmid in 79.7 – 100%. Among *Klebsiella* strains only the multiple resistant ones harboured R-plasmids, the resistance of monoresistant strains was mainly (72.5%) of chromosomal character. For plasmid characterization we used the "classical" methods (Inc groups, molecular weight estimation) and the method elaborated by us (based on phage-restriction). Besides the determination of R-plasmids, we demonstrated the presence and function of other plasmids playing a role in virulence. These findings provided us the possibility to study the mechanism of pathogenicity and virulence. The correlation between V type colicin and pathogenicity was proved by statistical analysis. The probable role of Col V plasmid in pathogenicity was proved by plasmid elimination and $LD_{50}$ determination. The virulence increasing effect of aerobactin was proved by the determination of serogroup, diagnosis and $LD_{50}$ values. Cloacin sensitivity experiments showed that the absence of cloacin sensitivity did not exclude the presence of a receptor, though the receptor of aerobactin and cloacin was common.

A significant difference was demonstrated in the incidence of the virulence markers examined (Col V, Hly, Aer) and plasmid carrier state in the K1, K5 antigen possessing and K1, K5 non-possessing strains. The correlation between toxin production and frequency of plasmid carrier state was reported. R- and Hly plasmid carrier and loop-test positive *E. coli* strains were siginificantly more frequent among the strains isolated from diarrhoeal pigs than among healthy ones. It has been demonstrated on wild-type, pathogenic, antibiotic resistant strains that both enrofloxacin and nalidixic acid are capable to curing R-plasmids in wild-type porcine *E. coli* strains in vitro.

A. SZENTIRMAI

**Physiological base of over-production of beta-lactam antibiotics**

*L. Kossuth University, Debrecen, Hungary*

The enzymatic steps of biosynthetic pathway of formation of penam and cephem skeleton were investigated in details by a number of research teams. Nowadays these well known results are in the textbooks. However, in later years a number of papers were delivered by molecular biologists about the formation of penicillin. Miller and Ingolia (1989) clones the clusters of genes for penicillin formation. Kurczatowski et al. (1982) and later Muller et al. (1991) localized the pathway of penicillin biosynthesis.

It seems clear that the copy number of genes and occurrences, also the high level of enzymes of biosynthetic pathway, are absolutely necessary, but not enough condition for over-production of penicillin nucleus. The intensive research on penicillin formation draws the attention of biologists to the regulation of biosynthesis of it.

More than fifty different well-known enzymes are working with extremely high effectiveness in the practically used, improved strains. It should be noted that 1 mg dry mycelium is able to produce more than 10 mg penicillin.

Revilla et al. (1986) have shown that glucose represses formation of L-α-aminoadipyl-L-cysteinyl-D-valine. A number of research teams studied the connection of penicillin formation with the pool-concentration of the starting amino acids.

Jaklitsh et al. (1986) have found that a high producer *Penicillium* strain contained a greater α-aminoadipate pool in the cells. Hönlinger and Kubicek (1989) found that the biosynthesis of penicillin-N in *Penicillium chrysogenum* is regulated by the α-aminoadipate pool size, the rates are increased when the intracellular pool of α-aminoadipate is raised further.

The research results of Affenzeller and Kubicek (1991) indicate a further control point of penicillin biosynthesis. Their results indicate that penicillin and protein biosynthesis use different pools of cellular amino acids. Their data suggest that the chanelling of amino acids between different cellular compartments, the exchange of substrates (amino acids) between the citosol and the ACV synthetizing compartment may be related to ability of microorganisms to synthesize penicillin.

It has been accepted that the genetically determined potencial of penicillin formation, the actual rate of penicillin production is markedly influenced by physiological parameters: aeration, carbon source, ammonium sulphate, etc.

We must take into consideration that in high producer *Penicillium* strains hundred times bigger amount of cysteine, valine and α-aminoadipic acids are formed for penicillin synthesis than for growth of fungal cells (mycelia). The formation of one molecule penicillin requires ATP (10), ammonia (3), sulphate (1), glucose (4), and NADPH (more than 8) moles. However, the biosynthesis of cephalosporins needs even more NADPH (11-12) and ATP also. All of them can function as co-substrates of biosynthetic pathway of beta-lactam antibiotics having pename or cepheme skeleton.

# BACTERIOLOGY

D. Cs. Szalay, I. Hajtós, R. Glávits and J. Takács

## Bovine thromboembolic meningoencephalitis in Hungary

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Capnophilic, Gram-negative pleomorphic coccobacilli were isolated from brain of 5-month-old calves died after showing nervous symptoms and from organs of a 4-month-old aborted bovine fetuses in two farms in Western Hungary. Based on the cultural, morphological and more important biochemical characteristics (i.e. oxidase and indole positive; catalase, urease and beta-galactosidase negative fermentation of glucose) the isolated bacteria were identified as *Haemophilus somnus*. There were minor differences in the haemolytic and ornithine decarboxylase activity as well as the sensitivity to antibiotics between strains isolated from brain and that isolated from the aborted fetus. A previous study showed also phenotypic differences among the *H. somnus* strains isolated from bovine pneumonia in Northern Hungary. The present study is the first report on the occurrence of bovine thromboembolic meningoencephalitis (TEME) and abortion caused by *H. somnus* in Hungary.

É. MOLNÁR

**Serotypes of** *Actinobacillus pleuropneumoniae* **biotype 1 in Hungary**

*Veterinary University, Budapest, Hungary*

Only serotypes 1 and 2 of *Actinobacillus pleuropneumoniae* biotype 1 were isolated in Hungary before 1989, serotypes 3, 7, 9 and 11 appeared after 1989. Serotypes of 484 *A. pleuropneumoniae* strains from 99 pig farms were identified up to June of 1991. The prevalence of the different serotypes was serotype 1, 58; serotype 2, 340; serotype 3, 10; serotype 7, 25; serotype 9, 23; and serotype 11, 28. According to the results of the coagglutination test further new serotypes may be isolated in the future. The serotypes were earlier identified with agglutination test but the primary identification is made nowadays by coagglutination. In this way 96-97% of the strains can be serotyped easier and quicker. In doubtful cases the agar-gel precipitation test, the indirect haemagglutination test and the counterimmuno electrophoresis are used as well. Cross-absorbing the immun sera is sometimes necessary.

L. MOLNÁR

**New drugs against** *Serpula hyodysenteriae*

*Veterinary University, Budapest, Hungary*

The agent of swine dysentery (SD) is *Serpula (Treponema) hyodysenteriae*. This bacterium – like the majority of spirochaetes – is a fragile microorganism and is sensitive to a number of chemotherapeutics. However, SD can persist in a large farm for decades and therefore the constant therapeutical or preventive treatment of the finishing stocks is recommended and widespread. As a consequence, resistance will develop to different drugs sooner or later. Therefore it is necessary to produce and test new potent drugs or drug combinations continuously. Sedecamycin belongs to the macrolide group of antibiotics. It is the product of the Japanese Takelan firm. In vitro it proved to be only moderately effective against various strains of *S. hyodysenteriae*, but it was more efficient in the therapy. It may play a considerable role in the therapy of swine before slaughtering. The monenzin had a moderate antibacterial effect against *S. hiodysenteriae*. In the in vitro tests it had an excellent synergic interaction with an antioxidant. If this combination has a similar effect in vivo, a new effective and cheap drug combination could be developed.

I. HAJTÓS

## Characterization of *Corynebacterium renale*-like strains isolated from male ruminants in Hungary

*Institute of Animal Health, Miskolc, Hungary*

In the last five years approximately 200 *Corynebacterium renale*-like strains were isolated from semen samples of rams and bucks, and from preputial secretion of bulls. These strains had strong urease and catalase activity, but were indole negative and fermented glucose in the OF test. Eighteen of the isolated 200 strains were studied in detail using 30 biochemical tests and sensitivity to antibiotics. On the basis of the most important criteria including caseinase, nitrate reduction, hydrolysis of Tween-80 and starch, fermentation of xylose and mannose, etc. described by Yanagawa and Honda (1978), our isolates could not been classified as *C. renale, C. pilosum* or *C. cystitidis*. Further studies are needed for exact classification of these 18 strains. *Corynebacterium* strains characterized in the present study were similar to the Australian "diphtheroid" organisms isolated by Southcott (1965), but our isolates failed to reduce nitrate.

G. CZIFRA, T. TUBOLY, B. SUNDQUIST and L. STIPKOVITS

## Evaluation of a blocking-ELISA for the detection of *Mycoplasma gallisepticum* specific antibodies using a monoclonal antibody

*Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary, and National Veterinary Institute, Uppsala, Sweden*

A peroxidase labelled monoclonal antibody recognizing an epitope on a 56 kD polypeptide (p56) of *Mycoplasma gallisepticum* was used in an enzyme-linked-immunosorbent assay (blocking ELISA) for detecting specific antibodies against *M.gallisepticum* in chicken sera of different origin. Undiluted sera collected from naturally or artificially infected birds were examined on microtiter plates coated with whole cell antigen prepared by detergent solubilization of *M.gallisepticum* (1226). Immunoglobulins from *M.gallisepticum* positive sera bound to different antigenic sites including this epitope, so the binding of the monoclonal antibody was blocked, resulting in no colour reaction. The opposite results, a strong colour reaction was obtained after incubation with *M.gallisepticum* negative sera or with no serum at all added before the incubation with the monoclonal antibody. Blocking-ELISA detected 84.7% and 72.6% of reactors in artificially or naturally infected flocks, respectively, while haemagglutination inhibition (HI) test proved to be positive only with 68.4%

and 48.6% of sera. All HI positive sera as 51.5% and 46.8% of HI negative sera showed positive reaction in blocking-ELISA. A strong correlation (r = 0.83) was found between HI and blocking-ELISA titres. Infection with closely related *Mycoplasma synoviae* did not give rise to false positive results. The blocking-ELISA was fast, easy to perform and well suitable for large scale diagnostic in poultry flocks.

K. FORGÁCH, A. VARGA, I. NÉMETH and L. STIPKOVITS

**Detection on IgG-type anti-*Mycoplasma gallisepticum* antibodies in sera of infected chicken with monoclonal antibody-based ELISA test**

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Serum samples of chickens infected naturally or artificially, as well as of chickens exposed to contact infection with *Mycoplasma gallisepticum* were examined for the presence of IgG isotype anti-*Mycoplasma gallisepticum* antibodies using isotype specific monoclonal antibodies in indirect ELISA test. The titres of IgG antibodies against *Mycoplasma gallisepticum* in serum samples taken on 7th-10th day postinfection from chickens infected artificially into the airsack at 2, 4, 6, 9 and 30 weeks of age reached values of 1:800 – 1:1600, 1:3200 – 1:6400, 1:12800 – 1:25600, and 1:6400 – 1:12800, respectively. In the serum samples of naturally infected chickens, the titres of IgG antibodies were between 1:6400 and 1:12800 at 30 weeks of age, thereafter they decreased gradually, during a 6-month observation period.

G. CZIFRA, T. TUBOLY, L. STIPKOVITS and B. SUNDQUIST

**Preparation and characterization of monoclonal antibodies to *Mycoplasma gallisepticum***

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Balb/c mice were immunized with *Mycoplasma gallisepticum* iscoms prepared from a virulent strain 1226 and heterokaryots were produced with the standard method. *Mycoplasma gallisepticum* and closely related *M.synoviae* were used as antigens to screen for the presence of specific antibodies in ELISA. Nine strongly reacting monoclonal antibodies (mAb) were further characterized with immunoblotting and divided into five groups according to the recognized polypeptides with relative molecular masses of 110, 64, 62, 56 and 50 kD, respectively. All the five groups proved to be species-specific when tested with 10 other avian

mycoplasma antigens, some mAbs can differentiate among different *Mycoplasma gallisepticum* strains. None of the mAbs can inhibit the haemagglutinating activity of fresh *Mycoplasma gallisepticum* broth culture, but two (B3, C6) agglutinated the stained *Mycoplasma gallisepticum* antigen in slide agglutination test.

B. NAGY, T. A. CASEY, S. C. WHIPP, H. W. MOON and E. A. DEAN-NYSTROM

## Studies on adhesins of verotoxigenic and enterotoxigenic *Escherichia coli* isolated from weaned pigs: the use of polyclonal and monoclonal antibodies

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Besides the adhesins (K88, K99, 987P, F41) and enterotoxins (LT, STa, STb) of the *Escherichia coli* strains responsible for enteric diseases of newborn pigs, there has been more and more interest devoted to the *E. coli* of weaned pigs producing oedema disease and diarrhoea. The toxin of the oedema strains belong to the family of verotoxins (VT2 or SLTIIv). Diarrhoeal strains produce primarily STb. Until recently, there were no informations regarding the antigens mediating intestinal colonization of these two groups of strains in weaned pigs. Bertschinger et al. (1990) reported the presence of new adhesive fimbriae (F107) on verotoxigenic *E. coli* (VTEC) isolated from oedema disease. Nagy et al. (1992) described new adhesive pili (2134P) on enterotoxigenic *E. coli* (ETEC).

Comparative studies reported here were directed on 10 ETEC and on 5 VTEC to learn about the prevalence, antigenic and morphologic differences of the two pili (fimbria). These studies were done on in vivo (in ligated gut segments) and in vitro grown bacteria, by immunofluorescence and electronmicroscopic methods using polyclonal and monoclonal antibodies. Results suggested that the VTEC and ETEC strains produced morphologically similar pili showing antigenic and biologic differences. The pili of ETEC strains (2134P) seemed to be specific for ETEC (could only be detected on ETEC monoclonals). Besides, there seemed to be an antigen commonly present on both ETEC and VTEC the identity or differences of which with the antigen F107 should be further studied.

M. HERPAY, É. CZIRÓK, H. MILCH, I. GADÓ and K. GY. LENDVAI

**Computer analysis of complex typing of *Escherichia coli* strains isolated from epidemics**

*B. Johan National Institute of Hygiene, Budapest, Hungary*

By using EPI-INFO computer program (CDC), we analyzed the properties of 3334 *Escherichia coli* strains isolated from different clinical materials between 1979-1990. From the outbreaks caused by *Escherichia coli* strains, the following serogroups could mainly be isolated: O2, O4, O6, O15, O18ac, O45, O75, O78 and not typable (NT). Epidemics mainly occurred in newborn departments. Strains of the same outbreak had sometimes different phenotypic characters. Some characteristics (phage pattern, H. antigen etc.) of *Escherichia coli* strains had changed by the end of the outbreak. These changes, however, were not in contradiction with the common origin of the strains. Clinical and pathogenic characteristics of the epidemics differed from each other. To find the etiological agent, application of different methods were needed.

I. TÓTH, V. KARCAGI, B. NAGY, I. GADÓ, M. CSIK and M. MILCH

**Examination of verocytotoxin producing capacity and determination of the presence of Shiga-like toxins, SLT1 and SLT2 genes in *Escherichia coli* strains**

*B. Johan National Institute of Hygiene, Budapest, and Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, Hungary*

Verocytotoxin (VT) producing capacity of 24 *Escherichia coli* strains were examined in vitro. Additionally 84 wild type strains were examined for the presence of Shiga-like-toxin (SLT) genes by SLT1 and SLT2 specific DNA probes. SLT1 and SLT2 structural gene sequences were labelled by the random primer method with $^{32}$p-dCTP. *Escherichia coli* strains belonged to 16, frequently VT producing serogroups. One strain of 026:H8 serotype produced VT and in the in situ hybridization experiments the presence of SLT2 gene was demonstrated. Phage was isolated from the VT producing strain, and to determine its converting capacity, an *Escherichia coli* K12 strain was lysogenized. Characterization the phage by electron microscopy is in progress.

E. NAGY

**Effects of different antibiotics on associations of aerobic-anaerobic bacteria**

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

Mixed bacterial infections involving aerobic and anaerobic bacteria are frequently encountered after surgery. The activity of antibiotics and their combinations against aerobic and anaerobic bacteria can be tested in vitro by means of association experiments. The changes in the colony counts of *Bacteroides fragilis* group strains and facultative bacteria (such as *Escherichia coli*, *Staphylococcus aureus* or *Enterococcus faecalis*) cultured together in 10 ml Wilkins-Chalgreen broth in an anaerobic environment were followed in the presence of different antibiotics.

The results showed that: (i) the MICs of metronidazole against *Bacteriodes* strains increased considerably in the presence of *E. faecalis*; (ii) beta-lactamase-producing *S. aureus* or *B. fragilis* protected the susceptible *E. coli* against the killing effect of mezlocillin; (iii) cefamandol had a much higher MBC against *S. aureus* in the presence of beta-lactamase-producing *B. fragilis* or *Prevotella melaninogenica*; (iv) the MBC of chloramphenicol was found to be 10 times higher against *Bacteroides* strains when these were cultured together with chloramphenicol-resistant *E. coli*.

J. FÖLDES

**Interaction of the gyrase inhibiting fluoro-quinolones with the transformation and transfection in the cells of *Bacillus subtilis***

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

The quinolones inhibit the gyrase in bacterial cells. The enzyme is topoisomerase II controlling with topoisomerase I the structure of the DNA. The former one reduces the size of the bacterial chromosome by negative supercoiling, the later one acts on the opposite direction. The quinolones disturb the balanced stage of the biological processes and on the same time induce a RecA protein for reparation. The transformation and transfection process seem useful for the study of the mode of action of the quinolones. The transformation and transfection of the competent cells of *Bacillus subtilis* 168 $I^-$ were performed with the DNA of the wild strain as of the SP50 phage. Coprofloxacin, ofloxacin and pefloxacin were added at subinhibitory concentration to the systems in different stages of the genetic process, the number of transformants was counted and the kinetic curve of the transfection was constructed. It was stated that the quinolones do not inhibit the uptake of DNA and the recombination process in the transformation. The number of the

transformants is in correlation with the decrease of the growth rate of the bacterial cells. The mechanism of the transfection is different. The gyrase inhibitors stop the phage synthesis in any stage of the transfection and continuously decrease the number of the produced phages. There is a quantitative difference also between the three quinolones in the action on the transfection.

M. KONKOLY-THEGE, A. DOBÁK, L. ALFÖLDY, Z. CSAJBÓK and B. LÁNYI

**In vitro and in vivo activity of sulbactam/ampicillin against** *Acinetobacter isolates*

*B. Johan National Institute of Hygiene, and Csepel Municipal Hospital, Budapest, Hungary*

In Hungary, antibiotic resistance is the most frequent among *Acinetobacter* isolates, consequently hospital-acquired infections caused by multiresistant strains after present a therapeutic challenge. According to the data of a multicentre study, 78.3% (90/115) of *A. calcoaceticus*, and 82.1% (23/28) of *A. lwoffii* isolates proved sensitive to sulbactam/ampicillin combination. The intrinsic activity of sulbactam – primarily a potent B-lactamase inhibitor – against *Acinetobacter* spp. is reflected in this promising in vitro efficacy. The minimum inhibitory concentration of sulbactam for 7 representative multiresistant strains, which were sensitive to the combination ranged 1-4 μg/ml. On the basis of these findings, a lifethreatining hospital-acquired *A. calcoaceticus* infection of a 19-year-old man with multiple injuries was treated with sulbactam/ampicillin. The infection had failed to respond to any antibiotic regimen for 5 weeks. After administration of sulbactam/ampicillin and adequate surgery the patient's status improved rapidly.

I. BARCS, R. OKAMOTO, T. OKUBO and M. INOUE

**False sensitivity to clindamycin in staphylococci producing clindamycin inactivating enzyme**

*B. Johan National Institute of Hygiene, Budapest Hungary, Gunma University School of Medicine, Maebashi, Japan, and School of Medicine, Kitasato University, Sagamihara, Japan*

A 44 kb resistance plasmid of *Staphylococcus epidermidis*, pBI109PGL encodes type V betalactamase, aminoglycoside (6')-acetyltransferase V and lincosamide inactivation nucleotidylation (lin) enzyme production, respectively. The *lin* enzyme determined by the pBI109PGL exhibits high level resistance to lincomycin but sensitivity to clindamycin by standard susceptibility methods. Substrate profile determination showed clindamycin to be a better substrate for the enzyme than

lincomycin. In cultures of the plasmid-harbouring strain, the level of clindamycin decreased below the inhibitory concentration in the first 4 h of incubation but the level of lincomycin persisted longer. The initial extended inhibitory effect of clindamycin is due to better membrane penetrating ability resulting in a higher intracellular concentration than that of lincomycin. Moreover, energy-dependent reduction in clindamycin uptake, probably due to active efflux of clindamycin but not of lincomyxin, was observed. A therapeutic effect of clindamycin is not expected in infections caused by *lin* producer strains because the bacteriostatic effect of the drug is rapidly eliminated after administration. According to these findings, staphylococcal strains with *lin* enzyme-dependent lincomycin resistance should also be considered resistant to clindamycin.

É.B. JÁKICS, S. IYOBE, K. HIRAI, H. FUKUDA and H. HASHIMOTO

**Occurrence of the *nfxB* type mutation in clinical isolates of *Pseudomonas aeruginosa***

*B. Johan National Institute of Hygiene, Budapest, Hungary, Gunma University School of Medicine, Maebashi, Japan, and Kyorin Pharmaceutical Co.Ltd., Shimutsuga, Japan*

The incidence of *Pseudomonas aeruginosa* strains, highly resistant to new quinolones, has increased more than 20%, within 3 years, in Gunma University Hospital (Japan). Resistance to quinolones is due to chromosomal mutations that alter the DNA gyrase or the bacterial outer membrane permeability. The nfxB gene responsible for norfloxacin (NFLX) sensitivity was examined using resistant mutants of 20 NFLX-sensitive strains and 12 resistant clinical isolations. Susceptibility patterns to NFLX, beta-lactam and aminoglycosides were used to determine mutation type. Mutants of NFLX-sensitive strains were transformed with the pNF111 recombinant plasmid which complements *nfxB* mutation. Comparison of parent, mutant and transformant MICs, revealed 15 cases where parent and transformant MIC were identical. Examination of NFLX uptake showed that the sensitive strains and the transformants accumulated NFLX, in spite of resistant mutants. The presence of pNF111 plasmid in transformants was confirmed by electrophoresis. The transformation of NFLX-resistant strains was successful in 3 cases of the 12. This study proved that the *nfxB* type mutation in *P. aeruginosa* can occur in vivo.

A. MARTON, R. MUNOZ and A. TOMASZ

**Epidemiological characteristics of pneumococcal resistance in Hungary and markers of the resistant strains**

*Heim Pál Children's Hospital, Budapest, Hungary, and The Rockefeller University, New York, USA*

The prevalence of antibiotic resistant *Streptococcus pneumoniae* is the highest in Spain and Hungary according to data published so far. The rate of resistance to penicillin and erythromycin increased from 1975 to 1989 from 20% to 50% and 10% to 50%, respectively. Isolates from pediatric specimens showed a higher resistance rate to penicillin, erythromycin, tetracycline, cotrimoxazole and chloramphenicol and a higher gedree of resistance to penicillin than these of adults. Penicillin resistant strains isolated from the upper respiratory tract were more prevalent than those from systemic infections. Geographic distribution of pneumococcal resistance to penicillin in Hungary is uneven. The highest resistance rate was found in North-eastern Hungary (in Country Borsod) and the lowest at the North-western border (County Győr). The serotypes of penicillin sensitive *S. pneumoniae* show a great variety, while serotype 19A is predominant among the resistant strains. An altered structure and electrophoretic mobility of the penicillin-binding protein (PBP) of the tested penicillin resistant strains, markers of reduced affinity to penicillin, were detected. Multilocus enzyme analysis of PBP proved identity of 4 of 5 penicillin resistant strains while surface protein A was identical. On the basis of these markers the Hungarian strains, resistant to penicillin differ from the Spanish and the American strains, and thus represent a unique clone.

É. CZIRÓK, I. GADÓ, G. V. LÁSZLÓ, M. CSIK, M. HERPAY, G. FALUDY and A. S. NAIDU

**Effect of bovine lactoferrin on *Escherichia coli* strains possessing different type of pili**

*B. Johan National Institute of Hygiene, Budapest, Public Health Institute, Hungarian Army Medical Corps, Budapest, Hungary, and Department of Medical Microbiology, University of Lund, Malmö, Sweden*

The effect of bovine lactoferrin (BLF) was examined on *Escherichia coli* strains harbouring human colonization factors (CFAI-H10407, CFAII-PB176). Mannose resistant haemagglutination (MRHA) and their agglutination in CFA specific serum were weaker when they were cultivated in the presence of 5 mg/ml BLF. BLF inhibited their adhesion to HEp-2 cells as well. On the basis of electron microscopic examination the number of pili on the bacterial surface was also reduced. In spite of

the expectation, bacteria became more hydrophobic in the salt aggregation test (SAT) due to the effect of BLF. The growth was poorer in BLF-containing medium but this effect could be observed in case of the isogenic CFA negative strains, too. Summarizing, BLF acts on surface of *Escherichia coli* and inhibits formation of fibrial structures of CFA.

I. MAYER, E. NAGY and J. FÖLDES

## Evaluation of synergistic effects of quinolones and cephalosporins against *Pseudomonas* strains

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

Fluoroquinolones possess broad-spectrum activity in vitro and in vivo against a great variety of facultative bacteria. However, the occurrence of strains resistant to these drugs used in monotherapy has already been reported. Combination of these drugs with beta-lactam antibiotics may prevent the emergence of resistant isolates or their selection during antibiotic therapy. In the case of mixed infections the combination provides broad coverage against pathogens. During this study, the in vitro effects of fluoroquinolones (ofloxacin, pefloxacin or ciprofloxacin) in combination with cephalosporins (ceftazidim, ceftriaxon or cefoperazone) were tested by means of disc diffusion, checkerboard titration and the killing curve method. The MIC, FIC and FIX values of the antibiotics and their combinations were determined. The 15 *Pseudomonas* strains used during this study were recent clinical isolates with different resistance patterns. The three in vitro methods did not give the same results as concerns synergism. The combination of pefloxacin and cefoperazone showed a synergistic effect on 40% of the multiresistant *Pseudomonas* strains tested by checkerboard titration or the killing curve method. The other combinations showed a synergistic effect in only 6-20% of the strains. The killing curve experiments revealed that 1/2 MIC of the quinolone + 1/2 MIC of the cephalosporin had a much higher killing activity than those of the drugs alone.

E. FODOR, M. MAGOS, E. TÓTH, ZS. KOCZIÁN, A. SZENTMIHÁLYI and J. FÖLDES

## Characterization of beta-lactamase-producing multiresistant *Pseudomonas aeruginosa* strains: bio-, sero- and genotyping

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary, and B. Johan National Institute of Hygiene, Budapest, Hungary*

The pathological significance of *Pseudomonas aeruginosa* in immunocompromised patients, in cystic fibrosis and in nosocomial infections is well established. The aim of this study was to characterize *Pseudomonas aeruginosa* strains isolated from patients in the intensive care units of surgical and paediatric clinics. Different methods were used, such as biochemical tests, serotyping, determination of resistance patterns and beta-lactamase (PBL) production. The PBLs were characterized by substrate profile and inducibiliy. Imipenem and cefoxitin were used as inducers and the PBL activities were determined in the culture supernatants, in the intact cells, and in the supernatants of the ultrasound-treated cells. Plasmid isolation was carried out by the alkaline method, and agarosegel-electrophoresis was used to investigate the plasmid profile. The results showed that most of the *Pseudomonas aeruginosa* strains belonged in serogroup III. Most of the strains were multiresistant. The highest PBL production was observed for the inducible strains, and the widest substrate profiles of the PBL were seen for those strains which were multiresistant. The plasmid analysis results indicate that antibiotoc resistance and PBL production in most *Pseudomonas aeruginosa* strains are chromosomally determined.

K. CSISZÁR

## The E test, a novel method of quantifying antimicrobial susceptibility testing of bacteria

*Nógrád County National Public Health Service, Salgótarján, Hungary*

The E test (AB Biodisk, Solna, Sweden) is a reliable, new method for quantitative determination of antimicrobial susceptibility. A total of 23 different bacterial strains were tested for Cefalor E test, as well as 18 *Pseudomonas aeruginosa* strains were examined for Piperacillin E test. On evaluation the E test seems to be simple to perform, is less time consuming and labourious than other methods, thus can be set up easily in the clinical microbiology laboratory.

S. CSUKÁS, M. PÁLFALVI and R. KISS

## Aetiological agents of chronic canaliculitis

*Institute of Microbiology, and Department of Ophthalmology, Semmelweis University Medical School, Budapest, and Department of Microbiology, National Institute of Food Hygiene and Nutrition, Budapest, Hungary*

Delicate branching filaments and cocci were seen by microscopic examinations in the pus from the canaliculus lacrimalis in patients with chronic canaliculitis. *Arachnia propionica* and *Staphylococcus warneri* were cultivated and identified. According to intraperitoneal inoculation of mice by the *Arachnia* strain, characteristic morphological changes of this microorganism were observed on the peritoneum and in the spleen giving an adequate explanation of direct microscopic findings in the pus.

ZS. KOCZIÁN, E. NAGY and J. FÖLDES

## Evaluation of Uriline ID and Uricult Plus systems to detect urinary pathogens

*Departments of Clinical Microbiology, A. Szent-György University Medical School, Szeged, Hungary*

Uriline ID (BioMérieux) and Uricult Plus (Orion Diagnostica) were developed with the aim of differentiating the most common urinary pathogens, in addition to determination bacteria in urine samples. Uriline ID permits the detection of three enzymes: beta-glucuronidase, by observation of the colonies under UV light, and tryptophanase and triptophan-deaminase, by observation of the immediate colour change after use of the reagents. The presumptive detection of *Escherichia coli* and *Proteus* sp. is possible with the Uriline ID system. Use of the Cled, MacConkey and Enterococcus selective media the Uricult Plus allows the differentation of Gram-pozitive and Gram-negative bacteria. During this study the two systems were compared separately with the conventional culture method. With the Uriline ID and conventional culture methods, the same 65 of 180 urine samples proved to harbour a significant amount of bacteria. In all 26 urine samples where the conventional method showed the presence of *E. coli*, the beta-glucuronidase-positive colonies also revealed a positive in situ indole reaction. Of 12 samples which harboured *Proteus* sp. only 4 gave a positive in situ trytophan-deaminase test with the Uriline ID system. Forty-four of 108 urine samples showed a significant amount of bacteria with both the conventional method and the Uricult Plus system. In 12 cases, *Enterococcus faecalis* was cultured on the Enterococcus selective media with the Uricult Plus system, whereas only the cocultured *Proteus* sp. was found in 4 cases with the conventional method.

E. URBÁN, J. PERÉNYI, I. KÖVESDI, E. RÁCZ and J. FÖLDES

## Interaction between the host cells and parasites by investigating the free radicals and the antioxidative enzymes

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

In many pathological processes, the production of the reactive oxigen intermediates (ROI) is considered as the reason for the damages and sometimes the death of the organism. The production of ROI is accompanied with a repair mechanism: detoxification and raising of antioxidative enzymes. For the study of ROI and antioxidative enzymes proved a useful model for the investigation of the relationship between intracellular parasites and host cells. This interactions was investigated in the peritoneal macrophage and GMK cell cultures infected with *Toxoplasma gondii*. In the protozoon infected cell cultures the following characters were measured: superoxide dismutase (SOD), catalase (CAT), reduced glutathion (GSH), glutathion peroxidase (GPO), and lipid peroxidation (LPO). In the infected macrophage cultures the activity of the SOD, CAT, LPO and the GSH showed a peak at 24 h. In the infected GMK cultures the SOD and CAT production reached the peak at 48 h followed by a decrease. The GSH content was growing continuously. The production of the GPO started only in the 48th h of the infections, after 72 h it decreased. The LPO activity was going on after 48 h. These experiments were completed with the measurement of the ROI ($OH^-$, $O_2^-$ and NO) activity.

N. BATHÓ, K. CSURI and J. MOLNÁR

## Synergisms in the antiplasmid effects in vitro

*Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

Promethazine eliminates various plasmids of *Escherichia coli* depending on the plasmid copy number. The antiplasmid effect can be increased in the presence of compounds that increase drug accumulation. Verapamil increases antiplasmid action of promethazine due to inhibition of drug efflux mechanism. Novobiocin exerts synergy with promethazine as a consequence of the inhibitory effect on plasmid gyrase.

A. M. DAM, S. KAEWPILA and L. G. GAZSÓ

## Feasibility study on the radiation treatment of pharmaceuticals

*Frédéric Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary*

Whereas the use of ionizing radiation for sterilization of medical supplies and equipments has become more and more general, a widescale application of radiation treatment for pharmaceuticals and basic materials has not yet been introduced. To promote the spreading of this convenient method several experiments were carried out. For two substances (Heparin-Na, Aprotinin sol.) the radiation sterilization doses were established. For setting up the radiation dose bioburden informations are needed. The radiosensitivity of contaminating microflora and the assurance level have to be also taken into account. The average initial count was fluctuating between $10^3$ and $10^6$ microorganisms/g and in some samples pathogenic microorganisms were also found. The irradiations by substerilizing doses resulted in gradual decrease of microbial count. The required average bioburden ($10^2$ microorganisms/g) could be achieved at 15 kGy or less. Besides of the radiation pasteurization doses, sterilizing doses for the two pharmaceuticals were also calculated according to AAMI recommendations. For completing the experiments the variation of enzyme activities was also tested. The microbiological counts of all pharmaceutical basic materials tested could be reduced to the required level and the pathogenic microorganisms could be eliminated by radiation treatment safely. The radiation pasteurization could be a very suitable method to treat sensitive basic materials.

A. LIPCSEY, J. PÁSZTI, A. SZENTMIHÁLYI and I. TÓTH

## Comparative study of *Pseudomonas aeruginosa* O11a,b and O11a,c serological subgroups

*B. Johan National Institute of Hygiene, Budapest, Hungary*

The O11 serogroup of the Lányi-Bergan *Pseudomonas aeruginosa* antigenic typing scheme has two subgroups. However, the chemical characterization of O11a,b and O11a,c strains did not show any differences between the two subgroups. The purpose of our work was to find the reason for this fact. Earlier we demonstrated that reference strains of subgroups O11a,b and O11a,c could not be distinguished on the basis of whole cell protein and outer membrane protein (OMP) patterns. In this study we examined the envelope protein patterns and the LPS (lipopolysaccharide) of these strains used by proteinase-K digested samples. We applied SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) at different gel

concentrations. Neither the envelope protein nor the LPS examination revealed any differences between these strains. Only their extracellular protein patterns were different.

ZS. MAGYAR and I. STŐGER

**Bacterial contamination of vaginal secretion in pregnant women before delivery (screening examination)**

*Central Laboratory of the J. Jáhn Hospital, Budapest, Hungary*

Between June 1, 1991 and March 31, 1992 2867 vaginal secretions were tested by cultivation within two weeks before delivery. *Candida* was isolated in 986 cases, and 1101 strains of pathogenic aerobic bacteria were found in 851 samples. Double infection was present in 202 persons. Twenty four women had three pathogenic microorganisms. The bacteriological tests preceeding delivery provides an opportunity for the immediate specific antibiotic therapy of the newborns infected during delivery.

L. SZTANKOV and A. FIZIL

**Immunmodulating activities of *Saccharomyces cerevisiae* polysaccharides**

*Human Institute for Serobacteriological Production and Research, Budapest, Hungary*

Glucans (water soluble and particulate forms) and mannan have been prepared from *Saccharomyces cerevisiae* yeast cell wall. Their ability to enhance the host resistance in normal and immunsuppressed mice to *Pseudomonas aeruginosa* F5 immunotype infection was evaluated. The highest degree of host resistance increasing was observed when the mice were treated five times repeatedly day-to-day, four days before challenge. Other groups of mice were injected i.p. and i.m. with different polysaccharides and the proliferation and the nitrite production level of the macrophages were investigated in vitro. The activity and the efficiency of polysaccharides are well indicated by the increase in the nitrite production level of macrophages. Correlation was between the increase of the resistance to *P. aeruginosa* infection and the intensity of the nitrite production but the nitrite production did not depend only on the count of the macrophages.

I. ZS. GODÓ, E. MAGYAR, I. ANDIRKÓ and F. ROZGONYI

**Effect of surface active agents on colony formation of *Staphylococcus haemolyticus* in soft-agar**

*Department of Microbiology, University Medical School, Debrecen, Hungary*

It was hypothetized that the formation of compact colony in soft-agar free of serum is characteristic mainly of strains of the species *Staphylococcus haemolyticus* among coagulase-negative staphylococci is due to hydrophobic interaction between cocci. The effect of a number of surface active agents on this phenomenon was examined. Neither 0.1% and 1% Tween 80 nor 5% and 10% aethylene glycol and polyaethylene glycol nor 0.1 – 4% trypsin influenced the colony morphology in soft-agar prepared in modified Staphylococcus 110 broth. Bovine lactoferrin and apolactoferrin at concentrations of 0.1 – 0.4% made compact colonies transient to diffuse ones. Thus, cocci are not adhered to each other in compact ball-like colonies by hydrophobic interaction or trypsin-sensitive proteins. It is possible that still unknown polysaccharide-binding proteins or other trypsin-resistant proteins are responsible for the formation of compact colonies by *Staphylococcus haemolyticus* in soft-agar.

M. R. NAGY and K. K. PÁLFY

**Microbiology of the sulphuretum type waters inside Lake Velencei**

*Central Transdanubian Environmental State Agency, Székesfehérvár, Hungary*

The reed-covered territories on the Bird Reserve in the western part of Lake Velencei enclose ecologically special water bodies with original water quality and floating moors under protection. The reconstruction works in the lake bed carried out for the recreation of the lake did not modify the original sulphuretum character of these enclosed water bodies. The nutrients transported by the inflow are transformed here by decomposition. These decomposition processes are of microbial origin, mainly anaerobic and – as a consequence of the character of the lake – are connected with the circulation of sulphur. The bigger part of the nutrients transported into the lake with inflow is released from the water as gas, so the inflow to the lake's large open water areas through these moors becomes poor in nutrients. In the closed water areas and sediments of the Bird Reserve the reductive processes are predominant. In case of equilibrium the algae-skin covering the surface of the

sediments produces oxygen and organic matters. As a live covering it separates the sediment and the water and regulates the biological processes between them. An equilibrium state develops among the microbiological processes responsible for the sulphate reduction, sulphide oxidation and sulphur reduction.

M. KÁDÁR

**Application of bacteriophages in water quality control**

*B. Johan National Institute of Hygiene, Budapest, Hungary*

As problems frequently arise in interpreting the results obtained with the traditional indicator microorganisms on the field of water quality control, more and more attention is paid to bacteriophages as possible index and indicator organisms. Quantitative detection of phages is applied for indexing faecal or sewage pollution, and for indicating virus removal efficiency of natural processes or water treatment methods. The investigation are aimed to target possibly homogenous, physically and ecologically relevant phage population, to select or construct bacterial host of possibly optimum sensitivity, and to develop effective and standardized assay methods.

GY. GALGÓCZI-FARKAS, L. G. GAZSÓ, E. BOKORI and E. J. HIDVÉGI

**Cadmium resistance of *Bacillus coagulans***

*Frédéric Joliot-Curie National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary*

*Bacillus coagulans* strain L25 110 was examined for the ability to accumulate cadmium from solutions, in view of a future application of this bacterium to the detoxification of industrial effluents. The purpose of first experiments was to describe the uptake of cadmium by bacteria in relation to their resistance to this metal. The bacterial strain was cultivated in TSB supplemented with cadmium (1-600 ppm). The rate of the uptake was studied as an effect of the cultivation time, the initial cadmium concentration and pH. After cultivation the cells were harvested, washed and added to the cadmium solutions. The suspensions were shaken for 24-48 h at 30 °C and from time to time samples were removed for supernatant Cd determination. The amount of cadmium ion was determined by atomic absorption spectrophotometry. Removal of the metal from the solution was observed during the late log phase and the stationary phase (99.3% of cadmium removed in 24 h). The amount of cadmium taken up by cells increased almost linearly with increase in the concentration of

cadmium up to 100 mg/l. At pH 3.0-7.0 cadmium accumulation was similar (93.2-95.4% removed in 4 h). The amount of metal taken up by 24-h-old cells increased rapidly during the first half hour (88.7% removal of Cd) and then increased slightly with time (97.8% removal of Cd in 24 h). Different agents were used to release cadmium from *B. coagulans* cells.

# AGRICULTURAL AND FOOD MICROBIOLOGY

J. FARKAS, É. ANDRÁSSY, L. MÉSZÁROS and D. BÁNÁTI

## Combined effects of reduced pH and water activity on the growth of untreated and irradiated *Listeria monocytogenes*

*Department of Refrigeration and Livestock Products Technology, University of Horticulture and Food Industry, Budapest, Hungary*

Microtitre plates were to assess the effect of combination of antimicrobial factors on growth of *Listeria monocytogenes* 4ab No.10. Twelve concentrations of NaCl (0.3-16.8/w/v, equal increments) were combined aseptically with eight hydrogen ion concentrations in approximately equal increments from 0.1 μmol/l (pH 7.1) to 6.5 μmol/l (pH 5.2) in tryptic phosphate broth. Hydrogen ion concentrations were installed with citrate-phosphate buffer mixes. Taking into account that a gamma radiation dose of 0.8 kGy resulted in 99% destruction of a 24 h culture of our test organism, wells of microtitre plates representing pH- and salt-gradients were inoculated with untreated and irradiated (0.8 kGy) cultures, respectively, such a way that the initial viable cell count was $3x10^3$/ml in each case. Inoculated plates were aseptically sealed with self-adhesive transparent foils and incubated at 35, 30, 10 or 5 °C. Time periods for growth were recorded by visual examination and it was estimated that "visible growth" represented at least a 1000-fold increase in cell numbers. The results have shown that radiation injury in survivors of gamma irradiation increased the pH-sensitivity and the combined anti-microbial effect of reduced pH and water activity, and it increased the minimum temperature of growth. Mathematical models describing the effect of hydrogen-ion and salt concentration on the reciprocal time to visible growth (the apparent growth rate) at 30 °C of untreated and radiation-injured inocula, respectively, were constructed after analysis of the results for a least squares fit to a quadratic model. Synergistic interactions between salt and hydrogen-ion concentration on growth were found, which were enhanced in case of the irradiated inocolum.

O. REICHART

## Reaction kinetic interpretation of the effect of disinfectants

*Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary*

In describing the thermal or chemical destruction of microorganisms, the most commonly used mathematical model is based on the analogy of the first order chemical reactions. Assuming that the inactivation of the microbes is due to a chemical reaction between a "critical structure" of the living cell and an other reactant molecule, the Eyring's theory of absolute reaction rates can be applied to this reaction. Considering the stoichiometric equation of this chemical reaction, the disinfection kinetics of the microbes can be described by the extension of the Eyring's model. The modified model is applicable to describe not only the heat inactivation, but the disinfection kinetics and the effect of the pH on the heat destruction as well.

ZS. KOMLÓ, L. FARKAS and A. SZENTIRMAI

## Effects of the bromthymol-blue on a *Xenorhabdus nematophilus* strain

*Department of Microbiology, L. Kossuth University, Debrecen, Hungary*

*Xenorhabdus* spp. are bacteria associated with entomopathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae*. The bacteria are pathogenic for the insect host when released into the haemolymph by the nematodes. They support nematode reproduction by producing nutrients and antimicrobial agents that inhibit the growth of a wide range of organisms. All *Xenorhabdus* isolates have been shown to produce two colony forms: the primary and the secondary form. The primary form is unstable in vitro and in vivo, producing the secondary form. Only the primary form produces suitable conditions for the nematodes and it has some phase characters: bromthymol-blue adsorption, antimicrobial activity, lecithinase, etc. A generally used method for the isolation of the symbiont bacteria is based on the bromthymol-blue adsorption. As we described earlier, the primary-secondary transition can be studied and intermediate forms can be isolated on the base of the amount of the adsorbed bromthymol-blue. By our experiences the bromthymol-blue has influence on the growth and the antimicrobial activity of the bacteria.

Z. NAÁR and M. KECSKÉS

**Competitive saprophytic ability of some antagonistic *Trichoderma* fungi**

*Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary*

The competitive saprophytic ability of 30 *Trichoderma* strains antagonistic against *Sclerotinia minor* (Jagger) – a polyphagous phytopathogenic fungus – has been tested in brown forest soil with agar disc method. The soil in Petri dishes was infested with conidial suspensions (serial dilution method) of 3 *Trichoderma* strains (*T. hamatum* – slow growing, *T. viride* – rapid growing, and *T. harzianum* – very rapid growing). Significant difference was found among the strains, but not among their amount of conidia. So only one dose ($10^6$ conidia/$cm^3$) was applied in the screening of 30 strains. The competitive saprophytic ability was associated with strain and not with species. Twelve of thirty strains were competitive against the microflora of the soil applied. Three of five mycoparasitic strains isolated from different propagules of fungi (sclerotia or rhizomorfa) were significantly uncompetitive. No clear regression was found between the competitive saprophytic ability and the cellulolytic activity measured with congo red method on CMC-containing media, and the growth rate on 25 °C.

É. KÁRPÁTI and T. SIK

**Host plant specific chemotaxis or rhizobia**

*Department of Biotechnology, University of Agricultural Sciences, Gödöllő, Hungary*

Rhizobia are migrating in the soil chemotaxis towards the root hairs of leguminous plants, to establish symbiosis. This movement is induced by root exudates of legumes. The experiments were aimed: (i) to prove the host plant specific chemotaxis of rhizobia; (ii) to identify chemotaxis genes in *Rhizobium meliloti*, particularly those of plant specific chemotaxis. A method by exponential dilution of root exudates, as attractants was worked out to detect host plant specific chemotaxis. Chemotaxis for food (amino acids, organic acids, sugars, etc.) and for plant specific attractants was clearly distinguished. It was proved that *R. meliloti, R. leguminosarum* and *R. leguminosarum* bv. *trifolii* are attracted by root exudates of alfalfa, pea and white clover, respectively. Root exudates of maize and wheat have no specific effect or rhizobia. To identify chemotaxis genes of *R. meliloti*, Tn5 induced motility, food and plant specific chemotaxis mutants were isolated and characterized. Mutant gene sites were complemented and mapped with known *R. meliloti* chemotaxis gene

clones. Mutations were localized by hybridization with Tn5 DNA probe on different EcoRl fragments of genomic DNA. This way food and plant specific chemotaxis genes could also be distinguished.

K. Posta

**Mobilization of manganase from calcareous soil by microbes and root exudates in maize**

*Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary*

Influence of rhizosphere microbes and vesicular-arbuscular (VA) mycorrhiza or manganase uptake in maize plants was investigated in pot experiments. Maize (*Zea mays* L.cv.Tau) plants were grown in sterilized calcareous soil for seven weeks in pots having separate compartments for growth of roots and vesicular-arbuscular (VA) mycorrhizal fungal hyphae. Soil was inoculated with rhizosphere microorganisms only (MO-VA) or with rhizosphere micro-organisms together with a VA mycorrhizal fungus *Glomus mosseae* (Nicol and Gerd.) Gerdemann and Trappe (MO+VA). Manganese was determined by atomic absorption spectrometry. Concentrations of Mn in shoots decreased in the order MO-VA > MO+VA > control treatment. In the rhizosphere soil, the total microbial population was bigger in mycorrhizal (MO+VA) than non-mycorrhizal (MO-VA) treatments but the proportion of Mn-reducers was 5-fold higher in the non-mycorrhizal treatment suggesting substantial qualitative changes in rhizosphere microbial populations upon root infection with the mycorrhizal fungi. The results demonstrate that change in rhizosphere micro-organism population is at least partly responsible for lower acquisition of Mn by mycorrhizal plants. Relatively high rates of $MnO_2$ mobilization by root exudates of non-mycorrhizal plants are also important for Mn mobilization in the rhizosphere which is strongly dependent on increased root length.

E. SZIGETI

## An applicable combined method for the inhibition of moulds and yeasts in the food industry

*University of Horticulture and Food Industry, Budapest, Hungary*

The aerobic spoilage of foods is mainly caused by moulds and yeasts. The inhibition of these microorganisms is possible with combined method only, due to the limited possibility of the application of radiation, chemicals and heat treatment. In our experiments the destruction and inhibition of three fungi (*Penicillium expansum, Candida parapsilosis, Pichia membranaefaciens*, isolated from cheese) were investigated. The aim of these experiments was giving parameters for a possible industrial application of UV radiation and combined chemical treatment. The surviving curves of UV radiated *P. expansum* and *P. membranaefaciens* consist of two parts. For the inhibition of the surviving fraction a mixture of propionic acid and potassium sorbate was used in several concentrations. The combined application of the two inhibitors results significant synergistic effect in the range of the low concentrations. This inhibition effect was greater than in the case of the control natamycin. As a summary of the results, for the inhibition of the surface spoilage of some food products, a short time UV radiation and chemical inhibition, followed by an anaerob packaging can be recommended.

GY. TURÓCZI and L. VAJNA

## Antagonism of *Trichoderma* species isolated from the rhizomorphs of *Armillaria* mellea s. l.

*Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary*

The most abundant sources of antagonistic microorganisms are the ecological niches where any kind of disease suppression can be observed. Such niches are the disease suppressive soils or the macroscopic parts of phytopathogenic fungi (i.e. sclerotia, rhizomorphs). Antagonistic microbes isolated from these niches can be expected to be more effective bio-control agents of plant pathogens than randomly isolated microorganisms. It is known since the thirties that trichodermas are frequent on the rhizomorphs of *Armillaria mellea* s. l., causing agent of root rot of woody plants. These trichodermas proved to be antagonistic to *Armillaria*. Vajna found in the course of a study on oak decline that trichodermas on *Armillaria* rhizomorphs were frequent in Hungary, too. *Armillaria* rhizomorphs from the areas of oak decline

were washed in sterile water and the pieces of rhizomorphs were incubated in moist chamber for 3-4 days. Most of the fungi growing on the cortex layer of rhizomorphs were trichodermas. Nearly half of the isolates were *T. viride, T. koningii, T. harzianum, T. hamatum* and *Gliocladium virens* could be isolated, too. The anatagonistic effect of isolates was tested to phytopathogenic fungi *Armillaria cepistipes, Phythium ultimum, P. debarianum, Fusarium culmorum, F. graminearum, F. oxysporum, Verticillium dahliae, V. tenerum, Botrytis cynerea, Alternaria alternata, Bipolaris* sp., *Phoma betae, Sclerotinia sclerotiorum, Rhizoctonia solani, Ceratocystis piceae*. Both the mycoparasitic ability of the isolates and the production of antibiotic substances and extracellular enzymes were recorded, and the two kind of antagonistic effect were compared. All of the isolates proved to be antagonist to the most tested fungi. The dominant mechanism of antagonism was the production of antibiotic metabolites in most instances, while a minor part of isolated parasited on the test fungi. Some *Trichoderma* isolates showed both antibiotic and mycoparasitic activity.

H. E. A. F. BAYOUMI and M. KECSKÉS

**Influence of nitrate concentrations on the nodulation of *Vicia faba* in the presence of *Pseudomonas fluorescens***

*Department of Microbiology, Universitity of Agricultural Sciences, Gödöllő, Hungary*

We investigated the effect of ammonium nitrate at five doses on the growth of *Rhizobium leguminosarum* bv. *viceae* strains using microfermentor technique, also their symbiosis with two Libyan varieties of *Vicia faba* in pot experiment, with or without co-inoculation of *Pseudomonas fluorescens* in the presence of 50%, 100%, and 150% or field applied rate of ammonium nitrate. The seedlings were grown in greenhouse for seven to eight weeks, the interaction of cultivars and *Rhizobium* in soil environment polluted with ammonium nitrate doses was measured as total plant, shoot (S), root (R) height, S/R ratio, nodule number, dry weight of plant biomass and nodules, total nitrogen content as well as estimated nitrogen-fixed per plant and efficiency of nitrogen utilization. The recorded data were compared to two controls (a) uninoculated seedling, (b) seedling inoculated with *Rhizobium* strains. The results were evaluated statistically, and it was found that: (i) 50% of applied field rate of ammonium nitrate enhanced the nodulation, and plant biomass, whereas the 100% and 150% doses inhibited the nodulation, but the plant biomass, and total nitrogen per plant were increased. (ii) The two Hungarian strains of *Rhizobium* were capable

to fix nitrogen symbiotically with two Libyan cultivars more than the English and Libyan strains. (iii) The co-inoculation of seedlings with *P. fluorescens* decreased the depression of the nodulation caused by high doses applied of ammonium nitrate. (iv) *Rhizobium* strains were variable in their response to ammonium nitrate doses.

T. PONYI, I. ZOLOTÁRJOV, É. KÁRPÁTI and T. SIK

## Chemical comparison of attractants from leguminous plant root exudates

*Department of Biotechnology, University of Agricultural Sciences, Gödöllő, Hungary*

Symbiosis is a complex process, in which the very first step is the chemotactic migration of rhizobia towards the plant root. This migration is induced by plant root exuded compounds. By measuring the chemotaxis inducing activity of the leguminous exudates with different *Rhizobium* species, it was shown that chemotaxis is host specific, so the plant attracts its own symbiont more efficiently than other *Rhizobium* or bacterium species. Host specific chemotaxis is induced by compounds of molecular weight less than 5000 Dal, determined by gel filtration. Our aim was the comparative analysis of these low molecular weight substances in the root exudate of alfalfa pea and white clover. Amino acids were determined and flavonoids were separated in the root exudate of leguminous plants. By chemical comparison of the exudates we had the chance to choose the possibly host specific compounds. These are, on one hand those existing in the exudate of young seedlings and may disappear later, on the other hand the ones distinct in different leguminous plants. The alfalfa, pea and white clover root exudates are greatly different in amino acid and flavonoid composition. Compounds characteristic of each leguminous plants were separated from the root exudates of early seedlings. We intend to prove host specificity of these compounds by chemotaxis measurement.

J. KUKOLYA and CS. DOBOLYI

## Measurement of FDA-hydrolysis for comparative studies of microbial activity in soils

*Department of Microbiology, University of Agricultural Sciences, Gödöllő, Hungary*

Spectrophotometric determination of the hydrolysis of fluorescein diacetate [3,'6'-diacetylfluorescein (FDA)] is a simple, sensitive and rapid method for determining microbial activity in soil. We determined FDA hydrolytic activity in

sample of 8 different soil types parallel with the quantification of bacteria, fungal propagules, streptomyces and 5 physiological groups of microbes: ammonium oxidizing bacteria, free-living nitrogen fixers, ammonifyers, denitrifyers and cellulolytic microbes. We used linear regression analysis for comparation of the results of the esterase activity measurement with the classical enumeration of different cultured microbes. The results of the statistical analysis: (i) positive correlation was found between esterase activity and the number of ammonifyers ($r = 0.735$), total number of bacteria ($r = 0.365$) and denitrifyers ($r = 0.280$); (ii) negative correlation was found in the case of the number of ammonium oxidizing bacteria ($r = 0.566$); (iii) there was no correlation at all between esterase activity and the number of fungal propagules ($r = 0.125$), streptomyces ($r = 0.031$), free living nitrogen fixers ($r = 0.182$) and cellulolytic microbes ($r = 0.006$). In further experiments we used the FDA-test for examining the side effect of the herbicide EPTC on soil microbiota. When 50 mg/kg EPTC dosis was used, "stimulative" effect could be found both in the esterase activity and the quantity of ammonifyers and total number of bacteria.

Z. NAÁR and M. KECSKÉS

**Saprophytic and mycoparasitic *Trichoderma* strains antagonistic against *Sclerotinia minor* (Jagger)**

*Department of Microbiology, University of Agricultural Sciences, Gödöllő*

Saprophytic *Trichoderma* strains (*T. hamatum, T. harzianum, T. koningii, T. longibrachiatum, T. pseudokoningii, T. viride*) were selected on in vitro mycoparasitic activity (damage of sclerotia and parasitism on hyphae of Sclerotinia minor). The longitudinal growth rate of effective antagonists (PDA medium) and *S. minor* (glucose-peptone-NaCl agar and PDA media) were measured at 10, 15, 20, 25, 30, and 35 °C. The antagonism of selected saprophytic and some mycoparasitic *Trichoderma* strains (isolated from different fungal propagules: sclerotia or rhizomorpha) against *S. minor* sclerotia in autoclaved soil were measured at 15 °C and 25 °C. It was found that the saprophytic *Trichoderma* strains were active parasites on sclerotia and on hyphae of *S. minor*, but the phytopathogenic fungus showed reverse parasitism on the hyphae of some *Trichoderma* strains also. The optimal temperature for growth was 25-30 °C for *Trichoderma* strains and 20 °C for *S. minor*. The amount of damaged sclerotia showed significant difference in sterile soil: at 15 °C and 25 °C was 6-26% and 22-70%, respectively. The saprophytic and the mycoparasitic character of *Trichoderma* strains had no significant effect on the activity.

V. T. PINTÉR, Á. KOSTYÁK and A. FÁBIÁN

## New methods of salmonella detection in foods

*Food Investigation Institute, Budapest, Hungary*

The ELISA technique and Rambach agar, a new selective medium were used for detection of salmonellae in seni-ready, raw meat products and hygienic swab samples. For ELISA test, Salmonella Visual Immunoassay Kit was applied. For cultivation enrichment media were used. The ELISA method detected: $10^5$ cells/ml in the enrichment media inoculated with the most common salmonellae. The kit is suitable for 90 tests for two months. The relationship in antigen structure of *Citrobacter freundii* did not cause false positive results. The test give negative results with concomitant microflora commonly existing in foods. The results of ELISA were similar to the traditional method based on biochemical reactions and agglutination. On the surface of Rambach agar the salmonellae grow in red, while other bacteria in variously coloured colonies. According to the informative description of Rambach agar 97-99% of the salmonella strains give the typical colour except *S. paratyphi*-A and -B and *S. typhi* which are colourless. In our investigation *S. cholerae-suis, S. abortus-ovis, S. gallinarum* and *S. typhi-suis* were colourless, too.

J. GASPARIK-REICHARDT, A. NÉMETH and B. RALOVICH

## Incidence of *Listeria* in a meat plant

*Hungarian Meat Research Institute, Budapest, Hungary*

The aim of our works was to determine the presence of *Listeria* in different parts of a pig slaughterhouse and meat processing factory and also to control the effectiveness of everyday cleaning and disinfection practice. With sterile cotton swabs 191 samples were taken either during the working period (120 samples) or after the usual cleaning and disinfection (71 samples) from hands, gloves, tools of the workers, surface of the carcasses, equipments, walls and drains of the plant. Fortytwo (35%) out of 120 samples proved to be positive; 16 positive samples originated from meat surface, the others from the surface of tools and equipments. The incidence of the positive samples was the lowest (4.6%) at the slaughtering line and the highest (83.3%) in the drying and chilling chamber. Among the samples taken on the slaughtering line only a very few were listeria-positive. These bacteria could be detected more often in the chilling rooms, and they were found almost in each samples of the processing rooms. Out of the 71 samples taken after cleaning and disinfection 11 (15.5%) were positive. Cleaning and disinfection decreased the

number of positive samples but listeriae still remained present. On the basis of these results it can be stated that the hygienic conditions in the plant were not appropriate and that the cleaning and disinfection practice was not effective enough.

J. GASPARIK-REICHARDT, A. NÉMETH and B. RALOVICH

## Incidence of *Staphylococcus aureus* in a meat processing plant

*Hungarian Meat Research Institute, Budapest, Hungary*

The aim of our work was to determine presence of *Staphylococcus aureus* in different parts of a pig slaughterhouse and meat processing factory as the sausages produced were several times objected because of that bacterium. We wanted to determine the critical control points from where the meat could be contaminated and also to check the effectiveness of everyday cleaning and disinfection practice. With sterile cotton swabs 147 samples were taken either during the working period (98 samples) or after the usual cleaning and disinfection (49 samples) from hands, tools of workers, surface of carcasses and equipments of the plant. During the work 19% of samples were positive in the deboning room and 36% were positive at the slaughtering line. After cleaning and disinfection, samples were taken from both the slaughtering line and processing room. On the first occasion 24% and in the second case 19% of the samples were positive. Accordingly, the hygienic conditions were not suitable in the plant, and the cleaning and disinfection practice was also insufficient.

B. BIRÓ, T. SZILI-KOVÁCS and J. SZEGI

## Some characteristics of $N_2$-fixing *Azospirillum* strains isolated from maize and wheat roots

*Research Institute for Soil Science and Agricultural Chemistry, Hungarian Academy of Sciences, Budapest, Hungary*

$N_2$-fixing bacteria were isolated from the rhizosphere and histosphere of agricultural plants (maize – *Zea mays* L. and wheat – *Triticum aestivum* L.). After testing the most important biophysical and biochemical characteristics (Gram-reaction, form, size, pigmentation, utilization of different carbon-sources, biotin requirement, anaerobic acidification with glucose and sucrose, nitrogenase activity, denitrification capability) some of them proved to be *Azospirilla* according to the classification of Tarrand et al. (1978). Data comparing with authentic Brazilian

strains (*A. brasilence* sp. 7. and *A. lipoferum* sp. 59.) showed that from our isolates 3 proved to be *A. lipoferum* and 7 *A. brasilense*. In the rhizosphere of maize (C4-plants) *A. brasilense* were more numerous and in case of the wheat (C3-plants) we could find mostly *A. lipoferum* strains. This finding shows only small correlation with literary data.

R. KISS and M. RODLER

## Examination of *Listeria* strains of food origin

*National Institute of Food-Hygiene and Nutrition, Budapest, Hungary*

As reported by several authors in the past years, foodborne infections play a considerable role in listeria infections. The following samples were examined: meat products conserved with seasoning, drying 125; brawn 10; cakes with egg, cream, honey, cheese 10; buffet meals 9; deep-frozen meatball 7; sausages, ham 6; smoked cheese 5; sheep's cottage cheese 2; environmental sample 2. Twenthy-five g sample were examined in 100 ml brain-heart broth made selective with CNA. After 2-4 days incubation at 30 °C, streaking was performed on Columbia agar with 5% blood and cultured for 24 h at 30 °C in increased $CO_2$ atmosphere. In addition to others, the following tests were used for identification: API 20 Strep, API Coryne, API Listeria (bioMérieux). A total of 147 strains were identified, 143 strains were examined with API CH 50 test, and 145 strains were serotyped with the following result: serotype 1/2: 115 (79.3%); serotype 4 ab: 30/20.7%. In 52% of 87 strains, lysis could be observed with the phage set of Durst.

L. LAKATOS

## Study of the genetic background of killer activity in *Candida glabrata*

*Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary*

Certain strains of *Candida glabrata* produce a proteinaceous killer toxin (zymocin) that kills the sensitive cells of the same and different species. In my experimental work I studied the genetic background of the killer activity in *C. glabrata*, i.e. I wanted to localize the genes coding for the toxin production and immunity whether they were found in the nucleus or in the cytoplasm. I also studied the effect of the mitochondrial function on the expression of the killer phenotype. I

used manganese ions and ethidium bromide as specific mutagens of yeast mitochondrial DNA for the induction of non-killer mutants. As the consequence of $MnCl_2$ treatment either $rho^-$ non-killer ($kil^-$) or $rho^+$ $kil^-$ mutants were isolated, while ethidium bromide induced $rho^-$ $kil^-$ mutants. All the respiration deficient mutants showed the non-killer phenotype that indicated the basic necessity of mitochondrial function for the killer toxin production. When killer strains were treated with respiratory inhibitors as oligomycin and diuron the killer toxin production was completely ceased. I tried to isolate killer plasmid(s) from the strains studied but failed to find any plasmid in agarose gel electrophoresis. I used somatic hybridization (protoplast fusion) to transfer killer genes to non-killer ($kil^-$ or $kil^0$) strains. The results obtained indicated the chromosomal inheritance of the killer activity.

K. SZAKMÁR

**Effect of environmental factors on the growth of yeasts**

*Department of Microbiology and Biotechnology, University of Horticulture and Food Industry, Budapest, Hungary*

The effect of pH, water activity, redoxpotential and temperature on the growth parameters of some yeasts of importance in the food industry was investigated. In the experiments the lag-period, specific rate of growth and the generation time were determined at four pH (3.0, 3.5, 4.0, 4.5), three water activity (0.995, 0.920, 0.850), three redoxpotential (from 0 to 400 mV Eh) values at 20 and 30 °C temperature. The microorganisms used in the experiments were *Saccharomyces cerevisiae*, *Yarrowia lipolytica* and *Zygosaccharomyces bailii*.

From the results multivariate mathematical models were constructed, which are applicable for describing the parameters of growth in the investigated range of environmental factors.

C. FEKETE, I. PAPP, A. TÓTH, A. POMÁZI and L. HORNOK

**Identification of new mitochondrial plasmids in nontoxigenic *Fusarium* strains**

*Agricultural Biotechnology Center, Gödöllő, and University of Agricultural Sciences, Gödöllő, Hungary*

*Fusarium* species known to produce trichothecene mycotoxins show great intraspecific variability. Not all isolates of a particular species are toxigenic, while some strains produce A types, others synthesize B types of trichothecenes. A survey

was started in order to identify toxin chemotypes of these fungi and reveal restriction fragment length polymorphisms (RFLPs) among strains differing in toxin production. The four major trichothecenes – deoxynivalenol, diacetoxyscirpenol, nivalenol and T-2 toxin – were identified by thin layer chromatography. Total DNA (tDNA), as well as mitochondrial DNA (mtDNA) were subjected to RFLP analysis. When undigested mtDNA samples were electrophoretized in agarose a series of bands appeared in certain strains of *Fusarium poae* and *Fusarium semitectum*. Nearly 40 isolates were examined and 30% of them, only nontoxigenic ones contained these DNAs. In more detailed experiments these DNAs turned out to be plasmids. They are localized in the mitochondria, their sizes range from 0.4 to 6.4 kb. A 1.9 kb plasmid from *F. semitectum* was shown by exonuclease III digestion to be linear and this was confirmed by restriction endonuclease mapping. When this plasmid was used as probe in Southern hybridization, no homology was detected between the plasmid and the nuclear and mitochondrial genome of the plasmid-bearing strains. Hybridization studies revealed no substantial sequence similarity among the different plasmids. The majority of the plasmid-bearing, nontoxigenic strains gave strong positive signals, when their *BamHI* digested tDNAs were probed with *Tox5*, a gene known to specify a key-enzyme in trichothecene biosynthesis. This suggests, that toxin production is in some way disturbed by these nonhomologous, autonomously replicated, linear mitochondrial plasmids, while they do not have a direct effect on the synthesis of the trichothecene skeleton itself. The similarity between these plasmids and those known to have deleterious effects on their host cell (e.g. senescence plasmids) seems to be worthy of further studies.

# MYCOLOGY AND INDUSTRIAL MICROBIOLOGY

E. RÁCZ, S. NEDELKOVICS and J. FÖLDES

## Characterization of *Candida* isolates by antibiotic sensitivity, bio-, sero-, and caryotyping for diagnostic and epidemiological studies

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

More than 1000 *Candida* strains were isolated in a four month period and isolates were characterized by cultural and morphological methods, by bio-, sero-, and caryotyping as well as by gas chromatography. *C. krusei* occurred the most frequently, followed by *C. albicans*, *C. pseudotropicalis* and *C. tropicalis*. Another 10 species were also identified. The majority of *Candida* was isolated from the urogenital tract followed by the trachea and the lower respiratory system. Ninety per cent of the isolates were sensitive to Nystatin, 50% to Fungizone, 25% to Nizoral, 20% to Fluconazol and 10% to Ancotil. The antagonism was observed between the antimycotica in the agar diffusion test, the most frequently between the Nystatin and Fluconazol. In serological tests cross-reaction was observed between the *C. albicans* and *C. tropicalis*, but not with the *C. krusei and C. pseudotropicalis*. The purified mannan antigen gave the same results as the commercial products. Karyotyping the yeast chromosomes was performed by pulsed-field gel-electrophoresis in one strain each of the isolated 14 species. The same strains were used in the gas chromatographic analysis.

B. RIBÁR and A. SZENTIRMAI

## Protoplast fusion and hybridization of *Penicillium chrysogenum* and *Schizosaccharomyces pombe*

*Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, Hungary*

In the course of our experiments we used a high-producer strain of *Penicillium chrysogenum* as a gene donor. As gene acceptor we used *Schizosaccharomyces pombe* var. *pombe* lysl-131. The best conditions were created for optimal protoplast yield of both strains. The *P. chrysogenum* had not been marked, so it was inactivated with malachite green. The efficiency of the inactivation was 92-99.9%. Owing to malachite green the protoplast membrane was fixed and no lysis took place upon adding water. After the fusion, all the regenerated colonies had penicillium-like morphology, whilst

*S. pombe*-like colonies increased upon fusion with *S. pombe* protoplasts (*P. chrysogenum* × *S. pombe*) as compared to the control self-fusion (*P. chrysogenum* × *P. chrysogenum*). In the following experiments we ultrasonicated the mycelia followed by protoplast formation and interspecific fusion. Small-sized $lys^+$ *S. pombe* colonies appeared in this experiment providing evidence for successful fusion. However, this lysl prototrophy was unstable, as the $lys^+$ first-generation colonies yielded $lys^-$ auxotrophic segregants upon restreaking.

G. BARTÓK, T. EMRI and J. KOZMA

## Regulation of pentose phosphate pathway in *Penicillium chrysogenum*

*Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, and BIOGAL Pharmaceutical Co., Biotechnological Research Center, Debrecen, Hungary*

Pentose phosphate pathway (HMP) and the Embden-Meyerhof-Parnass (EMP) pathway have a definite rate in *Penicillium chrysogenum*: in the case of idiophase three times higher amount of glucose is metabolized via HMP than by EMP route. We reported earlier that the fructose-2,6-biphosphate (F-2,6-$P_2$) can be found in *P. chrysogenum* too and the concentration of F-2,6-$P_2$ depends on the extracellular concentration of glucose. The F-2,6-$P_2$ is known to be a very efficient regulator of EMP pathway. It has been shown in plants that the F-2,6-$p_2$ regulates not only glycolysis but the pentose phosphate pathway respectively: F-2,6-$P_2$ inhibits the 6-phosphogluconate dehydrogenase. We demonstrated this regulatory mechanism in *P. chrysogenum* investigating of partially purified (DEAE-Cellulose chromatography) enzyme.

M. BOGÁTI, N. MOLNÁR and A. SZENTIRMAI

## Glutamate-dehydrogenase of *Penicillium chrysogenum*

*Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, and BIOGAL Pharmaceutical Co., Debrecen, Hungary*

This enzyme, apart from the fact that it plays a key role in the nitrogen metabolism of this fungus has interesting kinetical properties. In addition to the research of its physiological characteristics, we have also started the enzymological analysis. The process catalysed by the enzyme is ammonia + $\alpha$-ketoglutarate + NADPH $\rightleftharpoons$ glutamate + NADP + water. The equilibrium is moved away to the synthesis. No NADH-dependent GDH activity was shown. On the basis of the

literature and our previous stability and linkage examinations we have purified the enzyme from an industrial strain of *Penicillium chrysogenum*. Steps of the purification were: 1. Disruption of the mycelia by X-Press at -20 °C; 2. Heat treatment; 3. Precipitation by ammonium-sulphate; 4. Purification by DEAE-Cellulose chromatography; 5. Water deprivation by CM-Sephadex 50. Yield: 50%, purification: 70-fold.

The enzyme was desintegrated to subunits during purification. The molecular weight of the monomers is about 50000 Dal, the isoelectric point is 4.6; the pH optimum is 8.0. The enzyme is hindered by its own substrates: α-ketoglutarate, and NADPH. Michaelis constants were [crude preparation/purified preparation/corresponding literary data]: α-ketoglutarate 2.3/?/2.2;1.4;1.6; NADPH 0.1/0.09/0.07; ammonium 5.7/6.0/2.4;2.5;2.4. Examining the regulation of the enzyme activity we established that the ATP impedes it both in the case of the crude and the purified enzyme. Using ADP or AMP we failed to produce this effect.

N. MOLNÁR, M. BOGÁTI and A. SZENTIRMAI

## Effect of carbon and nitrogen sources on the biosynthesis of glutamate-dehydrogenase of *Penicillium chrysogenum*

*Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, and BIOGAL Pharmaceutical Co., Debrecen, Hungary*

The literature and our previous examinations suggest that glutamate-dehydrogenase (Glu-DH) plays a key role in the ammonium-uptake and assimilation of *Penicillium chrysogenum*. Since in the course of penicillin production in the production phase the N-source is ammonia, it is important to know the precise mechanism of uptaking. But in addition to the N-source, the carbon-source also plays an important role in the changes of Glu-DH level. Therefore besides the previously examined glucose we started to research the effect of the fructose and saccharose. In our experiments the following results were obtained: 1. The Glu-DH is formed continuously. 2. The enzyme-level is far greater in young, growing mycelia than in the old one. 3. Glucose is a better carbon source than sucrose and fructose. After the end of growth and depletion of glucose Glu-DH is formed with an unchanged pace throughout 10-15 h. 4. Using N-source (ammonia or glutamate) beside the glucose, the enzyme-level decreases after glucose runs out. In addition to changes of GDH-level we also measured the glutamate pool. Our results show that the regulation of Glu-DH synthesis is probably attached to the glutamate and the repressive effect of ammonium is effective through the glutamate. 5. Using fructose we got similar result,

but the repression ensued immediately in the presence of N-sources. 6. N-sources also show their repressive effect in the presence of sucrose, but in this case the enzyme level instead of increasing, decreased in the control culture.

I. PÓCSI, T. PUSZTAHELYI, A. ALEXA, M. BOGÁTI, L. KISS and A. SZENTIRMAI

## The formation of N-acetyl-β-D-hexosaminidase is repressed by glucose in *Penicillium chrysogenum*

*Institute of Biochemistry, L. Kossuth University, Debrecen, BIOGAL Pharmaceutical Co., Debrecen, and Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, Hungary*

The formation of N-acetyl-β-D-hexosaminidase is under carbon catabolite repression in growing *Penicillium chrysogenum*. The enzyme accumulates inside cells during the stationary phase of growth and is released into the culture fluid when the cell wall lysis has started and progressed. On the other hand, N-acetyl-D-glucosamine is not utilized by the organism even in the complete absence of other carbon-sources and does not influence either the mycelial growth or the hexosaminidase formation. The enzyme splits N,N-diacetylchitobiose in addition to the synthestic PNP-GlcNAc and PNP-GalNAc substrates, which together with the time-course of enzyme formation strongly supports that fungal N-acetyl-β-D-hexosaminidase may have a role in degrading intermediate cell wall breakdown products under starvation conditions. The physical and enzyme kinetic properties of both intracellular and extracellular N-acetyl-β-D-hexosaminidases are really the same indicating that the enzyme is released into the culture broth without any modification. Extracellular: pl = 4.9; Mr = 141 000; intracellular: pl = 5.0; Mr = 138 000. Kinetic characterization of *P. chrysogenum* N-acetyl-β-D-hexosaminidase:

| Parameter | Compound | Intracellular enzyme | Extracellular enzyme |
|---|---|---|---|
| Km(M) | PNP-GlcNAc | $(4.6 \pm 0.1) \times 10^{-4}$ | $(4.7 \pm 0.3) \times 10^{-4}$ |
| | PNP-GalNAc | $(1.34 \pm 0.06) \times 10^{-3}$ | $(1.3 \pm 0.1) \times 10^{-3}$ |
| Vmax(nkat) | PNP-GlcNAc | $0.236 \pm 0.003$ | $0.273 \pm 0.008$ |
| | PNP-GlcNAc | $0.218 \pm 0.005$ | $0.251 \pm 0.008$ |
| Vmax-ratio[a] | | 1.083 | 1.088 |
| Ki(M) | TP-GlcNAc[b] | $(1.15 \pm 0.02) \times 10^{-2}$ | $(0.92 \pm 0.04) \times 10^{-2}$ |
| | PNTP-GlcNAc[c] | $(1.06 \pm 0.03) \times 10^{-3}$ | $(1.06 \pm 0.03) \times 10^{-3}$ |

a:Vmax(PNP-GlcNAc-ase)/Vmax(PNP-GalNAc-ase).
b:Non-competitive type of inhibition.
c:Competitive type of inhibition.

J. KOZMA and A. SZENTIRMAI

## Aging of *Acremonium chrysogenum*

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The alternative (cyanide resistant) respiration (AR) pathway is known to be existed in higher plants since 1937. It was demonstrated in many microorganisms including fungi. We demonstrated that the alternative respiration pathway works in *Acremonium chrysogenum* (synonym: *Cephalosporium acremonium*). The activity of cyanide resistant respiration increased during the fermentation period. It seems to be independent of the carbon source applied (glucose or soybean oil) although there was a considerable difference between the activities measured in mineral and complex medium. The most important property of the alternative respiration pathway is non-phosphorylation: it is able to oxidize the reduced coenzymes without ATP production. On the other hand, the AR pathway generates a huge amount of free radicals. The free radicals might be caused the aging of the fungus. The increasing activity of AR pathway was not the only sign of the aging process. The second sign was the decreasing of the specific respiration (oxygen consumption) of the fungus. The third sign was a yellow pigment production at the end of the fermentation. Finally, the well known morphological development of *A. chrysogenum* should be mentioned: the mycelia start fragmentation and form arthroconidia.

ZS. HORVÁTH, E. K. NOVÁK, A. SZÁNTHÓ, P. OSVÁTH and I. VINCZE

## Common allergen in *Alternaria* and *Phoma*

*B. Johan National Institute of Hygiene, Budapest, and Szabadsághegy Pediatric Institute, Budapest, Hungary*

In screening of astmathic children with skin test (PRICK) and for fungus specific IgE (RAST), associated positivity to *Alternaria alternata* and *Phoma betae* allergens were frequently found. The possibility of common allergen(s) in these two dark pigmented fungi arose. The $2 \times 2$ contingency analyses of Prick tests, and RAST results gave chi square values 101.4, and 70.8 for the association. In contrast, however, to these tightness, out of 18 sera with double positivity in 6 cases the pretreatment with *A. alternata* or *P. betae* allergens gave no decrease in the RAST reaction with the other allergen. In 4 sera, however, min. 50% decrease was caused in their reaction against the other allergen by both pretreatments, while in the remaining 8 cases the pretreatment only with *A. alternata* allergen caused a decrease in the reaction with *P. betae* (*P. betae* pretreatment had no effect on their *A. alternata* reaction). Thus in producing allergies out of the known ca. 50 *Alternaria* and ca. 70

*Phoma* species those sharing intergeneric common allergen(s) are less frequently involved, while in most cases those with no or only unilateral allergenic relationships are represented (in these cases double or multiple allergizations cause the associated alternaria- and phoma-positive reactions).

ZS. HORVÁTH, H. E. ASHOUR and E. K. NOVÁK

## Isolation of heavy metal tolerating fungi

*B. Johan National Institute of Hygiene, Budapest, Hungary*

In view of the increasing environmental pollution with heavy metals and its elimination, fungi and their tolerance became of interest. The isolation from air of fungi was attempted by exposing Sabouraud plates each containing heavy metal ions ($As^{5-}$, $Ba^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Cr^{3+}$, $Cu^{2+}$, $Hg^{2+}$, $Mn^{2+}$, $Mn^{2+}$, $Ni^{2+}$, $Pb^{2+}$) in 1 mM concentration. Among the isolated 82 strains 52 tolerated also the 10 mM (0.5-2.0 g/l) concentration (4 *Alternaria*, 22 *Aspergillus*, 4 *Cladosporium*, 14 *Penicillium*, 3 *Rhodotorula*, 1 *Stachybotrys*, 1 "yeast", 3 "sterile mycelium"). Moreover, out of them 15 molds and 1 yeast in crossed investigation showed tolerance for at least two ions at this level (*Alternaria* sp. I, 47, 52; *Aspergillus niger* 12, 21, 37; *Aspergillus tamarii* 5; *Aspergillus* sp. 23, 53; *Cladosporium chlorocephalum* 30; *Cladosporium oxysporum* 51; *Penicillium chrysogenum* 41; *Penicillium expansum* 80; *Penicillium ovetense* 28; *Penicillium purpurogenum* 20; *Rhodotorula glutinis* 40). Isolation of fungi with considerable heavy metal ion tolerance from laboratory (non polluted) air is not a heavy task, i.e. the "constitutive" or spontaneous tolerance of fungi is relatively high.

K. HALMY and J. SERFŐZŐ

## *Microsporum vanbreuseghemii* as an infrequent pathogenic fungus inducing mycotic disease in Hungary

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*Microsporum vanbreuseghemii* (the perfect form is *Nanizzia* grubyi) as a pathogenic agent of mycotic diseases was first described by Georg et al. in 1962. The authors isolated and identified the fungus from a women patient with a circularly extending, scaly, at the borders inflammed skin symptome of superficial mycosis. On SGA and Mycosel agar the mycelium developed in three weeks. Its surface was

slightly granular, its colour was initially drab, finally pink, while the back side was first yellowish and later rusty brown. In the microcultures, the shape of macroconida is sausage-like of approximately 10×60 μm size, divided into 4-6-7 boxes. The wall of the macroconidia is moderately thick, and there are granules on the surface. Beside the macroconidia there are also numerous pyriform microconidia of about 2×4 μm size. It seems characteristic that the culture is promtly pleomorphised.

J. ZALA, ZS. HORVÁTH and E. K. NOVÁK

## Incidence of yeasts in human materials (1986-1991)

*B. Johan National Institute of Hygiene, Budapest, Hungary*

A time to time overview on the spectrum of yeast species isolated from human specimens may provide informations on the possible changes of the human pathogenic or commensalist yeast flora (yeast fungia). Thus after the earlier (1957-1976 and 1975-1984) summations, now the data of the last 6 years are summarized. In this period from the 2627 clinical materials worked up in details (cultivation + lege artis identification) 501 proved to be fungus positive (19.1%), out of which 406 gave yeast cultures (81.0% of the positives). In the species spectrum the pronounced prevalency of *Candida albicans* was observed, as it gave 60.0-82.2% of the strains depending on the year. *Candida tropicalis*, the second in frequency, however, oscillated between only 4.1 and 11.4%. The other 19 species showed 0.25-3.2% frequency all over the 6 years. Among the specimens the peritoneal dialysis fluids appeared as a new source (in 1989 15 *Aspergillus*-positive cases). From the data two more general and important conclusions can be drawn. There were no significant changes in the frequencies compared to the earlier periods. The introduction of quick identification tests or direct differentiating primocultural media for a species beyond *C. albicans* seems to be non-economic.

S. NEDELKOVICS, E. RÁCZ and J. FÖLDES

## Investigation on the possibilities of serotyping in *Candida* infections

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

The laboratory diagnosis of systematic *Candida* infections with special respect to the diseases treated with antibiotica or cytostatica is of great importance. The most reliable procedure is the investigation of pathologic specimens. The alternative technology might be the serology: the detection of circulating antigens and antibodies. The reliability of the serological methods depends on their specificity and

sensitivity. Different preparative methods were applied for the isolation of crude and purified antigens of *C. crusei*, *C. albicans*, *C. tropicalis* and *C. pseudotropicalis*. The mannan-type antigens were also isolated. Antibodies were raised in rabbits against the four species of *Candida*. Antigens were detected in the urines and sera, antibodies in the sera. The indirect haemagglutination (IHA) and its inhibition tests as well as the direct and indirect ELISA tests were applied. The results of the experiments prove the availability of the crude and purified antigens for the quantitative determination of the circulating antigens and antibodies. The purified mannan antigens are specific but cross reaction was observed between the *C. albicans* and *C. tropicalis*. The test of inhibition of IHA and the sandwich ELISA are working well in the detection of circulating antigen. The ELISA is more sensitive in order of nanograms. The results of the tests for antibodies are not always of diagnostic value.

L. GYÜRE and T. SCHMIDT

**Fluconazole capronate in experimental vaginal candidosis in rodents**

*BIOGAL Pharmaceutical Co., Debrecen, Hungary*

In the course of fluconazole capronate for antifungal activity we have used vaginal infections with *Candida albicans* and *Candida tropicalis* in estradiol-treated mice and estradiol-treated ovariectomized rats. *Candida albicans* OKI 63/10 was grown overnight on Sabouraud agar at 35 °C. Deep-frozen cell suspension of *Candida tropicalis* were harvested and made up at $2 \times 10^7$ $ml^{-1}$ in saline. Female mice of NMRI strain 23-25 g were injected subcutaneously a week with 0.5 mg estradiol benzoate. Female Wistar rats 160-170 g were ovariectomized at least 12 days before infection and 1 mg estradiol benzoate was given subcutaneously once a week. Three days after the first dose of estradiol animals were inoculated intravaginally with 0.03 ml (in mice) and 0.1 ml (in rat) of suspension containing $5 \times 10^6$ blastospores. Fluconazole capronate and fluconazole were given to groups of 6 animals at each dose level by oral route. The drugs were given twice daily for 3 consecutive days starting in 24 h after infection, and on the 4th day an additional treatment was given. Vaginal samples were taken with a wire loop, on days 1, 4 and 7 after infection, plated onto Biggy agar, and incubated for 48 h at 35 °C. The colony counts of the treated animals were expressed as a percentage of the mean log of the colony counts from the control group. The dose-effect curve were plotted on probability paper and the effective dose 50% ($ED_{50}$) determined. It has been concluded that fluconazole capronate possesses nearly the same $ED_{50}$ as fluconazole did.

B. LENKEY and A. SZ. PITI

## Application of cyclodextrin complexes in agar dilution

*L. Kossuth University, Debrecen, and BIOGAL Pharmaceutical Co., Debrecen, Hungary*

Various biologically useful compounds which are insoluble in water form water-soluble complexes with cyclodextrins or with their derivatives. These complexes are widely used in aqueous medium. However, these cyclodextrin complexes may be unusable because the cyclodextrins are able to prevent release and biological activity of encapsulated hydrophobic molecules. The effect of water-soluble dimethyl-beta-cyclodextrin complexes of well-known antifungal agents such as ketoconazole and tolnaftate was examined by agar dilution method with strains: *Candida albicans, Candida tropicalis, Candida pseudotropicalis, Rhodotorula rubra, Trichophyton mentagrophytes, Epidermiphyton floccosum, Aspergillus niger, Aspergillus fumigatus.* The free antifungal agents and their cyclodextrin complexes showed the same toxicity and their minimum inhibitory concentrations (MIC) were equal in both agar dilution method and "sandwich" assay. This preliminary study documents that the guest molecule in the cyclodextrin complex is able to show its genuine characteristic, consequently the hydrophobic biologically useful molecules which are incorporated into the cavity of cyclodextrin are applicable in biological experiments.

L. SZOMSZÉD, É. EMBERSICS and K. ZALAI

## Optimization of medium in ergot alkaloid fermentation

*Gedeon Richter Chemical Works Ltd. Budapest, Hungary*

Ergot alkaloids have been produced from sclerotia of *Claviceps purpurea*. There are more than 40 components from different strains known, many of which can be produced by direct fermentation of the fungus leaving out the parasitic cycle coupled to rye. The requirements for the composition of the medium may differ very much for each strain; a common feature is that the level of alkaloid concentration reached at the end of the fermentation may essentially vary with changes in the concentration of the medium components. The effect of four medium components – succinic acids, sodium chloride, inorganic phosphate (added as kalium dihydrogen phosphate) and methionine – on the alkaloid formation has been investigated during cultivation of *Claviceps fusiformis* producing elimoclavine on a synthetic medium using the modified Box-Wilson method of experimental design. By an unusual approach, performing and evaluating one single design, we have got an information on the effect of changing 3-3 components.

K. OLASZ

## Quick method for determination of activities of oxido-reductase enzymes

*Gedeon Richter Chemical Works Ltd., Budapest, Hungary*

There are occasions when the generally accepted methods of quantitative determinations of cell mass/amount in a culture media can not be used because of some features of the cell or of the culture media. For example, this is the case at the fermentation of a *Mycobacterium* sp. for sitosterin side chain degradation, so it is quite difficult to identify the true physiological condition of a culture although it is a very important parameter in industrial fermentations. We found that the activities of the oxide-reductase enzymes are suitable for characterization of the culture. Based on the literature and on our own experiments we have chosen a tetrazolium reagent which changes its light absorbance proportional to the oxido-reductase enzyme activity. This change of absorbance can be measured easily, and making use of this measurement, we can quickly characterize complex biological systems. The colour change, which is a measure of the oxido-reductase activities in the culture is visible to the naked eye, so it allows an even faster estimation of the state of the culture.

M. SZEGEDI, I. CSAPKAI and K. ZALAI

## Quick method to detect contamination in seed cultures

*Gedeon Richter Chemical Works Ltd., Budapest, Hungary*

The sterile medium, ready for inoculation, and/or the substrate to be converted in it may represent a high value especially in the case of fermenters with large volume. The quality and microbial purity of the seed culture determines fundamentally the result and effectiveness of the fermentation process. This makes essentially important of having methods for detection or elimination of occasional contamination of the seed cultures. Usually this task cannot be solved by conventionally dispersing the sample over solid media because by the time (12-24 h) of receiving the result the inoculum itself may have grown too old. The danger of delayed detectability exists even in cases when the infecting microorganism (e.g. *Escherichia coli, Staphylococcus* spp. etc.) grows much faster then the useful strain (e.g. *Claviceps, Mycobacterium* spp.) or when such kind of differences in the rate of propagation can be induced by using special selective media. Differences in optical density can be detected between two samples if one of them contains the slowly growing pure culture and the other has been taken from similar culture infected with fast growing microorganism. The difference can be measured by a BIOSCREEN C

microbiological analyser (Labsystems Oy, Helsinki). Our method makes possible the reliable detection of infecting microorganisms and in case of pure culture the producing fermentation can be inoculated at the optimal time. To increase the sensitivity of our method we have developed and used appropriate selective media for the different strains.

I. GYÜRE, I. FALKA and A. SZENTIRMAI

## Ester resolution by mycobacterial esterase(s)

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Enzymes with esterase activity proved to be very useful tools for the resolution of organic esters, usually formed in organic syntheses to prevent carboxyl and/or hyroxyl groups, due to their regio- and stereo-specificity. Although some mycobacterial strains were recently patented to have proper esterase activities for effective optical resolution of some organic compounds, only few papers involving detailed data on mycobacterial esterases have been published. We are investigating the esterase activity of *Mycobacterium* sp. NRRL B-3805 strain, principally from the viewpoint of its application for the resolution of phenylalanine esters used as model compounds. We observed that there are no considerable differences among esterase activities of crude extracts from cultures grown in different liquid mediums. Enzymes with esterase activities are of cell-bound since less than 5% of their activities were found in the supernatants of cultures. The pH optimum of crude extracts are about 8; the esterase activity is heat-sensitive. The stereospecificity of crude extract from the cultures grown in potato-dextrose medium was examined: it depends on the lengths of alkyl chain and the chirality of Phe-esters.

| Alkyl chain | Relative rate of hydrolysis (%) | | |
|---|---|---|---|
| | L-isomer | D-isomer | L/D |
| methyl | 51.8 | 13.9 | 3.7 |
| ethyl | 74.0 | 11.0 | 6.7 |
| butyl | 100.0 | 5.1 | 19.6 |
| iso-propyl | 24.9 | 1.8 | 13.8 |
| | (100% : 3.4 mU/mg of protein) | | |

The esterase activity can be separated into 2 peaks by gel filtration; the second one is responsible for 95% of the hydroxylase activity towards L-Phe butyl ester the best substrate from the view-point of resolution. This peak probably represents more than one enzymes having esterase activity since at least 8 bands of esterases hydrolyzing alfa-naphtyl acetate can be detected from crude extract by polyacryl amide gel electrophoresis.

A. MÁDI, E. SZÁLLÁS, A. SZENTIRMAI, M. M. PUSKÁS, GY. BATTA, Z. DINYA and F. SZTARICSKAI

**Optimization of antibiotic production by *Xenorhabdus nematophilus* 703, separation and structural investigation of the active materials**

*Department of Microbiology and Biotechnology, and Research Group for Antibiotics of Hungarian Academy of Sciences, L. Kossuth University, Debrecen, Hungary*

*Xenorhabdus nematophilus* a bacterial symbiont of the insectpathogen nematoda (*Neoaplectana*) produces a mixture of biologically active compounds, accordingly, this environment-brotheral strain may be efficiently utilized for biological plant protection against insects. We succeeded in increasing the antibiotic production by the strain 703 originated from the USA in lab-fermenter by means of the (i) increase of the dissolved oxygen level; (ii) exchange of the soya bean granulate component of the culture medium for soya peptone; (iii) direct inoculation with bacterium cells grown on a solid culture medium by washing; (iv) setting off the shaken inoculum culture; (v) reduction of the number of trans-inoculations. Thus it was possible to produce such an activity in the fermenter itself which was detected before only in shaken cultures. The usual work-up procedure of the culture was: centrifuging, extraction of the residue and supernatant with ethanol, ethyl acetate and n-butanol, respectively, followed by evaporation in vacuo and column chromatography resulted in different active materials. Further purification was made by gel permeation chromatography and HPLC.

E. SZÁLLÁS, A. FODOR and A. SZENTIRMAI

## Cultivation of insect pathogenic nematodes in synthetic media

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Insect pathogenic nemathodes are living in close symbiotic association with *Xenorhabdus* species. We studied the growth of nematodes (*Neoaplectana carpocapsae* var. *mexicana*, *Neoaplectana carpocapsae* ALL) using different synthetic media in presence or absence of symbiotic bacteria. The reproduction of insect pathogenic nematodes was faster and the growth yield was also higher in presence of bacterial symbionts. When cultured together with their symbiotic bacteria, the infectivity of nematodes was increased against the test organisms (*Galleria mellonella* var. *larvae*). Less amount of nematodes were enough to kill the caterpillars if they were propagated in presence of *Xenorhabdus nematophilus*. The nematodes can grow well in submerged culture, if they are inoculated into 72-h-old *X. nematophilus* culture.

L. FODOR, J. KOZMA and A. SZENTIRMAI

## Isolation of methanogenic bacteria

*Gedeon Richter Chemical Works Ltd., Budapest, and Department of Microbiology and Biotechnology, L. Kossuth University, Debrecen, Hungary*

We tried to isolate pure *Archeobacterium* cultures from anaerob mixed culture by using physiological and conventional microbiological methods. The mixed anaerob culture was maintained on methanol as a carbon source in semi-continuous fermentation. (1) We tried to eliminate the non-methanogenic (true) bacteria changing the millieu factors based on the special nutrient requirement of methanogenic bacteria. We obtained a highly purified and enriched methanogenic cultures using mineral (Low Phosphate Buffered Medium, LPBM) medium. However, the metabolic activities (the rate of methanogenesis, methanolysis of methanol) of the enriched cultures dropped down to the one third of the original one. (2) Growth of non-methanogenic bacteria was inhibited by antibiotics: rifampicin, 50 mg/l; vankomycin, 40 mg/l; doxycyclin, 30 mg/l; ampicillin, 50 mg/l. (3) Two methanogenic bacteria were isolated from the enriched cultures (*Methanosarcina* sp., *Methanobrevibacter* sp.). We used LPBM medium solidified with agar-agar and polysilicate plates. Serial dilutions and all the manipulations were done in JOUAN anaerob box and incubator.

# VIROLOGY

A. LENGYEL, É. ÁDÁM and I. NÁSZ

## Latex agglutination with adenoviruses

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Latex beads were coated either with different purified adenovirus antigens or with antibodies directed against the antigens mentioned. The sensitivity of reaction between latex particles coated with different types of purified adenovirus hexon, penton or fibre antigens and polyclonal and monoclonal antibodies, as well as human serum specimens was compared with the results obtained by other serological methods. Of antibody-coated latex preparations made with genus-, subgenus- and type-specific monoclonal and polyclonal antibodies the best results were observed with a genus-specific monoclonal antibody. Using this even a 0.1 μg/ml concentration can be detected, thus it may be supposed that it can be used for early diagnosis. Latex suspensions coated both with antigens or antibodies proved to be stable, their reactivity did not change within several years.

É. ÁDÁM, I. NÁSZ and A. LENGYEL

## Investigation of cross-reactivity patterns and competitive binding ELISA experiments with monoclonal antibodies directed against adenovirus hexons

*Institute of Microbiology, Semmelweis University Medical School, Budapest, Hungary*

With the help of 23 selected monoclonal antobodies (MAbs) directed against human adenovirus (AV) types 1 and 35, as well as bovine AV (BAV) type 2, epitopes of AV18 (subgenus A), AV19, AV26 (subgenus D), and AV41 (subgenus F) were studied on the basis of the cross-reactivity patterns (RPs) in indirect ELISA. Recent results were compared with the RPs of the previous experiments, and it was confirmed that the genus specific epitopes were present on all the hexons studied up till now, while the other epitopes could be found as intertype specific ones on the different hexon types. No difference was found between the serotypes of subgenus A, the antigenic structure of the members of subgenus D, however, was shown to be different. The detected epitopes of AV19 were similar to that detected on AV8, AV9, AV10, and AV13 in the previous experiments, but the epitope structure of AV26 and AV27 was found to be different from the epitope structure of the other members of

subgenus D, as well as from each other, too, The largest number of epitopes was detected on the AV19, and the smallest number was recognized on AV27 hexon. These results indicate significant differences in the antigenic relationships among the members of subgenus D. AV41 hexon reacted only with the MAbs having broader reactivity patterns (from the panel of MAbs against AV1). With the other MAbs, similarities were found among the hexons of subgenera A, B, D, E and AV41 of subgenus F (MAbs directed against AV35), as well as among AV18 (subgenus A), subgenera C and E, a part of subgenus D and AV41 (MAbs directed against BAV2). In competitive binding ELISA, it was verified that similar RPs of MAbs directed against different AV hexon types mean identical epitopes recognized in combinations on the different hexon types.

GY. SZŰCS and M. UJ

**Enteric adenoviruses in rotavirus-negative stool specimens**

*Baranya County Institute, National Public Health Service, Pécs, Hungary*

Enteric adenoviruses type 40 and 41 are the only adenoviruses which have been consistently associated with gastroenteritis in infants and young children, and may be the second major cause of gastroenteritis after rotaviruses. In our laboratory, after infection onto Graham 293 cells, rotavirus-negative faecal samples collected from children under 3 years of age were screened for adenovirus content by immunofluorescence assay as a routine since 1991. Of 640 samples tested in the last 15 months 155 (24%) contained infectious adenoviruses. In 39 cases, cells with unique pattern of immunofluorescence resembling enteric adenoviruses were observed. Of these, 16 were proved to be enteric adenovirus infection by type-specific monoclonal antibodies in ELISA (Adenoclone – Type 40/41 EIA Kit; Cambridge BioScience, Worcester, MA, USA). In our samples the overall prevalence of subgenus F adenoviruses is about 10% which is certainly under-estimated. More sensitive molecular methods (e.g. hybridization, PCR) are needed to determine the real epidemiological importance of these viruses in acute infantile gastroenteritis in Hungary.

I. VISONTAI, A. POROS and M. KOLLER

**Cytomegalovirus antigenaemia: application and significance of a rapid test in the clinical virology**

*B. Johan National Institute of Hygiene, Budapest, Hungary*

Active cytomegalovirus (CMV) infection of three bone marrow recipient patients was followed up by virus serological tests. Working with serially taken blood samples attempts were made to detect virus antigen in shell vial cultures of human fibroblast cells by using fluorescent antibody technique, and to detect CMV antigen in the circulating white blood cells (CMV antigenaemia). In both methods anti-CMV monoclonal antibodies were used. The immunohistochemical staining of the leukocytes was made either by indirect immunofluorescence in cell-drops dried and fixed on microscope slides or by immunoperoxidase staining of cytocentrifuge preparations. Comparing the results obtained by the different techniques the antigenaemia test was found to present positive results in the earliest stage of the infection, being the most convenient in view of the antiviral chemotherapy. The immunoperoxidase staining was found the most reliable when the cell preparation was made with cytocentrifuge. The results of the antigenaemia test correlated with the test-results obtained in the rapid virus isolation experiments.

A. KÁTAI, M. BENKŐ, B. BÖTHIG, U. KÜNKEL and E. SZÖLLŐSY

**Intratypic differentiation of poliovirus type 1 and 2 strains isolated from patients**

*Csongrád County Institute, National Public Health Service, Szeged, Hungary, and Robert Koch Institute of the Federal Health Service, Berlin, Germany*

Between May 1989 and May 1990 poliovirus type 1 (PV 1) strains were isolated in Szeged, in a period incompatible with the vaccination pattern in force in Hungary. Since these results have been supported by molecular biological methods carried out in Berlin. On the basis of their rct/40, E marker characters 42 out of the tested 52 virus strains proved to be similar to the wild reference poliovirus type 1 Mahoney strain, however, according to their oligonucleotid-fingerprint patterns they seemed to originate from the vaccine strain. During the period between January 1991 and April 1992 the distribution of the newly isolated poliovirus strains were the following: PV 1, six; PV 3 one; and PV 2 twenty-seven. Most of the lately isolated strains originated also from the period not suiting the vaccination pattern.

K. MEGYERI and I. ROSZTÓCZY

**Effects of interferon treatment on interleukin 6 and interferon production of Sendai virus-induced mouse L cells**

*Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

Two sublines of L929 cells with different IFN-producing capacities produced IFN and IL-6 simultaneously in response to Sendai virus infection. The IL-6 activity from the "high producer" subline always exceeded the amount of IFN present, expressed in IU/ml. In contrast, the "low producer" synthesized less IL-6 than IFN. Treatment of cultures with IFN enhanced the production of both cytokines. The difference in IL-6 production between the primed "high producer and low producer" L cell sublines was about one order of magnitude larger than for their IFN production. The injection of poly(A) plus RNA into *X. laevis* oocytes resulted in the translation of quasi-proportional amounts of IFN and IL-6 activities with respect to those detected in culture supernatants. In the case of IL-6 the observation was confirmed by blot hybridization analysis. Inhibition of protein kinase C did not affect the modulation by IFN. Staurosporin, one of the protein kinase inhibitors applied, adversely influenced IFN and IL-6 production. In spite of their different chromosomal localizations and different mechanisms of induction, interleukin genes simultaneously stimulated by Sendai virus seem to be coordinately regulated by IFN.

A. MARKOVITS, G. PREMECZ, G. BAGI, T. FARKAS and I. FÖLDES

**Ethanol-induced antiviral activity in human amnion cells**

*Microbiological Research Group of the B. Johan National Institute of Hygiene, Budapest, Frédéric Joliot-Curie National Research Institute for Radiology and Radiohygiene, Budapest, and Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, Szeged, Hungary*

Treatment of human amniotic cells (UAC) with ethanol transiently triggers the breakdown of inositol phospholipids and induces translocation of protein kinase C. Our previous works have shown that activation of this signal transduction mechanism in UAC results in an antiviral state. Studying the $^{3}$H-uridine incorporation into the vesicular stomatitis virus (VSV) RNA it was demonstrated that administration of ethanol inhibits the replication of VSV. The ability of various short-chain alcohols (methanol, ethanol, propanol, butanol) to stimulate the antiviral state in UAC correlates with the increase solubility of the alcohol in the lipid membrane of cells. Increasing chain length of the alcohol molecules a higher antiviral effect was observed even at lower alcohol concentrations. In conclusion, these results

demonstrate that ethanol at pharmacological concentrations affects phosphatidylinositol turnover and this in turn induces an increase in the activity of membrane bound protein kinase C. These biochemical events are associated with an ethanol induced antiviral state.

GY. VERESS, T. CSIKY-MÉSZÁROS, J. CZEGLÉDY and L. GERGELY

**Oral contraceptive use and human papillomavirus infection in women without abnormal cytological results**

*Institute of Microbiology, University Medical School, Debrecen, and District Gynecologic Outpatient Clinic, Debrecen, Hungary*

Both experimental and epidemiologic data support the idea that oral contraceptive (OC) use may have a stimulating effect on a certain point of cervical carcinogenesis. The current investigation tries to answer the question whether OC use might have an influence on early human papillomavirus (HPV) infections. A total of 425 women without abnormal cytological results were examined colposcopically and by the use of filter in situ hybridization (FISH) for the presence of human papillomavirus (HPV) types 6, 11, 16 and 18. Eighty-one cervical specimens (19.1%) were found to be positive for one or more of the HPV types in FISH. HPV positivity was significantly higher among women under 25 than among the older patients. The use of OCs was inversely correlated with the presence of ectopy or dysplasia in this group of women. On the other hand, HPV positivity was not significantly higher among OC users than among non-users in any colposcopic group. Neither the type of pill used, nor the duration of use had any significant effect on HPV positivity. Further investigations are needed to evaluate the effects of OC use on more severe HPV-induced cervical lesions.

J. KÓNYA, GY. VERESS, J. CZEGLÉDI and L. GERGELY

**Application of in situ hybridization to detect human papillomavirus in Caski cell line and exfoliated cervical cells**

*Institute of Microbiology, University Medical School, Debrecen Hungary*

Certain human papillomavirus (HPV) types are often detected in cervical and vaginal epithelial cells. HPV type 6, 11 and type 16, 18 are associated with benign and malignant tumours of the cervix, respectively. In situ hybridization is a simple method of viral nucleic acid detection. It makes possible conserving the original cell or tissue

structure and investigation of large series. The effect of fixation, proteolytic digestion, stringency during hybridization and post-hybridization washing was studied in Caski cells containing HPV 16 and fibroblasts. Application of cross-linking fixative (formaldehyde), moderate proteolytic digestion and high stringency hybridization made possible to distinct different HPV types. In situ hybridization performed on colposcopically normal exfoliated cervical cells will be presented.

J. MÉSZÁROS, E. HORVÁTH, R. POLYÁK-NAGY and Z. PÉNZES

## Simultaneous immunization against Newcastle disease and avian infectious bronchitis

*Veterinary Medical Research Institute, Hungarian Academy of Sciences, Budapest, and Control Institute for Veterinary Biologicals and Drugs, Budapest, Hungary*

According to other researchers the Newcastle disease virus and the avian infectious bronchitis virus often interfere when administered in the same vaccine. To avoid the interference, the viruses either should be administered separately or the titer of the components should be set properly. To evaluate the interaction, chickens were vaccinated using attenuated vaccines with various titres against Newcastle disease and infectious bronchitis (VITAPEST and BRONCHOVAC-1). The vaccines were administered in aerosol or spray form. Day-old chickens were vaccinated simultaneously with VITAPEST and BRONCHOVAC-1 using an Atomist sprayer. On the 33rd day the chickens were challenged with $10^{6.0}$ $EID_{50}$ Hertz 33 virulent Newcastle disease virus. The immunity was 93.3%. Similar result was obtained with chickens vaccinated only with VITAPEST spray. Chickens vaccinated simultaneously developed good immunity against infectious bronchitis, too. In field trials, with simultaneous immunization of day-old chickens there was no significant difference in immunity to Newcastle disease between groups vaccinated with VITAPEST or VITAPEST-BRONCHOVAC-1. Consequently, simultaneous immunization is possible, but the titre of the VITAPEST vaccine should be 3 $\log_{10}$ exponent higher than that of the BRONCHOVAC-1 vaccine.

J. TANYI, R. GLÁVITS, G. SÁLYI, E. KOSA and J. SZABÓ

**Viral pancreatitis in guinea-poult (*Numida meleagridis*)**

*Veterinary Institute, Debrecen, Central Veterinary Institute, Budapest, and University of Veterinary Science, Budapest, Hungary*

In a Hungarian guinea-poult flock housed extensively 5% mortality rate was observed. As a causative agent, a virus was isolated from the organs of the affected animals that could reproduce the disease experimentally. The clinical symptoms were the same both in naturally and experimentally infected animals: weakness, anorexia, recumbency opistotonus and occasional tonico-clonic convulsions. A certain proportion of the survivors showed retardation. Two-fivefold enlargement of the pancreas with high yellow discoloration, tense, solid palpation, focal haemorrhages and necroses was detected at necropsy. The histologic and EM investigation revealed degenerative changes and necrosis mainly of exocrine secretory gland epithelial cells. Ikosahedral viral particles 66-72 nm in diameter were observed in the cytoplasm of these cells, and lysis and dystrophic alterations of the cytoplasmatic organelles. In subacute cases irreversible atrophy of glandular tissue and restorative fibrosis were detected. The enzyme activity (amylase, trypsin, total protease and lipase) decreased in the faecal specimens of the infected animals, but increased significantly in the tissues of pancreas. The isolated virus was classified as a reovirus on the basis of the morphologic, physico-chemical and cultural properties.

L. FODOR and M. RUSVAI

**Efficacy of farms-specific vaccines containing bacterium and virus strains in controlling pneumonia in cattle**

*University of Veterinary Science, Budapest, Hungary*

On the basis of previous bacteriological and virological examinations farm-specific combined vaccines were produced and tested in two cattle herds. Both vaccines contained inactivated parainfluenza-3 (PI-3) virus and heat treated *Pasteurella haemolytica* A1 and *Pasteurella multocida* A cells with aluminium-phosphate adjuvant. Each calf received 5 ml vaccine at the age of 4-6 weeks and two weeks later. The antibody titre against the bacterial strains in the vaccines became 2-3 indices ($\log_2$) higher, anti-PI-3 antibodies rose 4-6 indices. The incidence of pneumonia, the loss and the medical costs decreased as a result of the vaccination.

I. ROSZTÓCZY and P. M. PITHA-ROWE

## Effects of various staphylococci on HIV-1 replication in cultures of human peripheral blood mononuclear cells

*Institute of Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary, and The John Hopkins Oncology Center, Baltimore, MD, USA*

Staphylococcal infections are frequent complications in patients with ARC and AIDS. The staphylococci can alter the functional activities of T lymphocytes and monocytes that are the primary sites of HIV replication in vivo in humans. Since HIV multiplication seems to be stimulated in activated T cells and monocytes, there is possibility for a positive feedback effect by staphylococci on HIV replication. In the present study the effects of various staphylococci on HIV replication were investigated in different populations of human PBMNC. Heat-killed preparations of various staphylococcal strains were added to the cultures at a ratio of 10 bacteria per cell 2 days before or 2 days after infection of the cells with 50 $TCID_{50}$ NL-43 strains of HIV-1. Replication of the virus was followed by monitoring RT activity in the culture media. The addition of *S. aureus* strain preparations increased RT activity by up to one order of magnitude in HIV-infected unseparated or separated plastic non-adherent, and in adherent mononuclear cells. The most efficient virus replication was observed in the non-adherent SRBC rosette-positive cells, while the SRBC rosette-negative cell population did not support HIV replication. Representative preparations of *S. epidermidis* were markedly different from *S. aureus* because they did not augment HIV replication in either cell population; instead, their presence occasionally resulted in a minor reduction of RT activity. Further analysis of *S. aureus* strains that had previously been found to exert different IL-2 and chemiluminescence-stimulating activities in HuPBMNC indicated that the HIV replication-enhancing activity followed a parallel pattern to the above properties.

I. NAGY, F. D. TÓTH, D. BÁNHEGYI, J. KISS, E. UJHELYI and G. FÜST

## Follow up study on neutralizing and enhancing antibody titres in HIV-infected persons

*Institute of Microbiology, University Medical School, Debrecen, St. László Hospital, Budapest, and National Institute of Haematology and Blood Transfusion, Budapest, Hungary*

Longitudinal serum samples from 7 persons were investigated for the presence of virus neutralizing and enhancing antibodies in the different stages of HIV-induced pathogenesis. Sera were tested for HIV neutralization and enhancement using MT-4

target cells. A gradual decrease in the titres of neutralizing antibodies was observed in 5 patients, in 4 of them enhancing antibodies were also elevated. A correlation was found between the decreased of T4 lymphocyte counts and the increase of titres of enhancing antibodies. Data suggest an important role of complement-dependent enhancing antibodies in HIV-induced pathogenesis. Measurement of neutralizing and enhancing antibodies may have a prognostic value in the natural course of HIV disease.

J. KISS, F. D. TÓTH, B. TELEK, L. REJTŐ, P. SURÁNYI and K. RÁK

## Detection and analysis of HTLV-I-related proviral DNA sequences in a Hungarian patient with adult T-cell leukaemia

*Institute of Microbiology, Second Department of Medicine, and Third Department of Medicine, University Medical School, Debrecen, Hungary*

Monoclonal integration of proviral DNA sequences related to HTLV-I was detected in the peripheral lymphocytes of a Hungarian male with ATL. The pMT-2 probe containing full length HTLV-I proviral DNA hybridized specifically only under low stringency conditions with the DNA from ATL cells. Restriction enzyme analysis with HindIII, SstI, and PstI revealed common bands in the DNA from the ATL patient and HTLV-I-infected cells. Analysis with EcoRI revealed one band in the DNA from ATL cells. Internal BamHI 1.0 kb band was absent in the ATL DNA. Restriction endonuclease mapping showed differences between ATL and HTLV-I proviral DNA mainly in the *env* region.

A. JOBBÁGY and I. FÖLDES

## Study of HTLV-1 antibodies in HIV-1 infected individuals

*Microbiological Research Group of the B. Johan National Institute of Hygiene, Budapest, Hungary*

Sera of 326 patients with various haematological disorders were tested for antibodies to HTLV-1 by immunofluorescence, syncytium inhibition and ELISA methods in 1985-1986. Out of the 326 cases only two patients with haemoblastosis reacted positively with HTLV-1 antigens, but none of the other ones with leukaemias and lymphomas, indicating that HTLV-1 infection was very rare in Hungary. As transmission of HIV and HTLV-1 infections is similar and – according to literary data – HTLV-1 infection is relatively frequent in HIV-infected

individuals, it seemed to be of interest to examine the putative occurrence of HTLV-1 infection among HIV-infected persons. Sera of 156 HIV-infected individuals (representing about one half of the known infected persons in Hungary) were tested with ABBOTT HTLV-1 EIA. None of them proved to be reactive. Similarly, none of the sera of 148 individuals found to be false positive with Vironostika HIV-1 test was reactive with the HTLV-1 test. As a conclusion, HTLV-1 infection – fortunately – is still very rare in Hungary, systematic HTLV-1 screening of donors, therefore, is not necessary.

I. FÖLDES, Á. GYURIS, E. TAMÁS and G. VAJDA

**In vitro anti-HIV effect of an African plant's extract (3804/91.H.P.)**

*Microbiological Research Group of the B. Johan National Institute of Hygiene, Budapest, Hungary*

Anti HIV-1 effect of a PBS extract of an African plant was investigated. Dilutions 1:200-1:800 of a stock solution (20 mg dry weight/ml) given to MT4 lymphoblastoid cells together with HIV-1 significantly decreased virus production controlled by measuring RT activity of the cell supernatant by indirect immunofluorescent and by syncytium inhibition assay. Cytotoxicity was observed only at 1:50-1:100 dilutions of the stock solution. The inhibition was specific for HIV, as it was not observed using herpes and rhabdoviruses. Antiviral effect of the extract could not be observed in case of permanently HIV-1 producing cells. By the use of concentrated HIV preparations a direct inhibition of RT activity was measured, which was not the case if AZT was applied instead of the extract. The effective component of the extract proved to be heatstable and of low molecular weight. This agent seems to be a promising candidate the treatment of AIDS/ARC.

Á. GYURIS, G. VAJDA and I. FÖLDES

**Establishment of an MT4 cell line continuously producing $SIV_{mac\ 251}$**

*Microbiological Research Group of the B. Johan National Institute of Hygiene, Budapest, Hungary*

The human leukaemic T cell line MT4 is sensitive for HIV-1, HIV-2 and SIV infection. As a result of successful infection of MT4 cells with $SIV_{mac\ 251}$ we have established a surviving and continuously $SIV_{mac\ 251}$ producing MT4 cell line. We have these cells for more than one year in culture. Growing charateristics, morphology, and virus production of this new cell line are regularly controlled. The

relative high and stable RT (reverse transcriptase) activity of the supernatant of these cells (compared to that of the HIV-1 cultured in H9 and MT4 cells) correlates well with high titres of $TCID_{60}$/ml values in infectivity assay. On electron microscopical pictures typical lentiviruses can be seen in large number near the cell mebrane of MT4 cells. These cells are useful for diagnostic purpose. We demostrate the results of Western blot analyses of monkey sera using MT4/$SIV_{mac\ 251}$ as antigen.

J. DEÁK, E. OSZLÁCS and J. FÖLDES

## Determination of adeno, herpes and Epstein-Barr virus specific IgM before and after removing the interfering serum factors

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

The early diagnosis of virus infections is based mostly on the detection of IgM antibodies. Rheumatoid factor (RF), as well the IgG may interfere with the IgM antibodies and in many cases result false positive or negative reactions. A total of 51 serum samples were examined. (1) The elimination of RF was carried out by kaolin treatment of sera samples. (2) The IgG and RF were removed simultaneously with GULLSorb reagent (Gull Laboratories, USA). (3) The results obtained were compared with antibody titres in the non-exhausted sera. It has been shown that (i) RF is removed by kaolin treatment; (ii) the GULLSorb reagent is suitable for removing both RF and IgG and eliminates false negative or positive results; (iii) the IgG in extremely high quantity (two cases: 3.9%) needs the repeated exhaustion of the samples.

J. DEÁK, E. OSZLÁCS and J. FÖLDES

## Comparative study for detecting CMV antibodies by different indirect immunofluorescent methods

*Department of Clinical Microbiology, A. Szent-Györgyi University Medical School, Szeged, Hungary*

Rapid diagnosis of human cytomegalovirus (HCMV) infections is of great importance in the transplantation of kidney and bone marrow as well as in patients after multitransfusions and looking for the etiology of spontaneous abortions. The investigations were carried out in three groups of patients: (1) Patients selected for kidney transplantation. (2) Women taking part in genetic councelling due to spontaneous abortion and/or suspected infertility. (3) Adults and children with

swollen lymph nodes and fever who were suspected of origin of virus infection. The HCMV specific antiodies were shown with indirect immunofluorescent (IIF) method, and by GULL rapid screening test detecting the IgGAM antibodies. There were 183 sera samples examined and 25 of them were positive with the screening test. Out of the 25 positive samples 17 were found positive for IgM. The parallel used rapid screening test for IgGAM and the more specific IgM determination proved to be reliable diagnostic methods resulting in a more rapid diagnosis than the traditional IIF method. These sensitive and specific technologies provide an unambiguous diagnosis.

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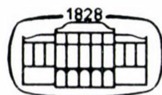


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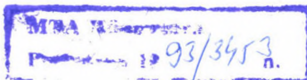

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