EXPERIMENTAL INFECTION OF LABORATORY ANIMALS BY THE PATHOGENIC NAEGLERIA GRUBERI STRAIN VÍTEK

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Abstract. The pathogenic strain Vítek of the amoeba Naegleria gruberi was isolated in Northern Bohemia from the liquor of an 11-year-old boy with acute amoebic meningoencephalitis, who had died shortly after infection (Červa et al. 1969). The new strain, cultivated in axenic culture, was used for animal pathogenicity tests in order to disclose that the virulence and pathogenic properties of the strain can cause a similar infection in the animals to that in the boy.

MATERIAL AND METHODS

The Vítek strain was cultivated under axenic conditions in a medium described in an earlier paper (Červa 1969). Suspensions for the inoculation of the animals were prepared from 3 to 5 day-old cultures. Whenever a higher dose of inoculum was required, this was obtained by careful centrifugation of the amoebae in the medium (1,500 rotation/min. for 5—10 min.). The amoebae in the basic suspension were counted in Bürker’s chamber to establish the final concentration. All operations were performed in silicon-treated glassware.

In all experiments we used white laboratory mice of the so-called strain II commonly used in Czechoslovakia, and albino guinea pigs of unidentified origin. Both these animal strains are agnathiotic and were not kept on a standard diet.

Histological preparations were made from tissue fixed in neutral formalin by standard paraffine techniques. The tissue samples were decalcified with 5% trichlor-acetic acid. Of the staining methods employed, these were chiefly Masson’s green trichrome or Heidenhain’s ferric haematoxylin.

For serological examination we used the CF test with extracellular antigen prepared from the Vítek strain. The antigen was prepared in a similar way as that from Acanthamoeba castellanii (Červa 1967).

RESULTS

Intracerebral inoculation was accomplished by injecting a dose of \(10^3\) amoebae (fluid volume 0.02 ml) into the brain of the mice (20 mice in the group), weighing 10—13 g each. Mortality of all mice in the group occurred within 2—7 days (average 5 days). Clinical signs of the disease occurred at about 24 hrs before death, showing mainly reduced activity, bristling of the pelt, disturbed equilibrium and, finally, loss of movement co-ordination. Postmortem examination showed extensive haemorrhages in the cranial cavity, the brain was of pulpy structure and was difficult to remove from the
skull cavity without major damage. Histological examination revealed a complete disintegration of most of the brain tissue due to the massive incidence of amoebae.

In a second simultaneously performed experiment, a group of deeply anaesthetized mice was inoculated intranasally with the same dose and suspension of amoebae as that used in the first experiment. After intranasal inoculation, the mice died after 7.4 days on the average; this is about 2 days later than after intracerebral inoculation. Shortly before death we observed typical signs of purulent meningitis, and a rounded mount appeared on the top of the head. Postmortem examination of the skull revealed a gap between the neurocranial sutures caused by increased intracranial pressure. Macroscopic examination showed extensive haemorrhages in the frontal lobes. Histological examination of the brains and the decalcified nasal cavities showed that practically the complete mucous membrane of the nasal cavities had been destroyed by reproducing amoebae and that the amoebae can migrate to the brain cavity through all openings of its skeletal base. A primary encephalitis develops in the frontal and basal parts of the brain and the meninges are affected only in the second place. Therefore, meningitis in these mice is not basically important.

The LD$_{50}$ of the Vitek strain tested in a single experiment by intranasal inoculation on groups of 6 mice weighing 10—13 g each, was 10$^4$ microorganisms per inoculum. The observations performed on guinea pigs were more detailed. Either anaesthetized animals (weight 200—300 g) were inoculated with an intranasal dose of 0.1 ml of a decimal dilution culture. Mortality of the guinea pigs is shown on Table 1. The temperature of

<table>
<thead>
<tr>
<th>Number of amoebae in inoculum</th>
<th>Number of guinea pigs</th>
<th>Average survival (days)</th>
<th>Number of survivors</th>
</tr>
</thead>
<tbody>
<tr>
<td>40,000</td>
<td>8</td>
<td>5.7</td>
<td>1</td>
</tr>
<tr>
<td>4,000</td>
<td>8</td>
<td>6.5</td>
<td>1</td>
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<tr>
<td>400</td>
<td>8</td>
<td>8.6</td>
<td>4</td>
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<td>40</td>
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The animals was taken with a high speed thermometer in the anus once a day. Temperatures above 39.5 $^\circ$ C were found to last only for the short period of 1—2 days. After higher doses of inoculum, the rise of temperature started approximately two days before the death of the animal. After lower doses of inoculum, the interval between the start of high temperatures and death was prolonged and there was also an evident prolongation of the time of survival. In this experiment, the longest time of survival of the guinea pigs was 15 days. The guinea pigs surviving intranasal inoculation did not develop high temperatures. When repeating this experiment without changing experimental conditions, we obtained different results. For example, three of the five guinea pigs died on day 14, 22 and 23 p.i. following intranasal inoculation with 250 amoebae. The increased temperature of these animals returned to normal values 12—14 days before death. In one of the animals, high temperatures lasted for 5 days. In another group of experimental animals, their temperature started to rise at the expected time, but the animals recovered without treatment. This indicates the necessity of detailed studies of the factors influencing the development of infection in experimentally infected animals. These studies may disclose some important facts explaining some questions of the pathogenesis of human amoebic meningoencephalitis.
In the guinea pigs, the first signs of the disease occurred as late as 2—3 days before death. The animals became inactive, their pelt bristled and, later, they could hardly stand on their feet. They fell sideways with their feet still moving until, finally, they became completely motionless. About 50\% of the animals died in opisthotonus.

Postmortem examination showed the completely haemorrhagic condition of the anterior part of the skull in front of the eye sockets, while neither the posterior portion of the skull nor the cerebrum and cerebellum were affected by pathological changes. The frontal lobes of the brain were either on one or on both sides changed into a pulpy substance which could be removed only with difficulties. This mass consisted largely of numerous amoebae and of leucocytic exudate with erythrocytes. While moving from the frontal processes to the cerebrum, the amoebae attacked simultaneously the meninges. Similar to human infection, the amoebae migrate chiefly along the capillaries, and the front line of the invading amoebae is not intercepted by cellular reaction of the host's tissue. At the time of death of the guinea pigs, the amoebae had reached the level of the anterior processes of the brain ventricles, sometimes penetrating the ependyma on their way to the lumen of the ventricle. The plexus chorioides was not attacked. The distance covered by the migrating amoebae at the base of the brain was slightly bigger than that in the upper parts, but never did we find amoebae in the tissue of the cerebellum. This shows that also in this case the primary process is that of a destructive amoebic encephalitis, while meningitis is only a concomitant process.

Series of transverse sections through the decalcified skulls of dead guinea pigs showed that primary reproduction of the amoebae occurred mainly on one side only, although the inoculum had been injected into both nostrils. At the time of death of the animals, the mucosa was still swollen and highly infiltrated by leucocytes; therefore, it was very difficult to demonstrate an occasional amoeba in it. The whole route of amoebic infection from their entrance through the openings in the skeletal base of the skull cavity up to the frontal lobes of the brain was clearly marked with a strong inflammatory reaction.

In order to reveal the presence of specific antibody, we examined the sera of all surviving guinea pigs with the CF test. Positive titres were found even in animals reacting to the infection with the shortest possible period of increased temperature. The titres of these animals were positive up to a dilution of 1 : 128. On the other hand, animals which did not react with increased temperatures to the highest doses of inoculum, were serologically negative. We were unable to demonstrate in cultivation the presence of amoebae or their cysts in any of the brains of the surviving animals.

In another experiment with a group of 10 animals, these were anaesthetized with urethane and inoculated with a massive dose of Vitex strain amoebae (0.1 ml) directly into the cerebellum. Four of these animals died within 24 hrs, another 4 after 2 days and the remaining animals within 3—4 days p.i. Histological preparations of the cerebella of these animals revealed the high incidence of amoebae close to the site of injection; this was associated with a strong cellular reaction and a typical multiplication of amoebae in the granular and ganglionic layer of the cortex of the cerebellum. The histopathological picture was similar to that found in some human cases of amoebic meningoencephalitis. We found also Purkynje cells harbouring amoebae in their plasma and frequently, a single cell was occupied by 2—3 parasites. Our experimental findings confirmed that pathogenic Naegleriae are capable of intraplasmic parasitism.

In order to obtain further information, an experiment was performed with chick embryos. The yolk-sac of 7 day-old chick embryos was inoculated with a dose of 100,000 amoebae. The embryos died within 9.6 days on the average. Macroscopical changes could not be found, but Naegleriae could be cultivated from brain tissue and the membrane of the yolk sac.
DISCUSSION

Experimental infection of laboratory animals with the Czech pathogenic *Naegleria gruberi* strain Vitek is similar to infection caused by the pathogenic strains of *Acanthamoeba castellanii*. Reproduction of the *Naegleriae* in the nasal mucosa is, however, more intense and so is also the rate of tissue penetration of these amoebae. More studies will be needed to discover whether these properties of the *Naegleriae* are only due to the shorter generation time or whether other factors such as the enzymatic equipment of the protozoan are involved. Although in the present experiments we have been concerned only with the nasal cavities and the brain, our findings indicate that the development of amoebic pneumonia is less important in intranasal *Naegleria* infection than in similar infections with *Acanthamoeba*. The simple reason may be the fact that the *Naegleriae* can penetrate the brain more easily by direct route and that an infection caused by them develops faster than that caused by *Acanthamoeba*.

An interesting feature is the two days' difference in the onset of mortality between an intracerebral and intranasal infection with the same number of amoebae. If we consider the numerous amoebae which do not reach the brain after intranasal inoculation, being aspirated into the lungs, swallowed, expelled from the nasal mucosa by the ciliated epithelium etc., it is evident that the migration of the amoeba to the brain is little obstructed by the nasal mucosa and that this is apparently incapable to prevent amoebic penetration.

Guinea pigs, although larger in size, are more susceptible to intranasal *Naegleria* infection than mice. In this, a *Naegleria* infection differs considerably from an experimental infection with pathogenic *Acanthamoeba* which, in larger, non-cortizone treated animals, are unable to reach the brain after intranasal inoculation. The temperature of the guinea pigs appears to rise simultaneously with the development of the primary inflammatory process in the nasal mucosa, while this process in the brain itself is accompanied by normal or subnormal temperatures. Interesting is also the protracted interval between increased temperatures and mortality after a low infective dose. The mechanism responsible for the protracted form of infection will have to be further investigated. The results may offer an explanation of some special cases of human infection with an unusually prolonged incubation period surpassing the general average of 5 days.

The surviving guinea pigs reacting with a temporary increase of temperature and with specific antibody production to intranasal infection appear to be the cases which survive a *Naegleria* infection in a natural way. On the other hand, the existence of an individual resistance to amoebic infection has been confirmed by the animals surviving the highest doses of inoculum without increased temperatures and without antibody production. A similar fact has been indicated also by the onesided development of the amoebic process in guinea pigs.

Histological preparations of brains fixed immediately after the death of the guinea pigs showed that Carter (1968) was wrong in assuming that the formation of large agglomerations of amoebae in the perivascular spaces without an inflammatory reaction had occurred in human cases after the death of the host. At this time, according to Carter, the amoebae were still capable of reproducing and penetrating the tissues, while these were unable to react to this process. This picture, however, is most typical of the histopathological manifestations in the frontal zone of growth of the amoebae and occurs, apparently, not only in experimental infection of laboratory animals, but also in man.

The negative findings in culture from brain tissue of animals which seem to have gone through at least the primary phase of the disease indicate that the Vitek strain is incapable of producing cysts in the brain tissue, differing in this from some pathogenic
strains of the genus *Acanthamoeba*. In view of this fact it is difficult to study the pathogenesis of experimental infection and to diagnose this infection in man. A positive finding could hardly be obtained in culture from material, which is either too old, frozen or damaged by other physical factors.

We have been able to demonstrate the intraplastic location of the amoebae in the Purkinje cells and that not only in material from human cases of amoebic meningoencephalitis, but also in the tissues of experimentally infected guinea pigs. It appears that the amoebae can enter the plasma of the Purkinje cells only because of the size of these cells. This remarkable phenomenon can be observed only for a very short time, since the host’s cells are quickly destroyed by degenerative changes. In the affected parts of the cerebellar cortex, the Purkinje cells were either absent or only their remnants were present. Carter (1968) found intracellularly located amoebae in neurons of the cortical layer of the brain of man.

In human infection, the distribution of the superficial encephalitis is consistent with the centripetal affection of the brain cortex from the meningeal spaces through which the amoebae seemed to have moved initially or have partly been transported passively by the flow of the brain fluid. The clinical picture is dominated by signs of meningitis. A different clinical picture was found in experimental animals undoubtedly due to the different arrangement of the organs in their skull. Encephalitis accompanied by meningitis progressed in a single wave from the anterior parts of the brain backwards and signs of encephalitis were clearly marked in the guinea pigs.

The reaction of the chick embryo to infection with the Vitek strain was similar to that with *Acanthamoeba*. The experiment with chick embryos served only the purpose explained in the pertinent paper (Červa 1970).

This overall preliminary evaluation of pathogenic tests with the Vitek strain will serve as a basis of other research projects to be included in our programme.

REFERENCES


Received 10 November 1970.

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EXPLANATIONS OF THE PLATES

Plate I.

Transverse sections through a decalcified skull of a guinea pig after intranasal inoculation with the Vitex strain. Direct enlargement of the histological preparations. Triehrome (×10).

Fig. 1. — Clearly visible leftsided development of the inflammatory infiltration of the nasal mucosa in the frontal part of the skull. Fig. 2. — Migratory route of the amoebae from the nasal mucosa to the frontal lobes (at the left). The right side is intact.

Plate II.

Fig. 1. — Longitudinal section through the brain of a guinea pig which died after intranasal inoculation with the Vitex strain showing both the onesided development of the process and the extent of the lesions leading to the animal's death. Direct enlargement of the histological preparation. Heidenhain's ferric haematoxylin (×5). Fig. 2. — Detailed view of Plate 1. Fig. 2. On the left side, the nasal mucosa (NM) and the tissue of the olfactory nerve (NO) are normal. On the opposite side of the skeletal septum (S), the tissue of the nasal mucosa and that of the olfactory nerve is greatly devastated by amoebic and leucocytic infiltration. Triehrome (×100).
Fig. 1

Fig. 2