FOLIA PARASITOLOGICA (PRAHA) 14 : 171—175, 1967.

Intracerebral Inoculation of Experimental Animals in Pathogenetical Studies of *Hartmanella castellanii*

L. ČERVA

Military Institute of Hygiene, Epidemiology and Microbiology, Prague

Abstract. There is no significant difference in the susceptibility of mice, rats and guinea-pigs to experimental intracerebral infection with A 1 strain of *Hartmannella castellanii*. A single living amoeba proved to be both the least infectious and the lethal dose for these animal species. The mean survival time of infected animals varies indirectly with the number of amoebae in inoculum and directly with brain tissue dimensions. The mass leukocytic—mostly polynuclear—infiltration is apparently the main defensive reaction of the host. The antibody response appears to be of little importance in this way of inoculation.

The isolation of *Hartmannella castellanii* strains pathogenic for laboratory animals (CULBERTSON et al. 1959, 1965b) indicated the possibility of finding a new parasitic organism capable of natural invasions of animals or man. The spontaneous occurrence of fatal human cases of hartmannellosis (CULBERTSON et al. 1965c, FOWLER et al. 1965) obviously support this supposition.

Very important experimental studies of pathogenic hartmannellae were published by CULBERTSON et al. (1959, 1963, 1965 abc), McCOWEN et al. (1959) and BOVEE et al. (1961). In the pathogenesis of experimental hartmannellosis a number of problems remains still obscure. These are the problems of virulence, definition of infectious and lethal doses, defence-mechanisms of the host and the problems of diagnostical detection of amoebae and chemotherapy of experimental infections.

We would like to add our experimental data to elucidate some of these basal problems. Our first communication comprises the observations of intracerebral inoculations of white mice, rats and guinea pigs.

MATERIAL AND METHODS

Amoebae. Both the A l strain and Neff-strain (NEFF 1957) of *Hartmannella castellanii* were obtained from Dr. J. H. Sandground of the Haskins Laboratories in New York.

Cultivation. Axenic cultures of these amoebae were grown for several years in a medium with

Proteose Peptone Difco as the basal ingredient. Our informative experiments with other media showed that most favourable and standard results were obtained in a combination of 2 per cent Bacto Casitone Difco and 0.5 per cent NaCl in distilled water (unpublished data). The addition of glucose had no favourable effect on the growth of amoebae in this medium. Cultures of A 1 strain were incubated at 37 °C. Under these conditions an inoculum of a single amoeba gave a rich growth between the 14th and 21st day. Only three or five days were sufficient for a luxuriant growth after larger inocula. Amoebae were easily visible on the internal surface of the tubes even by a small magnification of the microscope. The Neff-strain was grown at room temperature. Reisolation of amoebae from organs of infected animals was performed, inoculating 2 or 3 mm^3 of tissue into the tubes with the Bacto Casitone (BC) medium to which 1000 O.U. of penicilline and 200 gamma of streptomycine pro ml had been added.

Animal inoculation. Agnotobiotic white mice of the Czechoslovak H-strain weighing 13—15 grams, Vistar white rats weighing 120—150 grams and guinea-pigs weighing 250—300 grams were used in our experiments. Intracerebral inoculations were accomplished by injecting 0.03 ml of fluid into the brain of mice and 0.1 ml of fluid into the brain of white rats and guinea-pigs narcotized with urethanum.

Counting. A direct counting method was used, utilizing Bürker's counting chamber. Serial dilution of amoebae suspensions was performed by means of silicon coated glass.

Serologic tests. Complement fixation reaction with a cellular antigen was used following the technique described in detail in a previous paper (Červa 1967).

Staining. Conventional histological staining method (haematoxylin-eosine) was not found satisfactory for paraffin sections. The nucleus of amoebae stains only with a slight intensity so that the identification of amoebae in sparsely invaded tissue may be very difficult. Better results were obtained by using iron-haematoxylin stain after Heidenhain or trichrome after Masson.

Fluorescent antibody technique. The fluorescent antibody technique proved to be a very useful method to identify isolated or defected amoebae in tissue. Details of this technique were described in a previous paper (Červa 1966).

RESULTS

For animal inoculations, 10- or 14-day-old cultures were used without influencing the virulence of amoebae by serial transfers in animals or tissue cultures. The concentration of amoebae in basal suspension was adjusted by means of centrifugation to 10^6 of organisms per ml. Fig. 1 shows the results of the titration experiment in mice.

The survival time of mice depends obviously on the number of amoebae in inoculum. The dispersion of individual survival time values increases with decreasing inoculum. After the minimal dose of a single amoeba 40 per cent of mice died within the nine days following inoculation. Considering the faults which may occur when inoculating relatively large microorganisms in diluted suspension (sedimentation in the syringe etc.) a single living amoeba is apparently both the least infectious and lethal dose in intracerebral inoculation of mice.

A control group of mice inoculated intracerebrally with a concentrated suspension of *Hartmanella castellanii*—Neff strain survived without any signs of illness. Amoebae could not be detected in their organs on the 14th day after inoculation. Fig. 2 shows the results of a similar experiment with guinea-pigs. As in the first experiment, the survival time of animals is dependent on the size of inoculum,

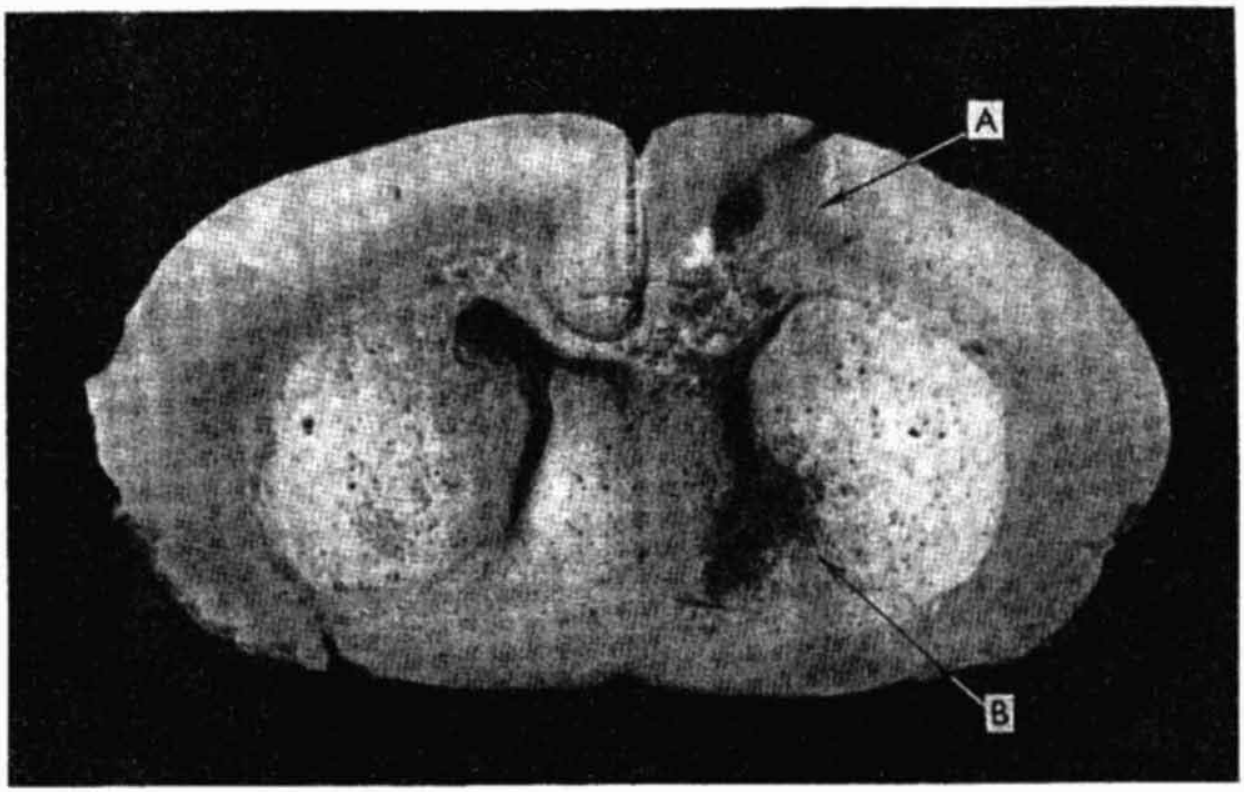

Fig. 1. Transversal section of brain of a guinea-pig dying after intracerebral inoculation. A — the area of inoculation, B — secondary amoebic focus.

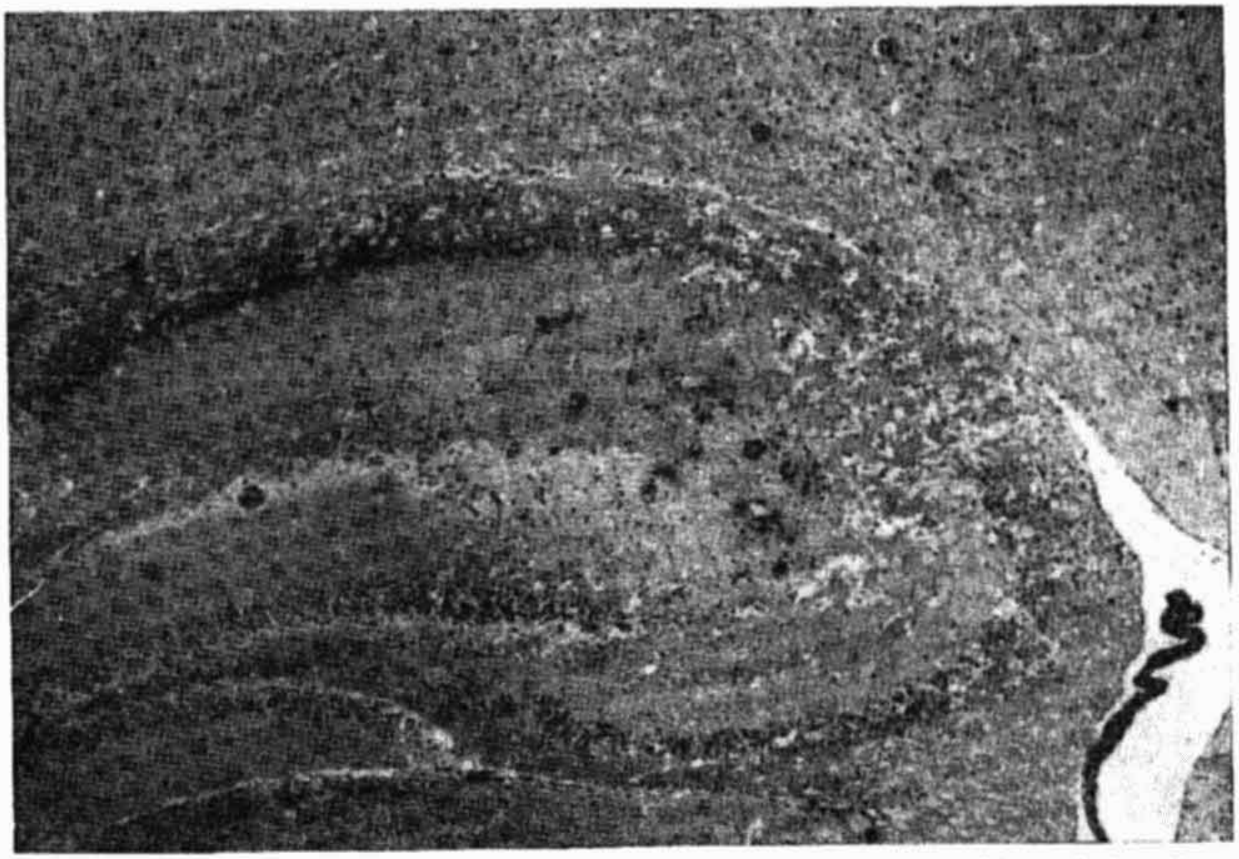

Fig. 2. The area of Cornu Amonis of a mice dying after intracerebral inoculation. Iron haematoxylin (×50).

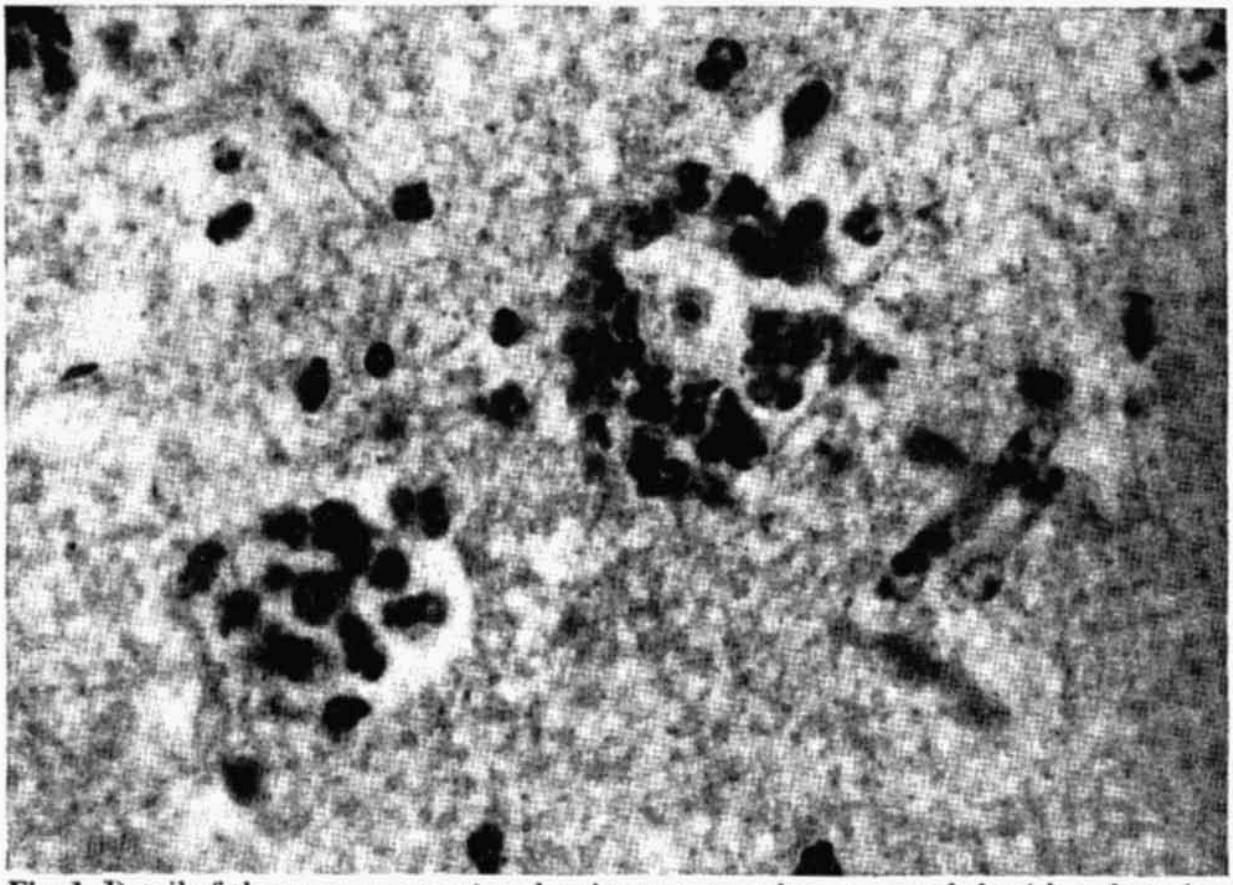

Fig. 1. Detail of the same preparation showing two amoebae surrounded with polynuclear leucocytes(×800).

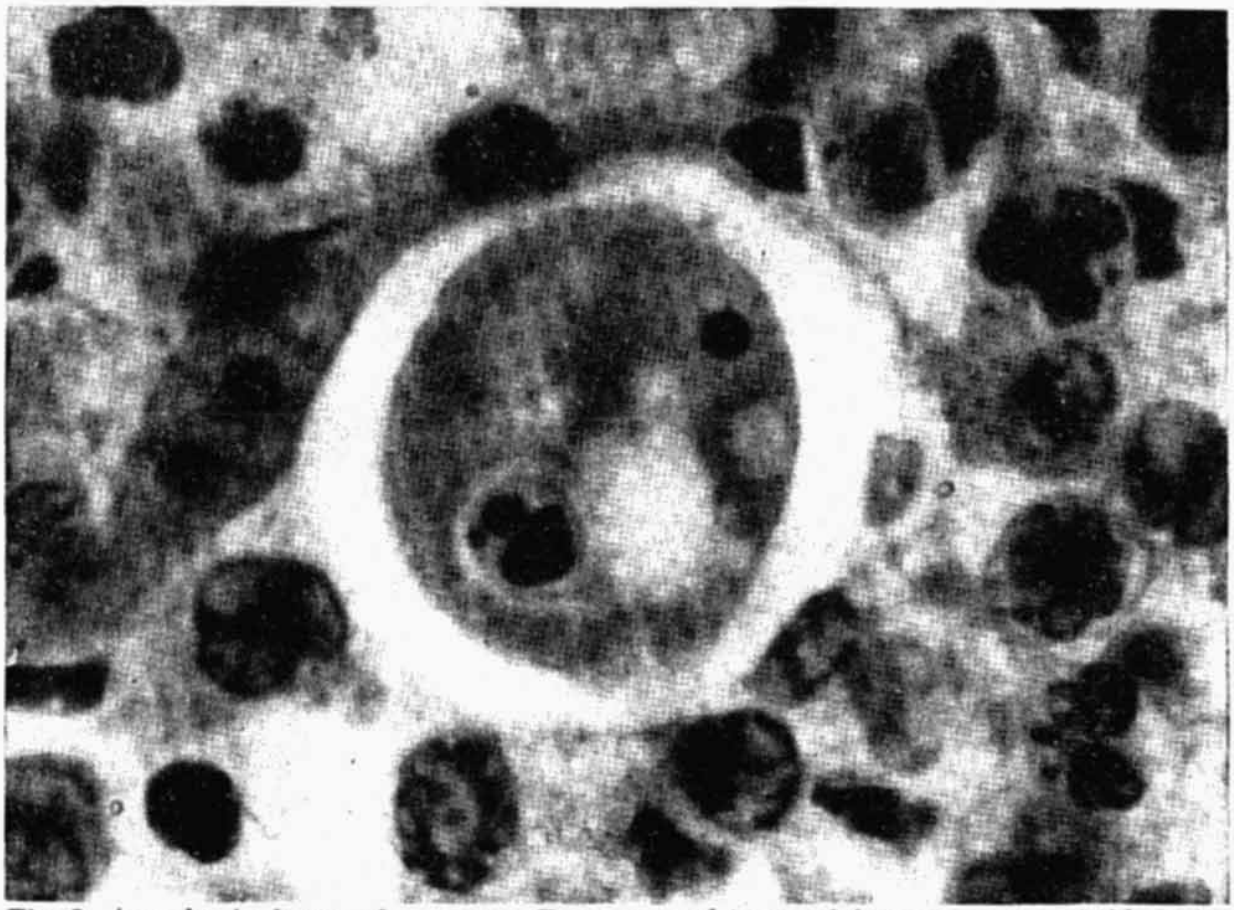

Fig. 2. Amoeba in brain of a mouse. Remnants of ingested leucocytes are visible in its plasma. Smear preparation stained with iron heamatoxylin (× 2 000).

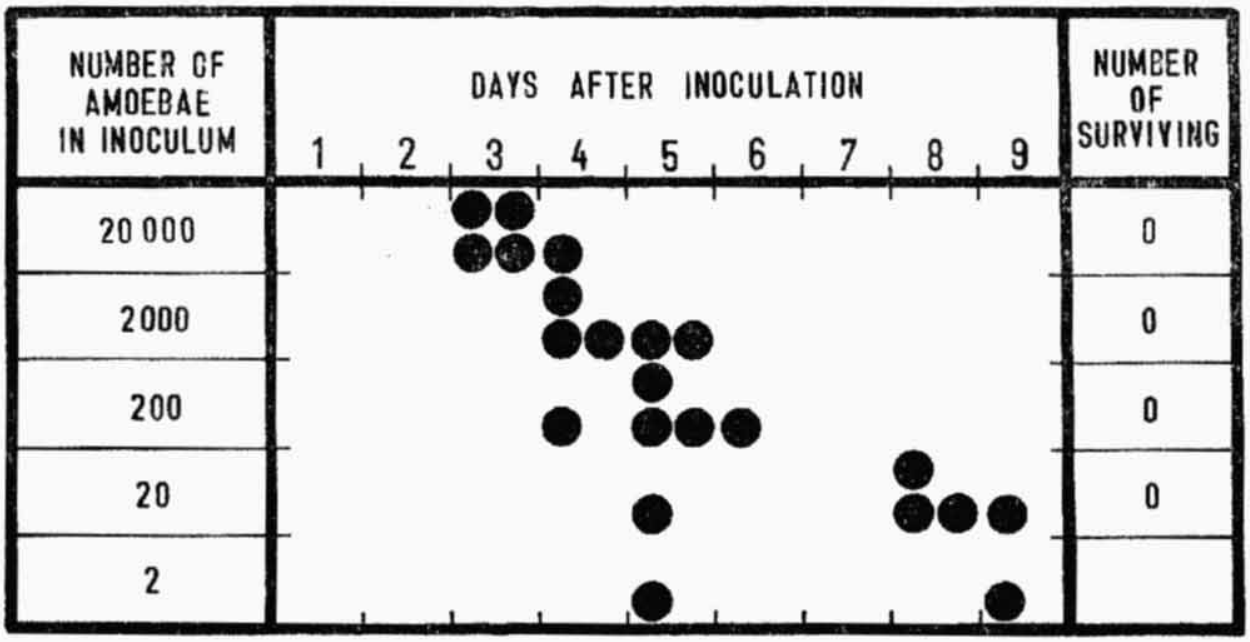

Fig. 1. Occurrence of death in mice after intracerebral inoculation of decimally diluted amoeba suspension.

but it is generally considerably longer. Receiving the minimal infectious dose, some of the animals survived for more than 3 weeks.

White rats inoculated with 10^3 of amoebae died between the 6^{th} and 8^{th} day. These results are identical with those obtained in the titration-experiment with guinea-pigs. The main signs following the development of amoebic lesions in the brain of mice: at the beginning an increase of activity and inclining of the head, later running in circles which passes into convulsive movements preceding death.

Amoebae were detected by means of cultivation in brains of all dead animals. Cultivations of lungs were positive by 20 per cent of dead mice. Other organs were always found negative. Inoculated guinea-pigs and rats do not manifest any signs of illness for a long time. Their body temperature remains normal. As late as 48 hrs before death a striking slackness appears. Loss of coordination in movements

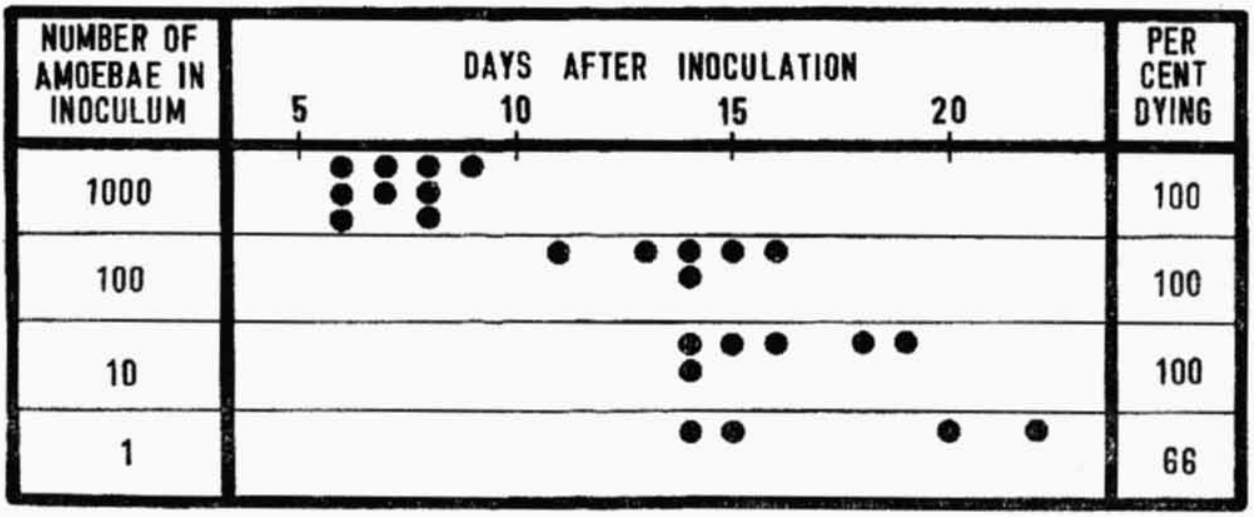

Fig. 2. Occurrence of death in guinea-pigs after intracerebral inoculation of decimally diluted amoeba suspension.

passes into complete paralysis, which is soon followed by death. In these animals amoebae were never found in other organs but the brain.

In histological preparations, extensive lesions in the area of inoculation were found. Multiplying amoebae may be always found in cornu Amonis and hypothalamus. The spreading of amoebae through the brain-tissue proceeds along the veins and cerebral ventricles and may give rise to secondary amoebic foci. Individual amoebae penetrating through the brain-tissue are often surrounded with rings or layers of polynuclear leukocytes. Heavily invaded areas of tissue are densely infiltrated with leukocytes. Characteristic vacuolization of the amoebae remains without any changes even in tissue preparations. Plasma of some of the amoebae holds ingested polynuclear nuclei or their remnants (Plate I and II).

The relatively long survival time of guinea-pigs enabled the tracing of antibody formation by means of CFR. The antibody titer of all guinea-pigs remained throughout the whole experiment (i.e. 23 days) completely negative.

DISCUSSION

Intracerebral inoculation of laboratory animals is widely used in virology as one of the most sensitive methods for the detection of viruses. The claims to virulence are very limited by this procedure. The fact that a single hartmannella causes death to mouse, rat and guinea-pig may be taken for evidence of the relatively high virulence of this organism. No signs were found by this method of a decreased virulence resulting from long lasting cultivation in vitro.

Histopathological picture of amoebic lesions supports the assumption that the main cause of death are the amoebae themselves. The existence of a concomitant pathogenic virus or other organism is not necessary for the explanation of hartmanella pathogenicity.

The character of lesions does not significantly differ in the three species of animals tested. There is also the probability of a similar generation time of amoebae in these animals. Differences in survival time of mice in comparison to rats and guinea-pigs are dependent chiefly on the volume difference of brains in these species. Death of the animals occurs as soon as a certain minimal part of brain is destroyed. The effect of the localization of brain lesions seems to be more perceptible after small inocula. The infestation of lungs in mice is probably mediated through invaded brain veins. Development of lesions in lungs after this way of inoculation has no important influence on the survival time of mice.

Multiplication of amoebae in brain tissue is obviously not a sufficient impulse for antibody production. This may have very unfavourable consequences for the serological detection of eventual clinical cases of hartmannellosis. Documentary photomicrographs of histological preparations of the fatal human cases of hartmannellosis published by FOWLER et al. (1965) show mass concentration of amoebae in brain without inflammatory reaction. This picture indicates that amoebae were