Short Communications

GROWTH OF THE PATHOGENIC A1 STRAIN OF ACANTHAMOEBA CASTELLANII IN THE CHICK EMBRYO

L. ČERVA

Army Institute of Hygiene, Epidemiology and Microbiology, Prague

Abstract. The pathogenic A1 strain of the amoeba *Acanthamoeba castellanii* grows in the tissue of the chick embryo, particularly in the central nervous system. The inoculation is best made into the yolk sac of 5 to 6 day-old embryos. After a small inoculation death occurs later than after intracerebral inoculation of mice. Chick embryos are suitable germ-free substrates for the titration of virulence of isolated strains of the organism, but are not suitable for diagnostic purposes.

The present study was designed to study the capability of adaptation of the pathogenic A1 strain of *Acanthamoeba castellanii* to the tissue of chicken organs and to detect whether these conditions are more favourable for the isolation or detection of virulence than those in the tissue of other laboratory animals which, being not always up to standard, yield results which are hardly comparable.

MATERIAL AND METHODS

The pathogenic A1 strain of *Acanthamoeba castellanii* was isolated in the U.S.A. from a culture of monkey kidney cells (CULBERTSON et al. 1958) and made available to us by the courtesy of Dr. J. Sandground of the Haskins Laboratories in New York. Previously we called this strain *Hartmanella castellanii* but, in view of recent new taxonomic knowledge, we are using now the generic name *Acanthamoeba* (PAGE 1967). For inoculation into the yolk sac we used 5 to 8 day-old chick embryos, for inoculation into the allantoic fluid 7 to 8 day-old embryos and for inoculation onto the chorioallantoic membrane and into the amniotic fluid 10 day-old embryos. During the experiments the embryos were incubated at +36 °C.

When not otherwise specified, inoculum suspensions were prepared from 7 to 10 day-old axenic cultures in BC medium (ČERVA 1966). The number of amoebae in the inoculum was adjusted according to the calculation in Bürker’s chamber. Each dilution used for inoculation contained 1000 I.U. of G penicillin and 100 gamma of streptomycin per 1 ml. Embryos were inoculated with 0.2 ml using standard virological techniques.

The presence of amoebae in the embryonic organs was identified by cultivation in BC medium. Under sterile conditions, tissue samples of about 1 mm³ were placed into the cultivation medium.

Chick embryos and their organs were fixed in 10% neutral formalin. Decalcification of older embryos was performed by adding 5% of trichloracetic acid to the fixation fluid. In addition to
haematoxylin eosin also Masson's trichrome and Heidenhain's iron haematoxylin were used for staining the paraffin sections.

RESULTS

Table 1 shows the course of infection of 8 day-old embryos inoculated into the yolk sac. The death of embryos in the first two groups indicates that the entrance through the yolk sac offers favourable conditions for the growth of the amoebae. A reduced dose of inoculum prolongs evidently the life of the embryos (see group 2).

<table>
<thead>
<tr>
<th>Number of amoebae in inoculum</th>
<th>Number of embryos</th>
<th>Survival after inoculation (days)</th>
<th>Percentage of deaths</th>
<th>Average survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000</td>
<td>5</td>
<td>4, 5, 5, 5, 6</td>
<td>100</td>
<td>5.0</td>
</tr>
<tr>
<td>1,000</td>
<td>6</td>
<td>5, 6, 7, 8, 8, 9</td>
<td>100</td>
<td>6.8</td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>—</td>
<td>0</td>
<td>On day 18, all living embryos in the culture were positive</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>—</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

The last embryo died on Day 9, i.e. at the age of 17 days. At this time the embryos are too large for further inspection in vivo. Embryos inoculated with still lower doses, maintaining their vitality up to the age of 18 days, were killed by cooling in the refrigerator and then inspected for amoebae. Cultivation revealed amoebae in the wall of the yolk sac, in the brain and the liver of the embryos; histological methods revealed them in the wall of the yolk sac and in the tissue of the central nervous tissue. In some embryos which died 6 days after inoculation, white cloudy foci (1—5 mm in diameter) were seen on the membrane of the yolk sac. Histological methods showed an agglomeration of amoebae in these clods penetrating the membranous tissue of the yolk sac. Amoebae were observed to enter also the lumen of the larger vessels to be carried rapidly to the embryonic organs. Since the formation of these visible clods on the membrane of the yolk sac is not a common feature in amoebic infection of the embryos, it cannot be used for diagnostic purposes.

Inoculation of massive doses of amoebae (10⁴ and more) into the amniotic fluid was responsible for the death of all embryos on Day 3 and 4 p.i. Microscopical examination revealed many amoebae in the amniotic fluid; upon cultivation amoebae were found in the yolk sac and in the embryo itself. We discontinued, however, to employ this mode of inoculation because it is successful only when used with older embryos; by reducing the number of amoebae in the inoculum to 10³, the life of the embryo is prolonged beyond the hatching period.

Also unsatisfactory were the results with inoculation into the allantoic fluid. Only 40% of the embryos died within 3—6 days although the inoculum contained several thousand of amoebae. The number of amoebae in the allantoic and amniotic
Fig. 1. Longitudinal section through the head of a chick embryo after inoculation into the yolk sac. The amoebic invasion into the brain tissue starts in the meningeal region (arrow). Direct enlargement of a histological section. Iron haematoxylin. x10.

Fig. 2. Survey of a developing amoebic focus accompanied by the formation of small haemorrhages. Longitudinal section through the embryo's head. Iron haematoxylin. x100.
Fig. 1. Detailed view of amoebae in the brain tissue of the embryo. Iron haematoxylin. x1000.

Fig. 2. Cross-section through the body of an embryo after inoculation onto the chorioallantois. Of the visible organs only the spine is affected asymmetrically by amoebic invasion. Direct enlargement of a histological section. Trichrome. x10.
fluid of the embryos decreases during the early stages of infection and in embryos
dying on Day 6 p.i., the results of microscopical inspection of the allantoic and am-
niotic fluid were negative. By contrast, in embryos close to hatching, which survived
up to Day 11 p.i., cultivation tests of their yolk sacs and of the embryos them-

selves gave positive results. The appearance of these embryos is completely normal.
Lower infective doses up to $10^3$ are not fatal to the embryos; in 10% of embryos,
amoebae could be detected as late as the hatching period.

In another experiment, the amoebic suspension was inoculated onto the chorio-
allantoic membrane. The results are shown in Table 2. The first fatal cases occurred
on Day 6, but a majority of embryos survived beyond the hatching period. The
chorioallantois seemed normal in appearance and macroscopical inspection showed

<table>
<thead>
<tr>
<th>Number of amoebae in inoculum</th>
<th>Number of embryos</th>
<th>Survival after inoculation (days)</th>
<th>Percentage of deaths</th>
<th>Average survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50,000</td>
<td>6</td>
<td>6, 6, 8, 8, 11</td>
<td>83</td>
<td>7.8</td>
</tr>
<tr>
<td>5,000</td>
<td>5</td>
<td>6, 8, 14, 14</td>
<td>66</td>
<td>10.0</td>
</tr>
<tr>
<td>500</td>
<td>6</td>
<td>11, 11, 12, 13</td>
<td>66</td>
<td>11.8</td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>6, 14</td>
<td>40</td>
<td>10.0</td>
</tr>
</tbody>
</table>

no conspicuous changes. Histological methods revealed small foci of amoebae in the
chorioallantoic tissue. The amoebae penetrate the vessels in the chorioallantois and
are carried to the predilect organs, the brain and the spine as shown in histological
sections. The results of inoculation of the chorioallantois were found less satisfactory
than those of the yolk sac.

The survival of the amoebae inoculated into the yolk sacs of 6 day-old chick
embryos was tested with regard to the age of the amoebic culture employed for
preparing the inoculated suspension. The results are given in Table 3.

<table>
<thead>
<tr>
<th>Age of culture</th>
<th>Number of embryos</th>
<th>Number of amoebae in inoculum</th>
<th>Survival after inoculation (days)</th>
<th>Average survival (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 weeks</td>
<td>14</td>
<td>7,000</td>
<td>4, 4, 5, 7, 9, 12, 12, 13, 13, 13, 14, 15</td>
<td>10.5</td>
</tr>
<tr>
<td>2 weeks</td>
<td>12</td>
<td>7,000</td>
<td>4, 4, 5, 6, 7, 9, 9, 9, 11, 11</td>
<td>7.4</td>
</tr>
<tr>
<td>4 days</td>
<td>12</td>
<td>7,000</td>
<td>4, 4, 4, 4, 4, 5, 5, 5, 5, 6</td>
<td>4.6</td>
</tr>
</tbody>
</table>
In addition to the results obtained in experiments with mice (Červa 1967), we received further proof of a close relationship between the growth phase of the culture used for preparing the inoculum and the apparent amoebic virulence.

**DISCUSSION**

The results of experimental infection of chick embryos with the A1 strain showed that the growth of this strain is not dependent on mammalian tissue only. The predilected site of amoebic reproduction is the tissue of the central nervous system of the embryo; this is reached by way of the vessels of the membrane of the yolk sac or of the chorioallantois, or by direct entrance into the embryo from the amniotic fluid. Reproduction is limited both in the amniotic and allatoid fluid. The capability of surviving in the allantoic fluid decreases along with embryonic growth, evidently in consequence of the increasing amount of renal products in this fluid. One of the problems to be solved is the further fate of chicks with confirmed amoebic infestation of the CNS during the hatching period, because these embryos showed no sign of affection.

The use of the chick embryo carries no advantage for the diagnostic detection or the growth of amoebae. Their inoculation into the yolk sacs of chick embryos may be of use only for the titration of virulence of pure isolated amoebic strains. In this case it is necessary to use the youngest possible embryos (5 to 6 day-old specimens) which may succumb to the infection before hatching has started. This procedure may help, in some way, to overcome the shortage of SPF and GF experimental animals.

The marked neurotropism of the amoebae, manifested also in the organism of the chick embryo, is of theoretical importance.

**REFERENCES**


L. Č. Vojenský ústav hygiény, epidemiologie a mikrobiologie, Praha-Střešovice, ČSSR

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