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INTRAVENOUS INFECTION OF MICE WITH NAEGLERIA FOWLERI*

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Abstract. *Naegleria fowleri* produced fatal meningoencephalitis in mice following intravenous (i.v.) inoculation. Amebae were present in the peripheral circulation for 120 minutes after i.v. inoculation with a dose of 10^7 trophozoites per mouse. Amebae were cultured from and observed in brain (days 1—21), lung (days 1—12), and liver and kidney (days 1—5). Infected mice exhibited weight loss, leukocytosis, reduced lymphocyte/neutrophil ratio, neurologic symptoms, and mortality. Histologically, the disease was characterized by an acute, hemorrhagic, necrotizing meningoencephalitis. Although amebae were detected in tissues other than brain, pathologic involvement of these tissues was minimal.

Naegleria fowleri, normally a free-living ameboflagellate, is an opportunistic organism capable of producing fatal meningoencephalitis in man. It also is able to produce a similar fatal disease in mice.

Epidemiologic and experimental evidence implicates the intranasal route of invasion. It would seem, then, that for experimental infections the preferred route of inoculation would be intranasal (i.n.) instillation. In general, this is true. However, one problem with this method of inoculation is knowing with certainty the number of amebae retained by the host, especially after ether anesthesia. Under ether, mice often sneeze out a portion of the inoculum, making it difficult to administer a consistently accurate dose. A way to possibly avoid this problem, and yet obtain similar results, would be to inoculate mice intravenously (i.v.). Injected via the lateral tail veins of the mouse, the entire inoculum would be retained by the host and hematogenous spread would carry amebae to the brain.

In this report we describe the hematogenous dissemination of amebae and host response to *N. fowleri* following i.v. inoculation.

MATERIALS AND METHODS

Cultivation and cell counting. The LEE strain (ATCC #30894) of *N. fowleri* was used throughout this study. Amebae were cultured axenically in cotton-stoppered, 2.8-liter, siliconized Fernbach flasks by using 1 liter of Nelson medium (John et al. 1977) inoculated with 10^4 amebae per ml. Cultures were adjusted to pH 5.0—5.5 and incubated at 37 °C in a gyrotory shaker (New Brunswick) at 100 rpm.

Amebae were harvested by centrifugation (1,200 × g, 5 min, 20 °C) at 72 h (early stationary growth phase). The cells were washed twice with Page ameba saline (Page 1967) and suspended in 0.15 M NaCl for counting and mouse inoculation. Amebae were judged viable by using trypan blue exclusion. Amebae were counted in a Coulter counter (model Z_{BI}) using procedures and settings described elsewhere (Weik and John 1977).

Mouse inoculation. Male DUB/ICR mice weighing 13 to 18 g (Flow Research Animals, Inc., Dublin, VA) were used in all experiments. Mice were allowed to adjust to their new environment for at least

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48 h before experimentation. They were given free access to water and feed (Purina Lab Chow). Mice were inoculated i.v. via a lateral tail vein with a 0.2-ml cell suspension containing 10^3, 10^6, 2.4×10^6, or 10^7 amebae per mouse of live *N. fowleri*. An i.v. inoculum of 2.4×10^6 amebae is the LD_{50} dose for the LEE strain of *N. fowleri* in 13 to 18 g male DUB/ICR mice (Haggerty and John 1978).

Cultivation of amebae from blood and tissue. Eight mice per group were inoculated i.v. with 10^3, 10^6, or 10^7 *N. fowleri* amebae per mouse. A 20 µl sample of blood was obtained from the clipped tail of each mouse at 5, 10, 20, 40, 80, 100, 120, and 160 minutes after inoculation. Blood samples were inoculated into Leighton culture tubes (Bellco Biological Glassware) containing 5 ml Nelson medium and penicillin (200 U/ml)-streptomycin (200 µg/ml). Cultures were incubated at 37 °C for 2 weeks and examined daily for amebae.

Amebae were cultured from tissues of mice inoculated i.v. with 2.4×10^6 *N. fowleri* per mouse. On days 1, 3, 5, 8, 12, 16, and 21 following inoculation, 4 mice per day were killed and tissues removed for culture. Brain, lung, liver, kidney, and spleen were removed aseptically, minced with scissors, and cultured individually in Leighton tubes containing Nelson medium and penicillin-streptomycin (as above). Cultures were incubated at 37 °C for 2 weeks and examined daily for amebae.

Determination of total and differential leukocyte counts and body weights of mice. Thirty-eight mice were each inoculated i.v. with 2.4×10^6 *N. fowleri*. On the day before inoculation, on the day of inoculation, and on days 2, 4, 6, 10, 14, 18, 22, 29, 35, and 42 after inoculation, blood from the clipped tails of 8 infected and 8 noninfected mice was obtained for total and differential leukocyte counts.

For total leukocyte determinations, 20 µl of blood was drawn using heparinized capillary pipetts and added to cuvettes containing 10 ml of azide-free isotonic diluent. Three successive Coulter counts were taken and averaged for each cuvette. The number of leukocytes/mm³ of blood was determined for each mouse, and the average total leukocyte count for each group of 8 mice was calculated.

Blood films for differential leukocyte counts were prepared at the same time blood was drawn for total leukocyte counts. Blood films were air-dried and stained with Giemsa stain. Films were examined under oil immersion, and the first 100 recognizable leukocytes were counted and recorded as lymphocytes, neutrophils, eosinophils, or monocytes. A differential leukocyte count was determined for each mouse, and the average count for each group of 8 mice was calculated.

Average mouse weights were obtained by weighing groups of 8 infected and 8 noninfected mice on the days given above. Each group of mice was weighed separately and the average body weight per mouse calculated.

Histology. Tissues for histologic preparation were obtained on days 1, 3, 5, 8, 12, 16, and 21 from mice inoculated i.v. with 2.4×10^6 *N. fowleri*. Brain, kidney, liver, lung, and spleen were fixed in 10 % buffered neutral formalin. The fixed tissues were embedded in paraffin and stained with hematoxylin and eosin (H & E).

RESULTS

Cultivation of amebae from blood and tissue. As shown in Table 1, mice that were inoculated i.v. with 10^3 *N. fowleri* were able to clear the amebae from the peripheral circulation within 5 minutes. As the inoculum was increased to 10^6 and 10^7 *N. fowleri*, greater time was needed to clear the amebae. An i.v. dose of 10^6 amebae/mouse was cleared between 5 and 10 minutes after inoculation. Amebae from the 10^7 inoculum were still present in the peripheral circulation of all mice at 80 minutes after inoculation. The amebae were progressively cleared to 160 minutes when none could be recovered by culture.

Table 2 gives the results for cultivation of amebae from tissues of mice inoculated i.v. with 2.4×10^6 amebae. The tissue from which amebae were recovered most frequently was brain. Amebae were cultured on all experimental days with 86% of brain tissue positive for amebae. Thirty-two percent of all lung tissue cultured was positive for amebae; amebae were isolated through day 12. Eighteen percent of kidney tissue and 11% of liver tissue was positive for amebae; amebae were cultured from both only through day 5. *Naegleria fowleri* was not cultured from spleen on any of the sampling days

Table 1. Percent of mice with *N. fowleri* in peripheral circulation following i.v. inoculation*)

Dose amebae/mouse	Minutes after inoculation							
	5	10	20	40	80	100	120	180
10^3	0	0	0	0	0	0	0	0
10^6	100	0	0	0	0	0	0	0
10^7	100	100	100	100	100	50	25	0

*) There were 8 mice at each dose level, and all mice were bled at each sampling interval.

Table 2. Percent of mouse tissues positive for *N. fowleri* by cultivation following i.v. inoculation*)

Days after inoculation	Tissues cultured**)					Average cumulative percent
	Brain	Lung	Kidney	Liver	Spleen	
1	100	75	25	25	0	45
3	75	50	75	25	0	45
5	100	25	25	25	0	35
8	100	25	0	0	0	25
12	100	50	0	0	0	30
16	50	0	0	0	0	10
21	75	0	0	0	0	15
Average cumulative percent	86	32	18	11	0	

*) Inoculum was 2.4×10^6 amebae/mouse (LD_{50} dose for the LEE strain of *N. fowleri* and 13—18 g male ICR mice).

**) Tissues were cultured from 4 mice on each day indicated.

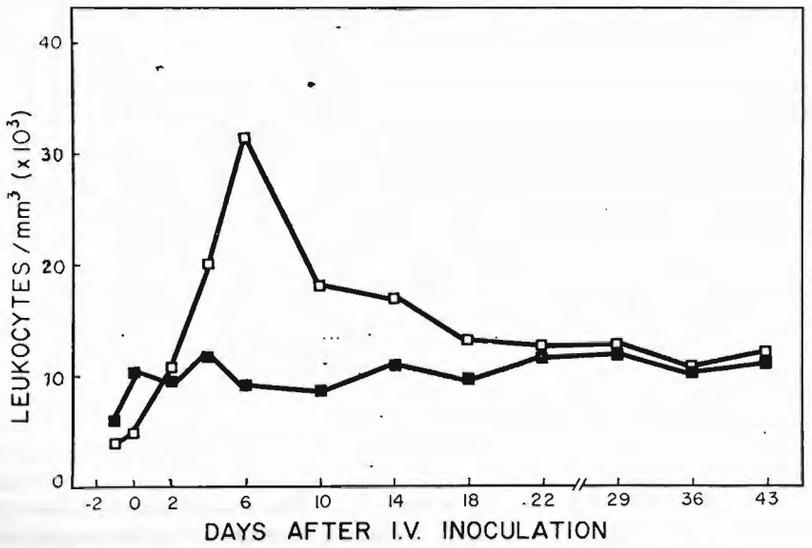

Fig. 1. Average total leukocyte counts for (□) mice infected i.v. with 2.4×10^6 *N. fowleri* and for (■) noninfected mice. Each point represents the average count from 8 mice.

although amebae were observed in H & E-stained tissue sections. The greatest recovery of amebae (45%) for all tissues occurred on days 1 and 3.

Total and differential leukocyte counts and body weights. The average total leukocyte counts for infected and noninfected mice are shown in Fig. 1. Total leukocyte counts for noninfected mice ranged from 6,310 to 11,900 cells/mm³ of blood during the experiment, with an average count of 10,550. Counts from infected mice increased soon after inoculation. A leukocytosis which occurred between days 2 and 18 attained a maximum of 31,500 cells/mm³ on day 6. Total leukocyte counts returned within the normal range after day 18.

The average differential leukocyte counts for infected and noninfected mice are presented in Fig. 2. The differential leukocyte counts from noninfected mice remained rather constant throughout the experiment. Lymphocytes ranged from 68—73%, neutrophils ranged from 19—23%, and monocytes ranged from 6—9%. Eosinophils were 2% or less.

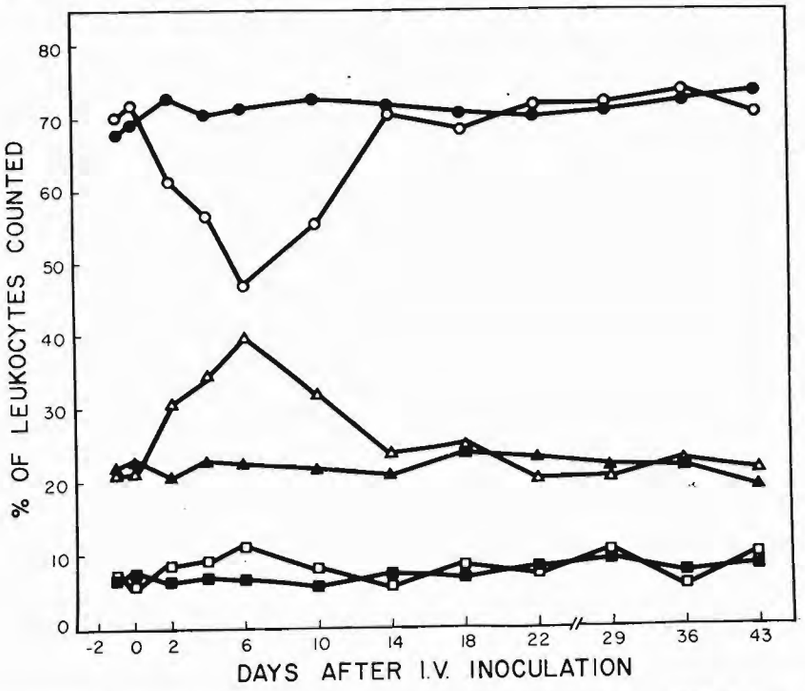

Fig. 2. Average differential leukocyte counts for mice infected i.v. with 2.4 × 10⁶ *N. fowleri* and for noninfected mice. Lymphocytes (○) infected mice, (●) noninfected mice; neutrophils (△) infected mice, (▲) noninfected mice; monocytes (□) infected mice, (■) noninfected mice. Each point represents the average count from 8 mice.

Differential leukocyte counts for infected mice began to change soon after inoculation. The percentage of lymphocytes decreased between days 2 and 14, reaching a minimum of 47% on day 6. An increase in the percentage of neutrophils occurred between days 2 and 14 with a maximum value of 40% occurring on day 6. Monocytes increased to 12% on day 6. Eosinophils remained within the normal range. The lymphocyte/neutrophil ratio decreased from 3.4 to 1.2 on day 6 and returned to normal on day 14.

The average total body weights for infected and noninfected mice are represented in Fig. 3. Noninfected mice gained weight steadily from an average of 18.7 g to 33.5 g during the 43 days. Infected mice showed a slight weight loss during the first 6 days. This was followed by a steady weight gain which, however, never reached the average weight for the noninfected mice.

Symptomatology and histopathology. In the i.v. inoculated mouse, clinical signs of infection usually appeared by day 3 with slight roughing of the fur. Over the next several days the fur became increasingly bristled and unkempt, and mice sat alone with arched or hunched backs. Changes in neuromotor activity began by day 6 and continued until death. During this time, mice exhibited various neurological signs including uncoordinated gait, unilateral movement characterized by circling or spinning, and partial or complete paralysis of the hindquarters. Deaths occurred after 8 days.

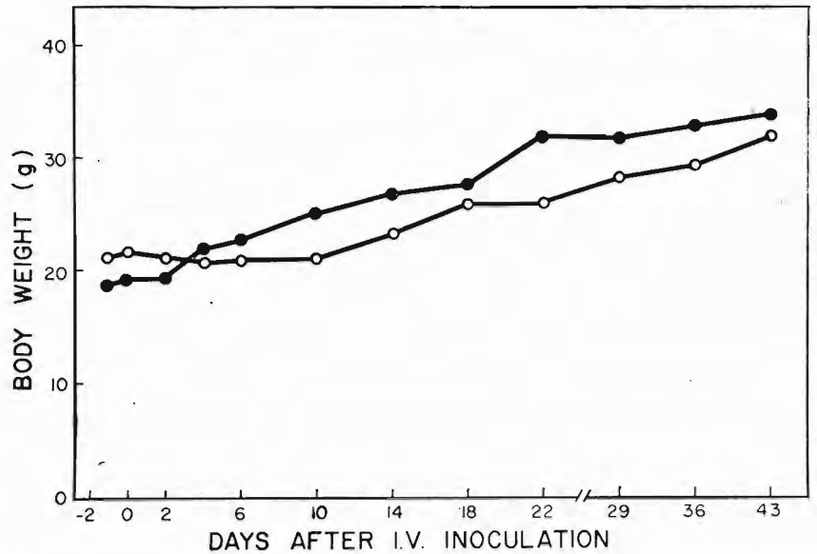

Fig. 3. Average total body weights for (○) mice infected i.v. with 2.4 × 10⁶ *N. fowleri* and for (●) noninfected mice. Each point represents the average weight from 8 mice.

Fig. 4 summarizes graphically the sequence of pathological events that occur in mice following i.v. inoculation with an LD_{50} dose of the LEE strain of *N. fowleri*.

Amebae were observed in hepatic sinusoids from days 1—5 (Plate I, Fig. 1). Additional hepatic involvement included areas of focal inflammation in the parenchyma and mononuclear cell infiltration along portal tracts (Plate I, Fig. 2). The lungs were congested and hemorrhagic through day 12 (Plate I, Fig. 3); amebae often were seen occluding pulmonary capillaries. An inflammatory infiltrate also was evident during this time. Amebae were found in capillaries of renal glomeruli through day 5. In the spleen, amebae were observed in capillaries on day 3 only. Inflammation and obvious tissue damage were absent in both kidney and spleen.

By the 3rd day following inoculation, amebic invasion of the brain was accompanied by hemorrhage and acute inflammation, comprised chiefly of mononuclear cells. Eight days after infection, meningoencephalitis was prominent (Plate I, Fig. 4). An acute

inflammatory infiltrate, often focal, was present in cerebral and cerebellar cortex. Amebae could be seen surrounded by inflammatory cells (Plate II, Fig. 1). Meningitis was extensive and diffuse (Plate I, Fig. 4). The inflammatory infiltrate of brain tissue and meninges was mainly one of mononuclear cells, although polymorphonuclear leukocytes were beginning to appear.

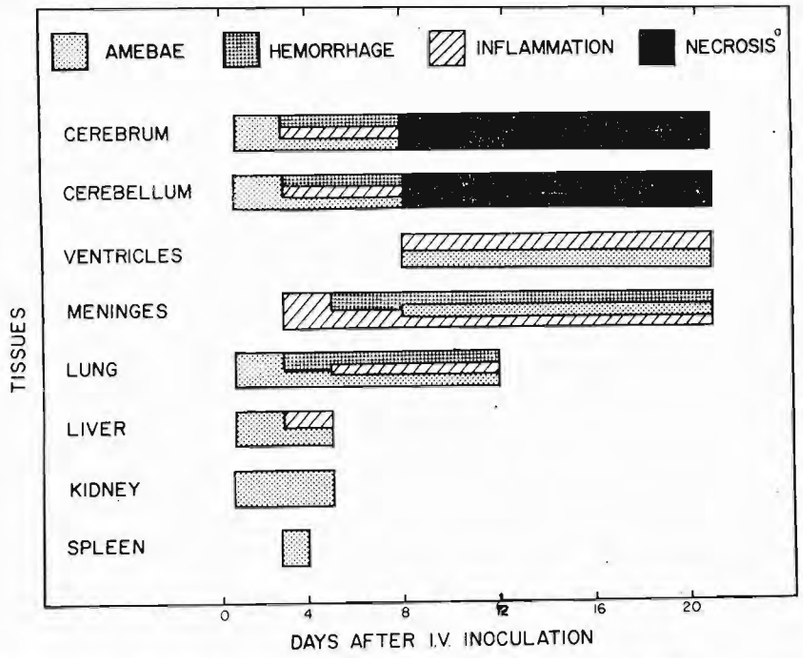

Fig. 4. Pathological changes in mice following i.v. inoculation with 2.4×10^6 *N. fowleri*. [a]Necrosis includes inflammation, hemorrhage, and the presence of amebae.

Days 12 through 21 were marked by hemorrhagic necrotizing meningoencephalitis. Polymorphonuclear leukocytes were the predominant cells in the necrotic exudate although lymphocytes, plasma cells, and macrophages also were present. Amebae, present in the dilated lateral and fourth ventricles and abundant in the tissue, contained phagocytosed erythrocytes and cellular debris (Plate II, Fig. 2). Amebae also were present in the choroid plexuses of lateral and fourth ventricles with associated acute inflammation.

DISCUSSION

Following inoculation into the lateral tail veins, amebae are carried via the inferior vena cava to the right heart, then to the lungs, back to the left heart, and finally to the brain by way of the carotid arteries. Since the brain receives about 14% of the total cardiac output (Milnor 1974), a large portion of the inoculum is not circulated through the liver, the major detoxifying and clearing organ of the body. Hence, amebae inoculated i.v. have direct access to the brain via hematogenous spread.

Organisms that gain access to the circulation generally are cleared from the blood

through phagocytosis by polymorphonuclear leukocytes and by fixed tissue macrophages of the reticuloendothelial system, especially Kupffer cells of the liver. Under normal laboratory conditions, the blood volume for adult mice is estimated at 5.5 ml/100 g of body weight (Bernstein 1975). Therefore, in order to recover at least one ameba in a 20 µl blood sample, there must be at least 42 amebae present in the circulation of a 13—18 g (calculated average of 15.5 g) mouse. The inocula of 10^3 (24 amebae/20 µl sample), 10^6 (2.4×10^4 amebae/20 µl sample), and 10^7 (2.4×10^5 amebae/20 µl sample) amebae per mouse provided an adequate supply of amebae to examine blood clearance following i.v. inoculation.

An i.v. dose of 10^3 amebae/mouse was rapidly cleared from the blood; amebae were not recovered at 5 minutes after inoculation. Doses of 10^6 amebae were cleared before 10 minutes and 10^7 before 160 minutes. The LD_{50} dose of 2.4×10^6 amebae/mouse used to evaluate host response to i.v. infection should have been cleared before 160 minutes. Thus, the amebae observed in H & E sections or cultured from tissue were ones that had established or survived in the tissue and were not amebae still in the circulation following i.v. inoculation.

As one would expect, brain was the tissue from which amebae were cultured most frequently. This was followed in decreasing order of frequency by lung, kidney, and liver. Although amebae were observed in H & E-stained sections of spleen, they never were cultured from minced suspensions of this tissue. Because of this curious observation, we attempted to culture amebae in the presence of minced and homogenized mouse spleen. In the presence of minced spleen, ameba growth was inhibited for about 24 hours. With the addition of spleen homogenate to the culture medium, ameba viability and numbers were dramatically reduced (data not shown). One can only speculate as to why the amebae did not grow in cultures containing a suspension of spleen tissue. Perhaps enzymes or other toxic components (even specific antibody) inhibited ameba growth.

It is normal for infected mice to lose a certain amount of body weight. Because they are sick they tend to eat and drink less than noninfected animals. Weight loss for *N. fowleri*-infected mice occurred early following i.v. inoculation. And though the mice began to gain weight after day 10, they never reached the levels of the same aged, noninfected controls. Culbertson et al. (1972) and Diffley et al. (1976) observed a gradual weight loss in guinea pigs infected subcutaneously and intramuscularly with *N. fowleri*. The weight loss noted in experimental *N. fowleri* infections can be attributed to decreased water intake and, to a lesser extent, feed consumption (Carter 1970).

Polymorphonuclear neutrophils play a central role in host defense against infection. An in many infections neutrophils act as phagocytic or killer cells through interaction with antibody, complement, and chemotactic factors. In acute naeglerial infection, one would expect an increase in circulating neutrophils to accompany the observed meningeal, cerebral, and cerebellar infiltration.

An increase in the percentage of circulating neutrophils occurred between days 2 and 14, reaching a maximum value of 40% (12,600 neutrophils/mm^3) by day 6. A leukocytosis of 31,500 leukocytes/mm^3 blood was recorded for infected mice on day 6. For noninfected mice the values on day 6 were 10,000 leukocytes/mm^3 with 22% neutrophils (2,200 cells/mm^3), nearly 6-fold fewer neutrophils than for infected animals.

Ferrante and Thong (1980) have described several mechanisms by which neutrophils are capable of killing *N. fowleri* amebae. In most instaces the amebae were surrounded by a number of neutrophils and presumably were killed by release of enzymes from the neutrophils of the group attack. The neutrophils also were able to pinch off and phagocytose portions of amebae. After several portions were pinched off, the amebae ruptured and died.

A mononuclear cell infiltration of the meninges and brain tissue occurred in response

to amebic invasion. Because of the dramatic increase in neutrophils (nearly 6-fold), the percentage of lymphocytes was observed to decrease from 70% to 47% on day 6. However, as a result of the leukocytosis, the actual numbers of lymphocytes increased about 2-fold on day 6. Likewise, the actual number of monocytes increased about 3-fold by day 6 even though the percentage of monocytes in the differential cell count did not vary substantially. A leukocytosis, neutrophilia, and apparent decrease in percentage of circulating lymphocytes all have been reported in naturally acquired (presumably by intranasal instillation) human primary amebic meningoencephalitis (Butt 1966, Callicott et al. 1968, Duma et al. 1971).

Following i.v. inoculation with an LD_{50} dose of *N. fowleri*, the incubation period was approximately 4 to 5 days and the clinical course about 3 days. Occasionally mice survived longer. At the onset of clinical infection the mice exhibited bristled fur, a disinclination to move, and a puffy face. As infection progressed they sat alone with eyes closed and backs hunched or arched. Usually they exhibited neurological signs such as circling, spinning, or posterior paralysis. Changes in neuromotor behavior occur much more frequently in mice inoculated i.v. than in those inoculated i.n. The area of brain affected by hematogenous entry is far greater than that of the olfactory lobes following intranasal entry. With this exception, the clinical features of infection are similar to those described for experimental animals inoculated i.n. Other investigators using routes of inoculation less direct to the central nervous system have reported no significant clinical symptoms (Carter 1970, Culbertson 1971).

Intravenous inoculation of mice with *N. fowleri* resulted in extensive hemorrhagic necrosis, primarily in the grey matter of the cerebral cortex, cerebellum, and brain stem. This was accompanied by diffuse sanguinopurulent meningitis. Invading amebae frequently were surrounded by inflammatory cells. The infiltrate was composed chiefly of mononuclear cells, most of which were lymphocytes. As the tissue became necrotic, the mononuclear cell infiltrate gave way to an inflammatory response consisting of polymorphonuclear leukocytes, macrophages, plasma cells, as well as mononuclear leukocytes.

The early mononuclear cell inflammatory response has not been noted in human cases of primary amebic meningoencephalitis. And in mice inoculated i.n. the cellular response in the brain is one predominantly of polymorphonuclear neutrophils (Martínez et al. 1973). It is unlikely that the i.v. route of inoculation is responsible for the early mononuclear leukocytic response. Rather, such an inflammatory response probably occurs in all naeglerial infections, regardless of route of inoculation, and in most studies (especially human cases) tissues have been examined at autopsy when neutrophils, macrophages, and plasma cells also were present in the necrotic exudate.

Meningitis was extensive, and at various time involved most surfaces of the cerebral hemispheres, cerebellum, and brain stem. Amebae were present in the dilated meninges especially during the latter stages of the disease when adjacent brain tissue was necrotic and hemorrhagic. The lateral and fourth ventricles were greatly dilated and on several occasions amebae were observed within the choroid plexuses. Perhaps amebae or their metabolic products served as irritants and stimulated production of cerebrospinal fluid which contributed to increased intracranial pressure as evidenced by the domed appearance of the skulls of infected mice.

Although amebae were inoculated i.v., pathologic involvement of tissues other than brain was minimal. Amebae were observed in capillaries of the spleen, but without inflammation. Carter (1970) described enlarged spleen, with amebae, necrotic foci, and polymorphonuclear leukocytic inflammation for mice inoculated i.n. However, the strains of mice and amebae were different and we have shown previously that the age, sex, and strain of mouse (Haggerty and John 1978), culture conditions, and strain

of *N. fowleri* (Haggerty and John 1980) greatly affect the outcome of naeglerial exposure and infection.

In the present study, amebae were noted in the renal glomerular capillaries of mice, but there were no inflammatory change. Similar findings were reported by Carter (1970) and Culbertson (1971) following i.n. and subcutaneous inoculation of mice with *N. fowleri*.

Hepatic involvement following i.v. naeglerial inoculation was limited to inflammation along the portal tracts and in the parenchyma. Amebae were observed in hepatic sinusoids without apparent inflammation. Carter (1970) and Culbertson et al. (1972) reported liver sinusoids containing amebae and diffuse inflammation, without necrosis, scattered throughout the lobules for mice infected via i.n. instillation.

After brain tissue, the lungs were most affected following i.v. inoculation with *N. fowleri*. During the early stages of infection, the pulmonary capillaries often were blocked by amebae and the alveoli exhibited hemorrhagic edema. Neutrophils and mononuclear leukocytes comprised the interstitial pneumonitis which occurred. Similar observations have been recorded for mice which had been inoculated i.n. (Carter 1970).

These results show that mice inoculated i.v. exhibited a similar course of infection both clinically and pathologically as that described for i.n. infected mice and for naturally acquired human infections. Since the mice inoculated i.v. died from meningoencephalitis in the same way as mice inoculated i.n., the i.v. route of infection offers a valid alternative method (although not preferred) of inoculation for studying experimentally induced primary amebic meningoencephalitis. The results also suggest that *N. fowleri* amebae probably are neurotropic and that human infection possibly could arise following exposure to amebae by routes other than i.n. instillation.

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ВНУТРИВЕННОЕ ЗАРАЖЕНИЕ МЫШЕЙ АМЕБОЙ *NAEGLERIA FOWLERI*

Р. Г. Мей и Д. Т. Джон

Резюме. Амебы *Naegleria fowleri* вызывали смертельный менингоэнцефалит у мышей после внутривенного заражения. Амебы встречались в периферическом кровообращении в течение 120 минут после внутривенного заражения дозой 10^7 трофозоитов/мышь. Амебы культивировали и наблюдали в мозге (1—21-й день), легких (1—12-й день) и печени и почках (1—5-й день). У зараженных мышей обнаружены следующие симптомы: убывание веса, лейкоцитов, понижение отношения лимфоцитов и нейтрофилов, неврологические симптомы и смертность. Гистологически болезнь характеризовалась острым, геморрагическим, некротизирующим менингоэнцефалитом. Хотя амебы были обнаружены в других тканях, чем в мозге, патологическое участие этих тканей было минимально.

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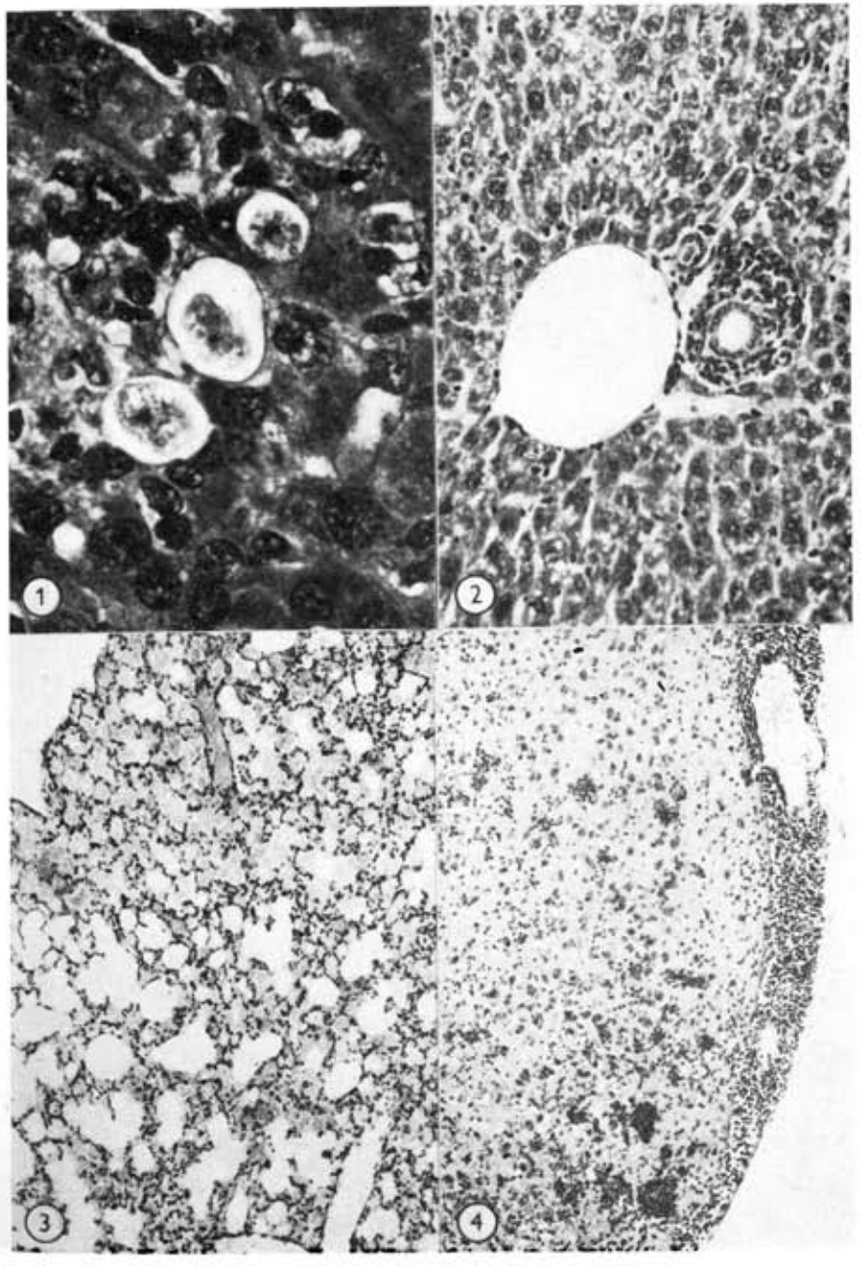

Fig. 1. Infected 3 days. Three adjacent hepatic sinusoids each containing an ameba. (H & E, ×760). **Fig. 2.** Infected 5 days. Acute monocytic inflammatory infiltrate surrounding bile duct of hepatic portal tract. (H & E, ×250). **Fig. 3.** Infected 8 days. Pulmonary congestion and hemorrhage with accompanying interstitial pneumonitis. (H & E, ×100). **Fig. 4.** Infected 8 days. Meningoencephalitis. Dilated meninges with acute inflammatory infiltrate; inflammatory foci within cerebral cortex. (H & E, ×100).

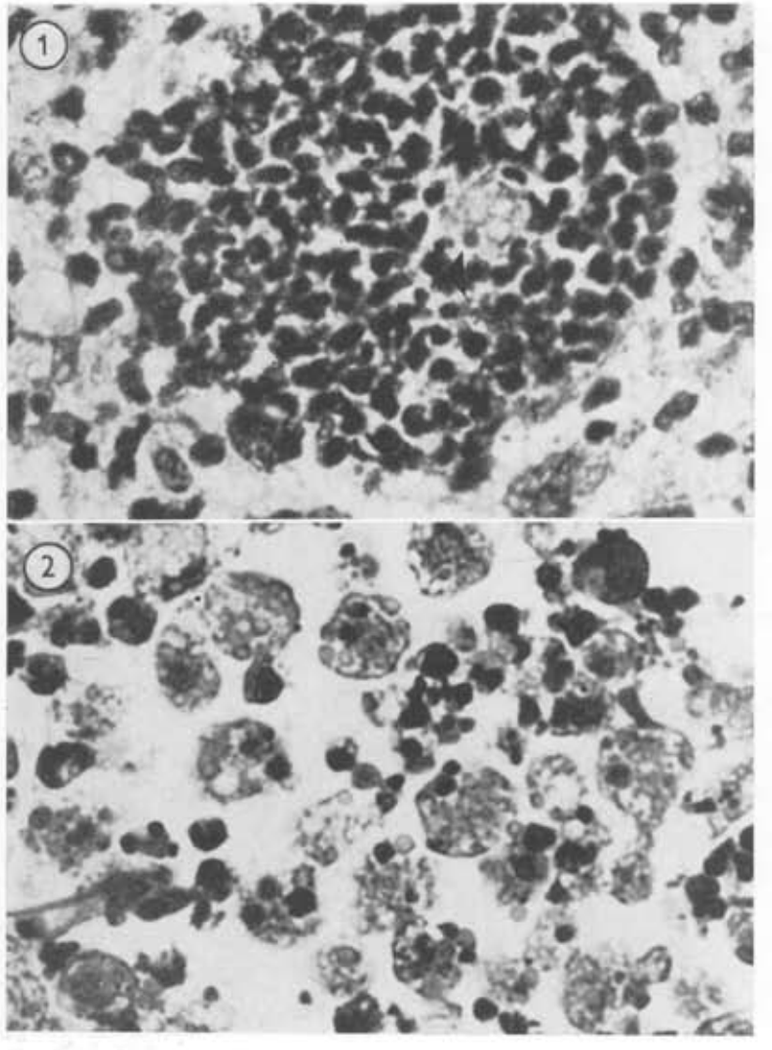

Fig. 1. Infected 12 days. An acute predominantly lymphocytic inflammatory response surrounding an ameba (arrow) within the cerebral cortex. (H & E, ×760). **Fig. 2.** Infected 21 days. Cerebellar cortex containing numerous amebae associated with inflammation and hemorrhagic necrosis. Amebae contain phagocytosed erythrocytes and cellular debris. (H & E, ×760).