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Intravenous Infection of Mice with *Naegleria fowleri*

by

Richard G. May

B.S., Virginia Polytechnic Institute
and State University, 1976

submitted in partial fulfillment of the requirements for the
Degree of Master of Science in the Department of
Microbiology of the Medical College of Virginia
Virginia Commonwealth University
Richmond, Virginia
July, 1979

This thesis by Richard G. May is accepted in its present form as satisfying the thesis requirement for the degree of Master of Science.

Date: July 13, 1979

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July 13, 1979

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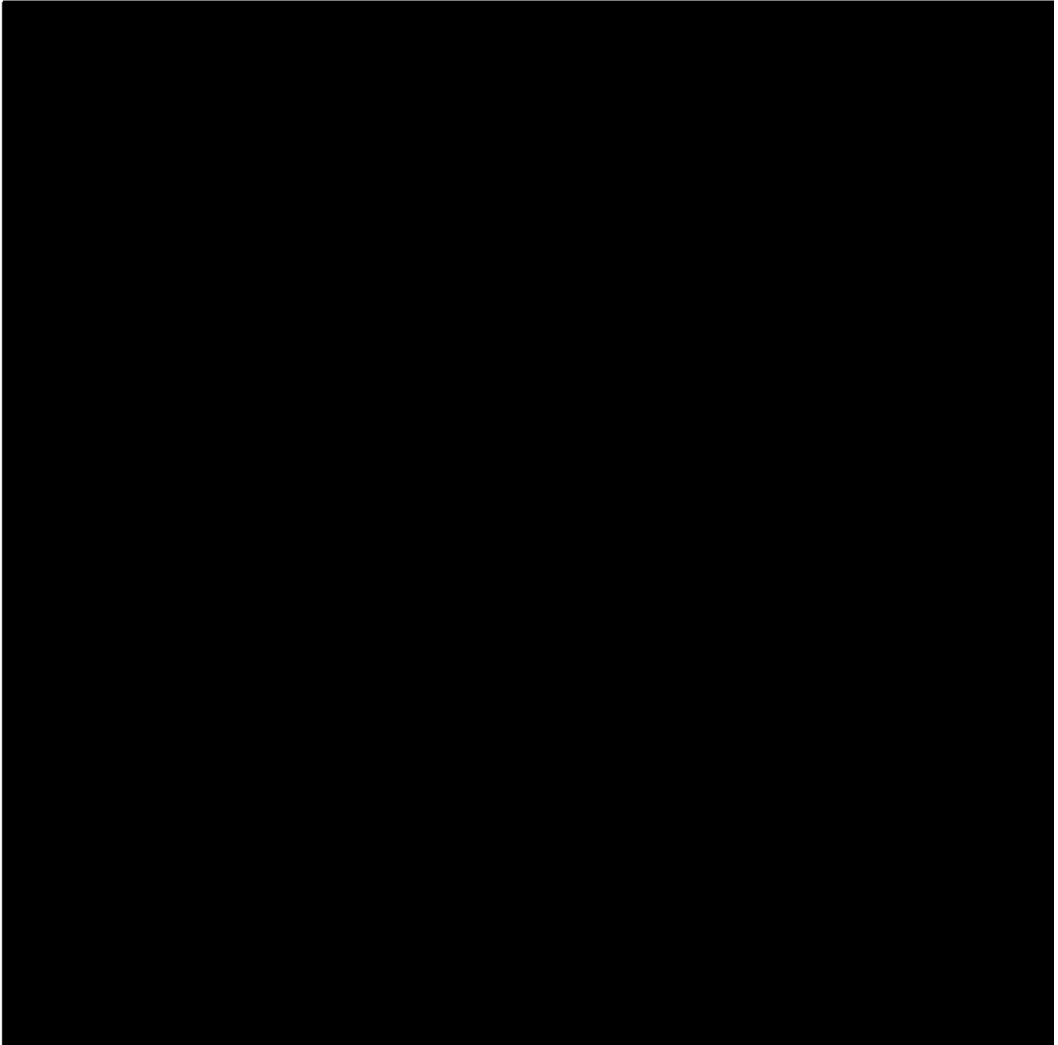

ACKNOWLEDGEMENTS

I would like to thank my friend and advisor Dr. David T. John for the guidance, criticism, ideas and endless time he so gladly contributed to my work during my two years at MCV. His encouragement, patience and especially his good humor made my stay quite enjoyable.

Doctors P. H. Coleman and G. W. Gander have provided valuable criticism and assistance during the preparation of this thesis. My committee members have earned my respect and thanks.

I would also like to express gratitude to my parents, David and Alice May, for their continuous support and encouragement throughout my educational endeavors.

Special thanks are extended to my friends who were always there when the end seemed so far away. They include Linda E. Bush, Richard and Mary Lane, Bob Dougherty, Bob Lipsky and Jean Young.

And finally, Laura Croes, for her most valuable assistance in preparing the final copy of this thesis.

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INTRODUCTION

Primary amebic meningoencephalitis is a fatal disease of man caused by the free-living ameboflagelate *Naegleria fowleri*. In general, the victims have been active, healthy, young adults with a recent history of swimming or other fresh water-related activity. Infection with *N. fowleri* is apparently by way of nasal introduction of amebae-containing water. After nasal installation, electron microscopic and histopathologic studies with experimental animals reveal that amebae reside in the olfactory mucosa and then invade and migrate through the submucosal structures into the nerve plexuses. Amebae pass through pores of the cribiform plate and into the subarachnoid space. Subsequently, amebae invade the olfactory bulbs and lobes and spread to more distant areas of the brain causing massive hemorrhage, necrosis and edema. Frequently, the amebae aggregate in the perivascular spaces where they provoke a predominantly neutrophilic cellular response. Within seventy-two hours after the onset of symptoms, a rapid deterioration of the patient ensues resulting in coma and death (Carter, 1972; Martinez et al., 1977).

Standard amebocides, such as diodohydroxyquin, chloroquin and metronidazole are ineffective in treating *N. fowleri* infections. Amphotericin B, an antibiotic used to treat systemic fungal infections, has been shown to be an effective antinaeglerial agent *in vitro*. Its ability to provide protection *in vivo* is unclear (Carter, 1969; Padilla and Padilla, 1974; Schuster and Rechthand, 1975). However, in Australia, Anderson and Jamieson (1972) used amphotericin B to successfully treat a 14 year old boy with confirmed primary amebic

memingoencephalitis. Duma et al. (1971), using the same drug and similar regimen, were unsuccessful with two patients at the Medical College of Virginia, Richmond. Other drugs, such as penicillin, suphadiazine, chloramphenicol, oxytetracycline HCl, streptomycin, methotrexate and emetine have had no effect on N. fowleri in vitro at levels in excess of those likely to be obtained therapeutically in the brain (Carter, 1968).

The taxonomic position of Naegleria places it in the kingdom Protista, phylum Protozoa and class Sarcodina. It is of the order Schizopyrenida and the family Vahlkamphidae due to its ability to transform from trophozoite to flagellate and because of its promitotic nuclear division. Naegleria reproduction involves nuclear division (karyokinesis), in which the nucleolus elongates and divides into two polar masses and the nuclear membrane remains intact, followed by cytoplasmic division (cytokinesis). The genus Naegleria is identified by organisms which are biflagellate and do not possess cytochromes. Within the genus Naegleria there are two species, the pathogen N. fowleri and the nonpathogen N. gruberi (Page, 1976). Willaert and Le Ray (1973) have described a third species, N. jadini. Synonyms for N. fowleri are N. aerobia (Singh and Das, 1970) and N. invades (Chang, 1971). Synonyms for N. gruberi are Amoeba gruberi, Dimastingamoeba gruberi and N. punctata (Fulton, 1970).

Naegleria fowleri can be differentiated from N. gruberi in many ways. The mitochondria of N. fowleri are dumbbell-shaped rather than oval as in N. gruberi. Naegleria gruberi cysts have numerous conspicuous pores through which excystment occurs while N. fowleri cysts have few inconspicuous pores. Naegleria fowleri cysts are less resistant

to drying than are cysts of *N. gruberi* (Carter, 1970). *Naegleria fowleri* is pathogenic, grows best at 37 C, although it will grow at 45 C, and is unable to grow in the presence of 0.5% saline. *Naegleria gruberi* is nonpathogenic, grows best at 25 C and grows well with 0.5% saline in the medium (Singh and Das, 1970). *Naegleria fowleri* cysts are more sensitive to chlorine than are the cysts of *N. gruberi* DeJonckheere and Van de Voorde, 1976). These species also differ in optimal pH for growth, growth media composition and size of the amebae. Concanavalin A agglutinates *N. gruberi* but not *N. fowleri* (Josephson et al., 1977). *Naegleria jadini* reportedly can be differentiated from *N. fowleri* by its reduced virulence and inability to grow at 37 C and from *N. gruberi* by its nonporus cyst wall (Willaert and LeRay, 1973).

Researchers have used mice, guinea pigs, monkeys and rabbits in their investigations of experimental primary amebic meningoencephalitis. Probably the most useful laboratory animal model involves the mouse. Mice have been used because investigators have shown that experimentally induced primary amebic meningoencephalitis in mice and naturally acquired primary amebic meningoencephalitis in humans have a similar incubation period, the disease is essentially confined to the central nervous system, similar clinical and pathological features occur and the outcome is invariable fatal (Carter, 1972; Culbertson, 1971; Duma, 1972 and Martinez et al., 1973). Also, mice are versatile, inexpensive, easy to handle and small enough to be housed in large numbers in a small area.

Culbertson et al. (1968) inoculated specific pathogen-free mice intranasally (I.N.) with *N. fowleri* (HB-1 strain) and observed amebic hepatitis and rhinencephalitis. Similar inoculations, intravenously

(I.V.) and intraperitoneally (I.P.), showed amebae to be widely disseminated throughout the mouse.

Carter (1972) studied the pathogenicity of N. fowleri administered by a variety of routes. Mice were inoculated by the I.N., I.V., I.P., intramuscular (I.M.), intragastric, subcutaneous, intrahepatic, anterior or intracerebral, posterior intracerebral and intrapleural routes. Clinical symptoms and death occurred in all the mice inoculated I.N. and anterior or posterior intracerebrally. A third of the mice died following I.V. or intrahepatic inoculation. No clinical or pathological symptoms of primary amebic meningoencephalitis were found in the mice that were inoculated by the remaining routes.

The flagellate stage of N. aerobia (N. fowleri) was inoculated I.N. into mice in which it produced fatal meningoencephalitis. Brain smears from the infected mice showed only the ameba stage, indicating that the flagellates reverted to amebae after I.N. inoculation. In all likelihood, it was amebae that actually invaded the host and were responsible for death of the mice (Singh and Das, 1972). Similar results were obtained when mice were inoculated I.N. with N. aerobia (N. fowleri) amebae (Singh and Das, 1970).

Martinez et al. (1973) inoculated mice with amebae of two different strains of N. fowleri, (LEE-1 and CJ-1). After the onset of clinical symptoms (ruffed fur, circling, hunching), the disease progressed rapidly to death. Examination of brain tissue showed that both grey and white matter were affected and characterized by hemorrhage, edma, disintegration of neural structures with wide-spread invasion by amebae. Amebae were observed adjacent to arterioles and capillaries. The nasal and olfactory mucosa was extensively infiltrated by motile

amebae.

Cerva, (1971) inoculated mice intracerebrally and I.N. with *Naegleria* (Vitek strain). After intracerebral inoculation, all of the experimental mice died. Shortly before death the mice showed symptoms of infection such as reduction of activity, uneven coat, disturbed equilibrium and finally loss of coordination. The mice that were inoculated I.N. died a few days after those inoculated intracerebrally. Histological examination of both groups of mice showed necrosis of much of the brain tissue and hemorrhage of the frontal lobes and also destruction of the mucous membranes of the I.N. inoculated mice.

Guinea pigs inoculated I.M. and subcutaneously with *N. aerobia* (*N. fowleri*) exhibited generalized loss of weight and strength. Hind-quarter I.M. injections caused enlargement of the regional lymph nodes. There was no amebic involvement of the brain; however, hepatosplenomegaly, enlarged kidney and amebic lesions of the intestines did occur (Culbertson et al., 1968).

Červa, (1971) inoculated guinea pigs I.N. with high, medium and low doses of *N. fowleri*. Guinea pigs given the low dose developed an elevated body temperature for an extended period of time and over half of the guinea pigs died. In the two higher dose groups, a rise in body temperature was noted only a few days before death. In a similar experiment Singh and Das (1972) inoculated two guinea pigs I.N. with *N. aerobia* (*N. fowleri*). Fatal meningoencephalitis developed soon afterwards.

Phillips (1974) inoculated adult, germ-free guinea pigs I.N., intraorally, into the conjunctival sac and into skin lesions. Most of the guinea pigs inoculated I.N. died with meningoencephalitis. However,

guinea pigs which were inoculated by the other routes remained in good health and were free of tissue damage at autopsy. Histological examination of the guinea pigs that had succumbed to N. fowleri infection (I.N. inoculated) showed destruction of the cerebellum, hemorrhagic meningitis, destruction of the frontal lobes, degradation of the meninges and a hemorrhagic condition of the anterior brain.

Monkeys have been inoculated I.N., I.V. and intrathecally with N. fowleri. Those receiving amebae I.N. or I.V. exhibited no evidence of Naegleria infection or central nervous system involvement. Monkeys that died as a result of N. fowleri inoculation intrathecally developed extensive lesions in the cerebellum with only small hemorrhages in the cerebrum. Amebae were isolated from the brains, spinal cords, lungs and liver. The monkeys that survived intrathecal inoculation exhibited fever, anorexia, leukocytosis, elevated levels of serum enzymes, and varying degrees of central nervous system involvement (Wong et al., 1975).

Naegleria fowleri (HB-1 strain) amebae have been injected into the marginal ear vein of adult rabbits. Rabbits that died exhibited extensive brain and liver damage (Culbertson et al., 1968).

Investigators have concluded that the natural route of invasion for N. fowleri is from the nasal mucosa through the cribiform plate and into the brain. Therefore, the logical way to inoculate experimental animals would be by I.N. installation. Unfortunately, it is often difficult to administer a consistently accurate dose I.N. The reasons are that (1) when under ether anesthesia the mice tend to sneeze out a portion of the inoculum, (2) some of the inoculum may remain on the external nares and (3) a portion of the inoculum may flow into the

nasopharynx and be swallowed or, even worse, be aspirated and contribute to possible pneumonia. So, although it is possible to calculate the dose given, it is difficult to determine the number of amebae retained by the host.

One way to avoid losing amebae and yet obtain results similar to I.N. installation would be to inoculate the mice I.V. The lateral tail veins are readily accessible for inoculation, the entire calculated inoculum is retained by the mouse and hematogenous spread carries amebae to the brain. The aim of my thesis is to examine the course of infection for mice inoculated I.V. with *N. fowleri*.

The overall results of this study are that mice inoculated I.V. with *N. fowleri* died from meningoencephalitis similar to that observed for mice inoculated I.N. Although amebae were detected in liver, lung, spleen and kidney, pathological involvement of these tissues appeared to be minimal.

MATERIALS AND METHODS

Ameba Strains, Cultivation and Maintenance

Naegleria fowleri (LEE strain), used throughout this study, was isolated in 1968 from human brain at the Medical College of Virginia by E. C. Nelson (Department of Microbiology, Virginia Commonwealth University). Amebae were grown in Nelson medium consisting of Page ameba saline (0.12gNaCl, 0.004g $MgSO_4 \cdot 7H_2O$, 0.004g $CaCl_2 \cdot 2H_2O$, 0.142g Na_2HPO_4 and 0.136g KH_2PO_4 per liter of distilled water) supplemented with 0.1% (w/v) Panmede liver digest (Paines and Byrne LTD., Greenford, England), 0.1% (w/v) glucose and 2% (v/v) calf serum (Grand Island Biological Co., Grand Island, NY). Stock cultures of *N. fowleri* were maintained in 25-cm^2 tissue culture flasks (Falcon Plastics, Oxnard, CA) at 30 C. Unagitated cultures of *N. fowleri*, used as inocula for Fernbach flasks, were grown in 75-cm^2 tissue culture flasks with 30 ml Nelson medium. The flasks were inoculated with 10^4 amebae/ml and incubated at 30 C for 72 h. Agitated cultures of amebae were grown in cotton-stoppered 2.8-liter Fernbach flasks with 1 liter of Nelson medium inoculated with 10^4 amebae/ml, adjusted to pH 5.0 - 5.5 with 1N HCl and incubated at 37 C for 48 to 72 h. Flasks were agitated in a gyrotory shaker (New Brunswick Scientific Co. Inc., New Brunswick, NJ) at 100 rpm.

Cell Harvesting and Counting

Amebae were harvested for mouse inoculation following growth in one-liter volumes of Nelson medium in cotton-stoppered 2.8 liter Fernbach flasks. Amebae were harvested by centrifugation at 2000 X *g* for 10 minutes at 20 C in a Beckman model J-21B centrifuge (Beckman

Instruments Inc., Palo Alto, CA). The amebae were washed twice in Page saline and resuspended to the desired final volume in sterile physiological saline.

All cell counts were made with an electronic particle counter (Coulter Counter, Z_{BI}, Coulter Electronics, Inc., Hialeah, FL) by adding 0.2 ml of ameba suspension to 9.8 ml of electrolyte solution consisting of 0.5% (v/v) formalin and 0.4% (w/v) NaCl in distilled water. Cuvettes were immediately Vortex-shaken at setting 7 for 5-10 seconds to dispurse cell aggregates and then read within 15 minutes. Prior to counting, cuvettes were inverted several times to resuspend settled amebae and then read after bubbles dispersed. Three successive counts were taken and averaged for each cuvette. Coulter settings for counting amebae were: gain 0, matching switch 20 K, bandwith selector extended, 1/amplification 4, 1/aperature current 1, lower threshold 10, and upper threshold maximum (Weik and John, 1977). For counts of 10^4 or higher, a coincidence correction chart was consulted for the adjusted true count which corrected for aggregates of amebae and coincident passage of amebae through the aperature.

Mouse Strain, Maintenance and Inoculation

Male DUB/ICR mice weighing 13-18 g (Flow Research Animals Inc., Dublin, VA) were used in all experiments. Mice were allowed to adjust to their new environment for at least 48 h prior to experimentation. Mice were kept in groups of no more than 10 per cage and exposed to a 12 h photoperiod. Room temperature was maintained at about 23 C. Mice were given free access to feed (Purina Lab Chow, Ralston Purina Corp., St. Louis, MO) and water, and cages were cleaned on alternate

days.

Mice were inoculated either intravenously (I.V.) or intranasally (I.N.). Mice inoculated I.V. received 10^3, 10^6, 2.4 X 10^6 or 10^7 live *N. fowleri* suspended in 0.2 ml physiological saline. Injections were made with a 1 cc tuberculin syringe and 25 gauge needle into a lateral tail vein. Prior to inoculation, cages containing mice were warmed slightly with a heating pad to help dilate the veins.

Mice inoculated I.N. were anesthetized with ether. The inoculum, containing 10^5 or 10^6 live *N. fowleri* suspended in Page saline, was instilled into a single nare using a 10 ul Eppendorf pipet.

Cultivation of Amebae from Blood and Tissue

Eight mice per group were infected I.V. with either 10^3, 10^6 or 10^7 *N. fowleri*/mouse. A 20 ul 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. Hemocaps, 20 ul heparinized pipets (Drummond Scientific Co., Broomall, PA), were used to collect the blood. Blood samples were inoculated into tissue culture Leighton tubes (Bellco Biological Glassware, Vineland, NJ) containing 5 ml sterile Nelson medium and penicillin (500 U/ml) -streptomycin(500 ug/ml). Cultures were incubated unagitated at 37 C for 2 weeks and examined daily for amebae.

Amebae were cultivated from tissues of mice inoculated I.V. with 2.4 X 10^6 *N. fowleri*/mouse. On days 1, 3, 5, 8, 12, 16 and 21 following inoculation, 2 mice per day were killed and tissues removed for culture. Brain, lung, liver, kidney and spleen were removed aseptically, minced with scissors and individually cultured in Leighton

tubes containing 5 ml Nelson medium and penicillin(500 U/ml) - streptomycin(500 ug/ml). Unagitated cultures were incubated at 37 C for 2 weeks and examined daily for amebae.

Viability and Growth of Amebae in the Presence of Mouse Spleen or Urine

To examine the viability of *N. fowleri* in spleen homogenate, a spleen was removed from a noninfected mouse, cleaned of excess fat, weighed and placed in sufficient cold, sterile Page saline to contain 0.1 g spleen/ml saline. The spleen was homogenized (Teflon tissue grinder, A. H. Thomas Co., Philadelphia, PA) for 30 seconds in an ice bath. Cold, sterile Page saline was used to prepare 1:10 and 1:100 dilutions of the homogenate. Using undiluted homogenate and 1:10 and 1:100 dilutions, 0.9 ml of each was brought to 37 C and 0.1 ml of *N. fowleri* (10^6 amebae) added. Controls contained 0.9 ml Page saline and 0.1 ml *N. fowleri* (10^6 amebae).

Tubes were incubated at 37 C; samples were removed at 30, 60, and 90 minutes and examined using a hemocytometer (Spencer Bright-Line, American Optical Corp., Buffalo, NY) to determine percent viable amebae and cell concentration. Exclusion of 0.01% trypan blue was used to judge the viability of amebae.

To evaluate growth of amebae in the presence of minced spleen (used in cultivation of amebae from infected mice), Nelson medium was prepared as previously described, inoculated with 10^4 or 10^5 amebae/ml and distributed in 25 ml amounts into 6 sterile 125-ml Erlenmeyer flasks. Two additional flasks contained medium without amebae. An entire minced spleen was added to each of two flasks and one-half a spleen was added to each of two other flasks. Two flasks, which were

spleen controls for Coulter particle counts, received only minced spleen. The remaining two flasks, containing only medium and amebae (no minced spleen), served as controls for normal ameba growth.

The effect of urine upon amebae was examined. A 0.1 ml sample of N. fowleri (10^6 amebae) was added to 0.9 ml of urine obtained from noninfected mice. The tube was incubated at 37 C and samples were removed at 30, 60 and 90 minutes and examined using a hemocytometer to determine ameba viability (using trypan blue) and cell concentration. Controls contained 0.9 ml Page saline and 0.1 ml N. fowleri (10^6 amebae).

Histologic Technique

Mice were inoculated I.V. with 2.4 X 10^6 N. fowleri/mouse and on days 1, 3, 5, 8, 12, 16 and 21 after inoculation mice were killed and tissues removed for histologic preparation. Sagittal sections of brain, beginning along the midline, were cut and, together with one kidney, one lobe of liver, one lobe of lung and half the spleen, for each day indicated, were fixed in 10% buffered neutral formalin for 1 week. Fresh formalin was replaced after 2 days of fixation.

Tissues were embedded in paraffin in Tissue-Tek plastic embedding rings (Fisher Scientific Co., Pittsburgh, PA) with lung and kidney together, liver and spleen together and brain alone (Histology Laboratory, Department of Pathology, Medical College of Virginia).

A rotory microtome (model 820, American Optical Corp.) and a chilled (in the freezer) plane-wedge microtome knife (American Optical Corp.) was used to section chilled (on ice), paraffin-embedded tissue blocks at a thickness of 6 um. Because paraffin-embedded tissue sections tend to become slightly distorted and wrinkled during

sectioning, a thermostatically controlled flotation bath (Boekel, A. H. Thomas Co.), set at 48 C, was used to soften, unfold and flatten sections prior to retrieval on microscope slides. Tissue sections were dried by placing the slides on a slide warmer (Precision Scientific Co., Chicago, IL) at 48 C.

Tissue sections were stained with hematoxylin and eosin, using a Tissue-Tek II slide staining set (Fisher Scientific Co.), by the following procedure.

Xylene I .... 2 minutes
Xylene II .... 2 minutes
100% ethyl alcohol .... 2 minutes
95% ethyl alcohol .... 2 minutes
Distilled water .... rinse
Harris' alum hematoxylin[a] .... 15 minutes
Distilled water .... rinse
Li_2CO_3, pH 8.0, solution .... 10 minutes
0.1% HCl in 70% ethyl alcohol .... 8 dips
Distilled water .... rinse
0.75% eosin Y[b] in distilled water .... 2.5 minutes
Distilled water, running .... 3-4 minutes
95% ethyl alcohol .... 1 minute
100% ethyl alcohol .... 1 minute
100% ethyl alcohol .... 1 minute
Xylene I .... 1 minute
Xylene II .... 5-10 minutes
Permount[b] and coverslip

[a]Harleco, Gibbstown, NJ

[b]Fisher Scientific Co.

The following staining procedures also were used in preparing brain tissue: Mayer's haemalum, Mallory phosphotunastic acid haematoxylin, Luxol fast blue, Giemsa, trichrome and periodic acidschiff.

Stained tissue sections were studied using all objectives of a compound microscope (Series 10 Microstar, American Optical Corp.). Photomicrographs of stained tissues were prepared by the Department of Visual Education (Medical College of Virginia).

Determination of Total and Differential Leukocyte Counts and Body Weights of Mice

Thirty-eight mice were each inoculated I.V. with 2.4 X 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 ul of blood was drawn, using heparinized capillary pipetts (Drummond Scientific Co.), and added to cuvettes containing 10 ml of azide free isotonic diluent (Fisher Scientific Co.) and hand-shaken. Three drops of Zap-isoton (Coulter Electronics), a high-speed lysing agent, were added to the mixture to lyse erythrocytes.

Cuvettes were immediately Vortex-shaken at setting 7 for 5-10 seconds to separate cell aggregates and to complete erythrocyte lysing. Prior to counting, cuvettes were inverted a few times to resuspend settled cells and read after the bubbles dispersed. Three successive counts were taken and averaged for each cuvette. Coulter settings for

counting leukocytes were: gain 0, matching switch 20K, 1/amplification 1/2, 1/aperature current 1/2, lower threshold 10.5, and upper threshold maximum. For counts of 10^4 or higher, a coincidence correction chart was consulted for the adjusted true count. The number of leukocytes/mm^3 of blood was determined for each mouse and the average total leukocyte count for each group of 8 mice was calculated and recorded.

Blood films for differential leukocyte counts were prepared at the same time blood was drawn for the total leukocyte counts. Blood films were air-dried and stained with Giemsa stain (Fisher Scientific Co.). 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 and recorded.

Average mouse weights were obrained by weighing groups of 8 infected and 8 noninfected mice on the days given above. Each group of mice was weighed separately on a Harvard trip balance (Ohaus Scale Corp., Flornam, NJ) and the average body weight per mouse calculated.

Transmission of N. fowleri from Mouse-to-Mouse

N. fowleri amebae were grown in one-liter cultures as described above. Thirty-two mice were each inoculated I.V. with 10^5 amebae; these were highly virulent amebae which had been serially passaged in mice 10 times. Inoculated mice were housed together with an equal number of uninoculated mice, i.e. 4 infected and 4 noninfected mice per cage. Twenty mice, housed in isolator cages in the same room, were

kept as uninoculated, nonexposed controls.

The uninoculated (but exposed) mice were observed for signs of *Naegleria* infection for 28 days after the death of 50% of the inoculated mice. At weekly intervals, 3 mice from the uninoculated group were killed and brain, lung, spleen, liver and kidney were cultured for *N. fowleri*.

At the end of the 28-day observation period, 20 uninoculated (but exposed) mice together with the 20 uninoculated, nonexposed controls were challenged I.V. with 10^6 amebae each. Mice were held for 21 days after inoculation, and the cumulative percent dead was recorded on a daily basis.

In addition, the above procedure was used to evaluate mouse-to-mouse transmission of *N. fowleri* following I.N. inoculation. Mice were inoculated with 10^5 amebae each; the challenge dose was 10^6 amebae/mouse I.N.

RESULTS

As shown in Table 1, mice that were inoculated I.V. with 10^3 *N. fowleri* each were able to clear the amebae from the perpheral circulation within 5 minutes. As the inoculum was increased to 10^6 and 10^7 *N. fowleri*, greater time was required 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 present in the perpheral circulation of all mice to 80 minutes after inoculation. The amebae were then progressively cleared to 160 minutes when amebae were not recovered by culture.

Mice were inoculated I.V. with *N. fowleri* and later killed for culture of amebae from various tissues. The results are presented in Table 2 and illustrated in Figure 1. The tissue from which amebae were recovered most frequently was the 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 9% of liver tissue cultured were positive for amebae; amebae were cultured from both tissues only through day 5. Amebae were not cultured from spleen on any of the experimental days although they were observed in H&E-stained tissue sections. The greatest recovery of amebae (45%) for all tissues occurred on days 1 and 3.

When *N. fowleri* is cultured in the presence of minced mouse spleen or fresh mouse urine, viability and growth were affected. The results for growth of *N. fowleri* in Nelson medium containing minced mouse spleen are presented in Table 3 and illustrated in Figure 2. Amebae that were

grown in the absence of minced spleen (control) reached a maximum cell yield of 2.1 X 10^6 amebae/ml at 72 h of growth. The mean generation time during log phase growth was 7.5 h and 9.1 h at 48 h and 72 h, respectively. When one-half or a whole spleen was minced and added to the cultures at the time of inoculation of N. fowleri, an increase in the mean generation time occurred. In the presence of one-half a minced spleen amebae reached a maximum cell yield of 4.8 X 10^5 amebae/ml at 72 h. The increased mean generation time was 9.8 h and 12.8 h for 48 h and 72 h growth, respectively. When a whole minced spleen was added to a culture of N. fowleri growth was inhibited during the first 24 h; then amebae resumed growth reaching a maximum cell yield of 8.1 X 10^5 amebae/ml at 96 h (72 h after growth initiation). Mean generation time at 48 h and 72 h after growth initiation was 9.0 h and 11.4 h, respectively.

Table 3 also presents the results of the addition of one-half or a whole minced spleen to cultures containing an initial inoculum of 10^5 N. fowleri/ml. The control cultures (no spleen added) reached a maximum cell yield of 2.1 X 10^6 amebae/ml at 72 h with a mean generation time of 16.9 h. Amebae that were grown in the presence of one-half or a whole minced spleen had a maximum cell yield of 5.5 X 10^5 and 5.2 X 10^5 amebae/ml, respectively, at 72 h with mean generation times of 28.8 h and 27.7 h.

The viability of N. fowleri incubated with dilutions of spleen homogenate are presented in Table 4 and illustrated in Figure 3. Amebae that were incubated without spleen homogenate maintained a viability greater than 98% with cell numbers constant over the 90 min incubation period. The results were similar for amebae incubated with spleen

homogenate diluted 1:100. When the spleen homogenate was diluted 1:10, viability decreased to 72% at 30 min then returned to a near normal value of 93% by 90 min. However, as the viability increased, the number of amebae/ml decreased slightly to 0.85×10^6/ml. Viability and amebae/ml were greatly reduced when undiluted spleen homogenate was added to the ameba suspension. The viability ranged from a low of 66% at 30 min to 75% at 90 min. Amebae decreased to 8.3×10^5/ml by 90 min.

Viability of *N. fowleri* in mouse urine also was examined. By 30 min of incubation, the viability of amebae in undiluted mouse urine had decreased to 20% and by 90 min it was 14%. Ameba concentration also decreased from 1×10^6 amebae/ml at time 0, to 3.5×10^5 amebae/ml by 90 min of incubation.

In the infected mouse, clinical symptoms of primary amebic meningoencephalitis usually appeared by day 3 with slight roughing of the fur. During 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 period, mice exhibited various neurological signs including incoordination of walk, unilateral movement characterized by circling in one direction and partial or complete paralysis of the hindquarters. Deaths occurred after eight days.

On *post mortem*, various tissues were examined for pathological involvement. A portion of normal liver is shown in Plate I, Figure 1. Hepatic portal tracts are comprised of branches of the portal vein, hepatic artery and bile duct. The portal tracts delineate lobules of liver tissue. A lobule has several portal tracts at its periphery and a main vein at the center. From the central vein radiate parenchymal

cells arranged in anastomosing and branching plates. The plates of parenchymal cells are exposed on either side to blood flowing in the hepatic sinusoids.

Amebae were observed in the hepatic sinusoids from days 1 - 5 (Plate I, Figure 2). Beginning on day 3 and continuing to day 5, focal polymorphonuclear leukocytic infiltrate with foreign-body giant cells occurred in the liver parenchyma (Plate I, Figure 3). Furthermore, a polymorphonuclear inflammatory infiltrate appeared around the portal ducts (Plate I, Figure 4).

The pulmonary capillaries during days 1 through 12 were often occluded by amebae (Plate II, Figure 5). Hemorrhage and consolidation of the interstitial spaces and aleveoli began three days after inoculation and persisted until day 12. Interstitial and alveolar leukocytic infiltrate occurred during days 5 through 12 (Plate II, Figure 6).

In the kidneys, amebae were found in the capillaries of the renal glomerulus beginning at day 1 and continuing until day 5 (Plate II, Figure 7). Inflammatory response or tissue damage was not seen in the kidney. In the spleen, amebae were found in the capillaries only on day 3; again, no inflammation or tissue damage was observed (Plate II, Figure 8).

A portion of normal cerebrum is shown in Plate III, Figure 9. In the cerebral hemispheres, gray matter (cortex) comprises the superficial layer. Most of the cells are pyramidal, stellate and fusiform in shape. The white matter, underlying the grey cortex, is composed of bundles of myelinated fibers supported by neuroglia.

Normal cerebellum is shown in Plate III, Figure 10. The cerebellum is a large and highly convoluted structure comprised of an outer

molecular layer composed of few small neurons and many nonmyelinated fibers. A single row of Purkinje cells (large pear-shaped perikarya) separates the outer molecular layer from the inner granular layer. The granular layer is composed of densely packed neurons and fibers. Within the granular layer are myelinated fibers of the white matter.

The entire central nervous system is covered by three membranes, the dura mater, arachnoid and the pia mater, collectively referred to as the meninges.

Amebae were present in the brain within 24 h after infection and were observed in the cerebellum and the anterior and posterior regions of the cerebrum. There was no obvious tissue damage or inflamation associated with the amebae.

Three days after infection amebae were found in many areas of the cerebrum, cerebellum and brain stem. Focal hemorrhage and acute inflammation were observed throughout the brain and were associated with amebic invasion. Hemorrhage also occurred in the cerebral and cerebellar meninges.

By 5 days after infection amebae were present in all areas of the cerebrum, cerebellum and brain stem. Focal hemorrhagic necrosis occurred throughout the brain (Plate III, Figure 11). Perivascular inflammation and a predominantly lymphocytic cellular response began to appear in brain tissue (Plate III, Figure 12). The cerebral and cerebellar meninges exhibited focal hemorrhage and were infiltrated by inflammatory cells.

Eight days after infection the cerebral and cerebellar cortex exhibited an acute inflammatory infiltrate which often times was focal (Plate IV, Figures 13 & 14). The meningitis was extensive and diffuse

rather than localized. The inflammatory infiltrate of brain tissue and meninges was mainly one of mononuclear cells, but polymorphonuclear leukocytes also were present.

Internal hydrocephalus was evidenced by the extreme dilation of the lateral ventricle (Plate IV, Figure 15) and fourth ventricle (Plate IV, Figure 16). Amebae were seen in the lateral ventricle (Plate V, Figure 17) and fourth ventricle (Plate IV, Figure 16; Plate V, Figure 18) in the vicinity of the choriod plexuses. A sanguinopurulent exudate was evident in the ventricles.

Inflammation and hemorrhagic necrosis accompanied massive amebic invasion of the cerebrum and cerebellum (Plate V, Figure 19 & 20). The inflammatory cells in the necrotic exudate consisted of macrophages, plasma cells, lymphocytes and polymorphonuclear leukocytes. The amebae were large, highly vacuolated and contained phagocytosed erythrocytes and cellular debris (Plate V, Figure 20).

Perivascular inflammation consisting principally of lymphocytes, was observed throughout the brain including cerebrum (Plate IV, Figure 16), cerebellum (Plate V, Figure 19) and brainstem.

Days 12 through 21 after inoculation were marked by continued sanguinopurulent meningitis. Amebae were present in the dilated lateral and fourth ventricles. Perivascular infiltration around blood vessels occurred throughout the brain (Plate VI, Figure 21).

Diffuse amebic invasion associated with acute inflammation and hemorrhagic necrosis was evident throughout the brain (Plate VI, Figure 22 - 24). Polymorphonuclear leukocytes constituted the predominent exudate although mononuclear cells also were present. Amebae were abundant in the cortical gray matter of the cerebrum and cerebellum

with or without surrounding inflammatory infiltrate which was principally of mononuclear cells (Plate VI, Figures 23 & 24).

Figure 4 graphically summarizes the sequence of pathological changes that develop in mice following I.V. inoculation with an approximate LD_{50} dose of N. *fowleri*.

Total body weights, total leukocyte counts and differential leukocyte counts were determined for infected and noninfected mice. The average total body weights for infected and noninfected mice are shown in Figure 5. Noninfected mice gained weight steadily from an average of 18.7 g to 31.8 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 of the noninfected mice.

The average total leukocyte counts for infected and noninfected mice are shown in Figure 6. Total leukocyte counts for noninfected mice ranged from 6,310 - 11,900 cells/mm^3 of blood during the experiment, with an average count of 10,550. Counts from infected mice increased soon after inoculation. A leukocytosis occurred between day 2 and day 8 and attained a maximum of 31,500 cells/mm^3 at 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 Figure 7. The average differential leukocyte counts from noninfected mice remained relatively constant throughout the experiment. The lymphocytes ranged from 68-73%, neutrophils ranged from 19-23% and monocytes ranged from 6-9%. Eosinophils and basophils remained less than 1%. The percentage of lymphocytes decreased between day 2 and day 14, reaching a minimum of 47% at day 6.

An increase in the percentage of neutrophils occurred between day 2 and day 14, reaching a maximum value of 40% on day 6. Monocytes increased to 12% at day 6. Eosinophils and basophils remained within normal ranges. The lymphocyte/neutrophil ratio decreased from 3.4 to 1.2 at day 6 and returned to normal at day 14.

Mouse-to-mouse transmission of naeglerial infection was tested by placing I.V. or I.N. inoculated mice in the same cage with noninfected mice. Amebae were not recovered from tissues of noninoculated mice that were exposed 1, 2, 3 or 4 weeks to mice infected I.N. Amebae were recovered only from the lungs of one mouse after 4 weeks of exposure to I.V. infected mice. Amebae were not recovered from the remainder of noninoculated mice that were exposed 1, 2, 3 or 4 weeks to I.V. infected mice. Mice which were exposed to I.V. or I.N. inoculated mice together with unexposed (control) mice were challenged with a lethal dose of *N. fowleri* I.V. or I.N. The mean time to death for the mice exposed to I.V. infected mice was 10.8 $\pm$ 2.5 days as compared with 8.4 $\pm$ 1.8 days for the unexposed mice. The mean time to death for the mice exposed to I.N. infected mice was 13.6 $\pm$ 2.0 days as compared with 10.9 $\pm$ 1.6 days for the unexposed mice. A summary of the statistical analysis for the differences in the mean time to death of exposed vs. nonexposed mice is presented in Table 5.

The amebae isolated from the lungs (described above) were identified as *Naegleria fowleri* by morphology, ability to transform into flagellates, growth in Nelson medium and pathogenicity for mice.

Table 1.

% of Mice with *N. fowleri* in Peripheral Circulation After I.V. Inoculation[a].

Dose amebae/mouse	Minutes after Inoculation 5	10	20	40	80	100	120	160
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

[a] There were 8 mice at each dose level and all mice were bled at each interval

Table 2.

% of Mouse Tissues Positive for *N. fowleri* by Cultivation Following I.V. Inoculation[a].

Days after Inoculation	Brain	Tissues Cultured Lung	Kidney	Liver	Spleen	Cumulative Average %
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
Cumulative Average %	86	32	18	9	0	

[a]Tissues were cultured from 4 mice for each day indicated following inoculation.

Table 3.

Growth of *N. fowleri* in Nelson Medium with Minced Mouse Spleen.

Amount of Spleen Added to Culture	Culture Age (h) 0	24	48	72	96	120
	Amebae/ml (X 10^4)					
Inoculum of 10^4 amebae/ml						
No Spleen	0.98	11.0	97.2	210.0	150.0	100.0
One-half (0.057 g) Spleen	1.1	6.5	30.0	48.0	44.0	15.0
Whole (0.106 g) Spleen	1.1	1.0	8.0	42.0	81.0	49.0
Inoculum of 10^5 amebae/ml						
No Spleen	11.0	31.0	55.0	212.0	170.0	-[a]
One-half (0.059 g) Spleen	9.3	14.0	46.0	55.0	52.0	-
Whole (0.176 g) Spleen	9.0	14.0	51.0	52.0	12.0	-

[a]-, not tested

Table 4.

Viability of N. fowleri Incubated With Mouse Spleen Homogenate.

Dilution of Spleen Homogenate	Incubation Time[a]					
	30 min		60 min		90 min	
	Amebae/ml ($X 10^6$)	% Viability	Amebae/ml ($X 10^6$)	% Viability	Amebae/ml ($X 10^6$)	% Viability
Control[b]	1.08	98.6	1.00	98.5	1.04	98.9
Undiluted[c]	0.97	65.9	1.04	69.9	0.83	74.6
1:10	0.98	72.1	1.04	91.7	0.85	92.9
1:100	1.08	98.6	0.98	98.9	1.09	98.1

[a]At time 0, cultures contained $\sim 10^6$ amebae/ml.

[b]Page ameba saline replaced spleen homogenate.

[c]Homogenate concentration was 0.1 g spleen/ml Page ameba saline.

Table 5.

Cultivation of tissues for *N. fowleri* from uninoculated mice housed with mice infected I.V. or I.N. with *N. fowleri* and a comparison by Student's "t" test of the mean time to death for mice exposed to *Naegleria*-infected mice following challenge with a lethal dose of *N. fowleri*.

	Routes of Inoculation for Infected Mice			
	I.V.		I.N.	
Cultivation of tissues from uninoculated mice[a]	LUNG-- *N. fowleri* amebae were isolated from 1 mouse 4 wk after exposure. (12 mice examined)		No amebae were cultured from any tissues. (12 mice examined)	
	Exposed[b]	Nonexposed[c] (control)	Exposed[b]	Nonexposed[c] (control)
Number of mice challenged	20	20	20	20
% deaths after challenge	75	100	80	100
% protection	25	0	20	0
Mean time to Death(days)	10.8±2.5	8.4±1.8	13.6±2.0	10.9±1.6
"t" value	3.055		4.342	
Level of significance[d]	0.01		0.001	

[a]The following tissues from uninoculated mice were cultured for amebae at 1, 2, 3 and 4 weeks after exposure to infected mice: brain, lung, kidney, liver and spleen.

[b]Exposed mice were housed together with infected mice.

[c]Nonexposed mice were housed in cages isolated from infected mice.

[d]Significance of the differences in mean time to death for exposed and nonexposed mice challenged with *N. fowleri*.

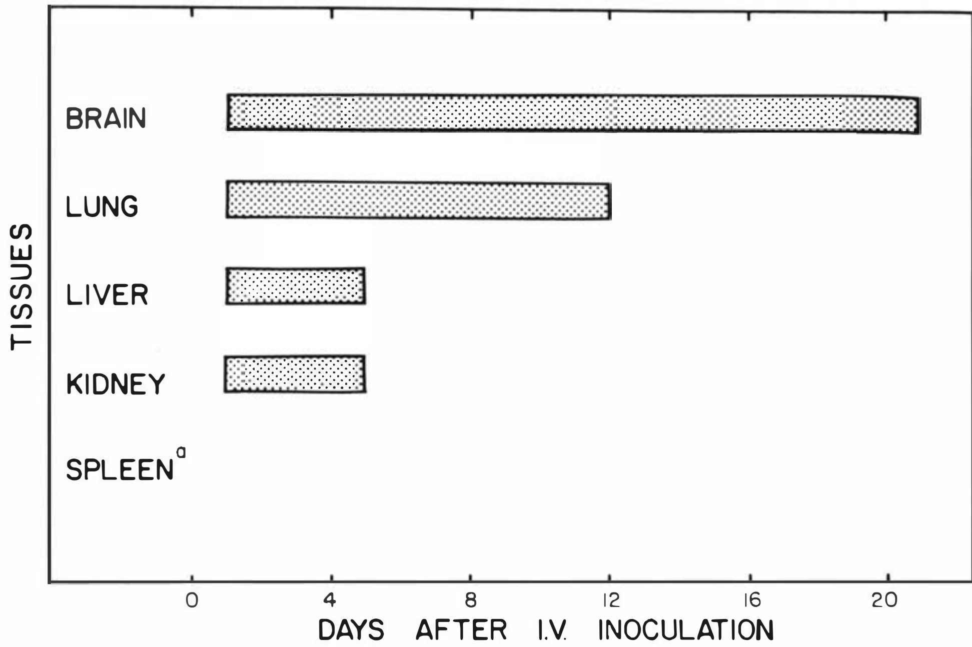
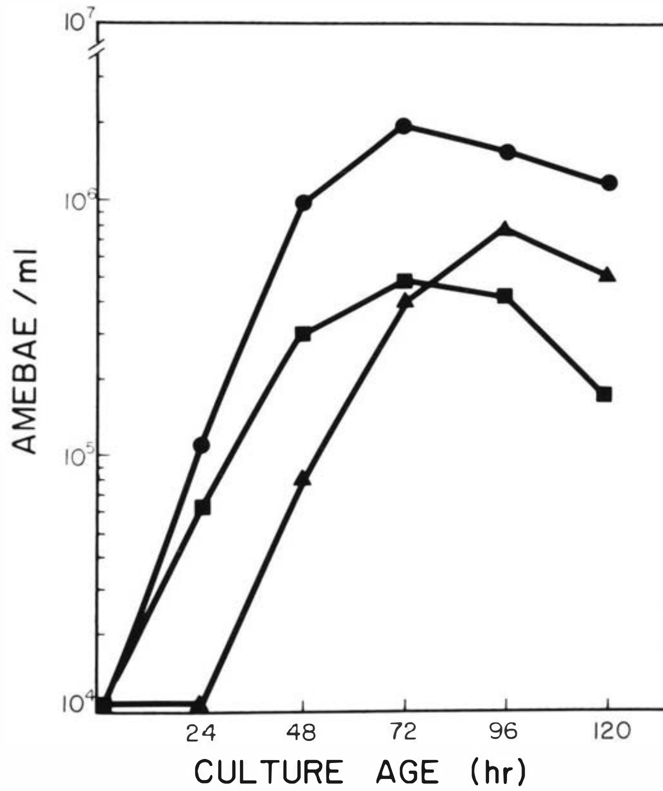

BRAIN
LUNG
LIVER
KIDNEY
SPLEEN[a]
TISSUES
0
4
8
12
16
20
DAYS AFTER I.V. INOCULATION

Figure 2. Growth of *N. fowleri* in Nelson medium with minced mouse spleen.

(●) Control (no spleen); (■) addition of one-half a spleen; (▲) addition of a whole spleen.



10^7
10^6
10^5
10^4
AMEBAE /ml
24
48
72
96
120
CULTURE AGE (hr)

Figure 3. Cell viability and concentration of *N. fowleri* incubated at 37 C with spleen homogenate. Cell viability (●) without homogenate, (■) with homogenate; cell concentration (○) without homogenate, (□) with homogenate.

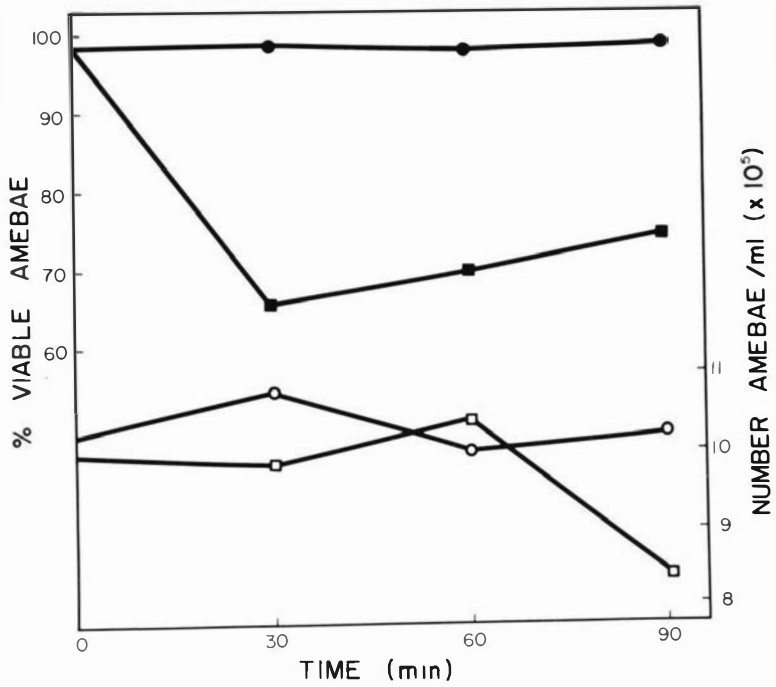

100
90
80
70
60
% VIABLE AMEBAE
11
10
9
8
NUMBER AMEBAE /ml (x 10^5)
0
30
60
90
TIME (min)

Figure 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.

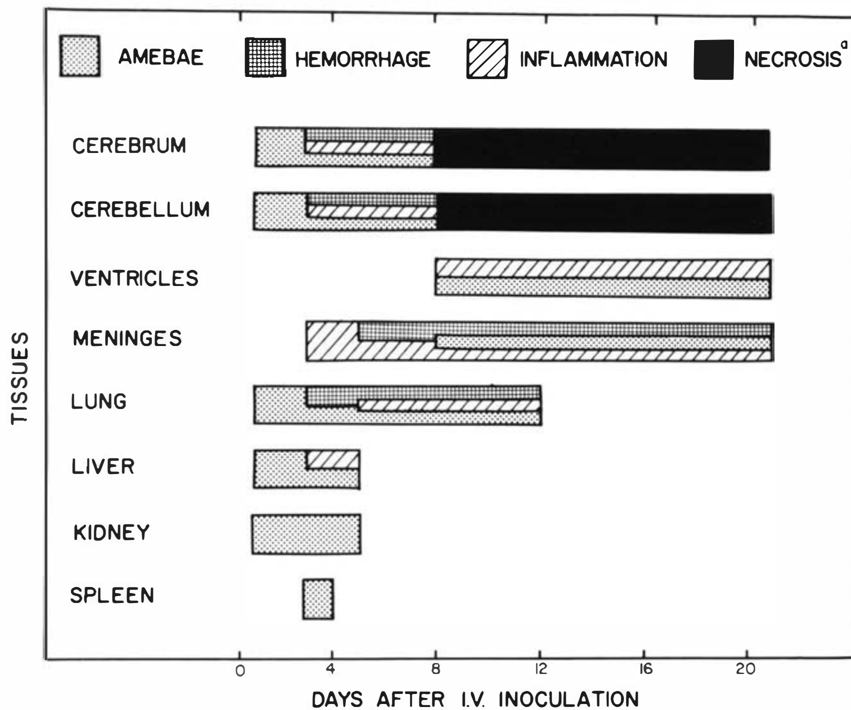

AMEBAE
HEMORRHAGE
INFLAMMATION
NECROSIS[a]
CEREBRUM
CEREBELLUM
VENTRICLES
MENINGES
LUNG
LIVER
KIDNEY
SPLEEN
TISSUES
0
4
8
12
16
20
DAYS AFTER I.V. INOCULATION

Figure 5. Average total body weights for (○) mice infected with 2.4×10^6 *N. fowleri* and for (●) noninfected mice.

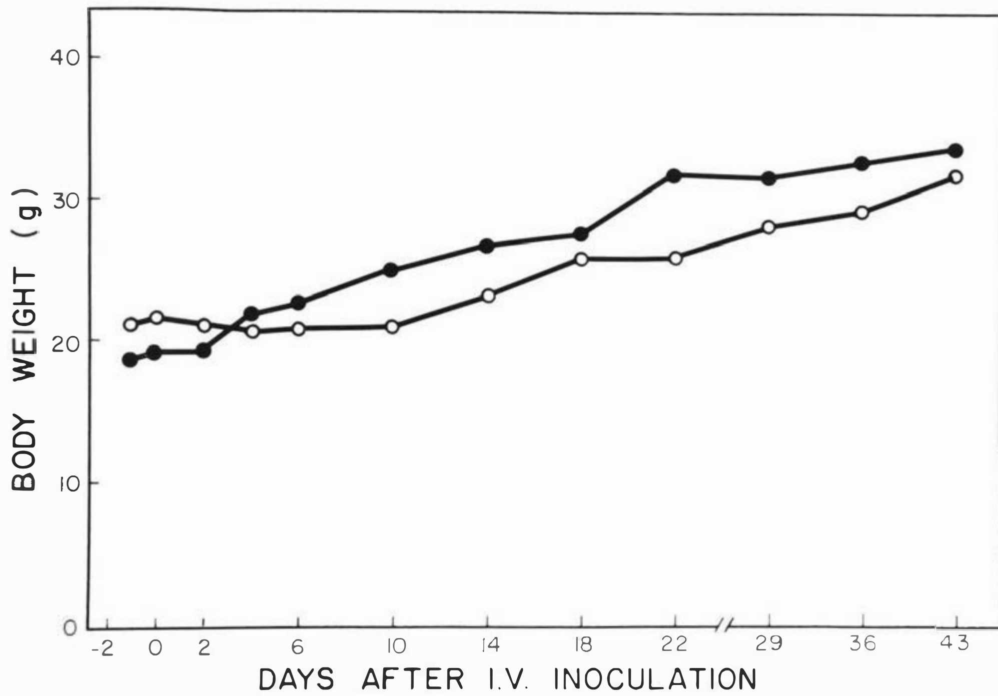
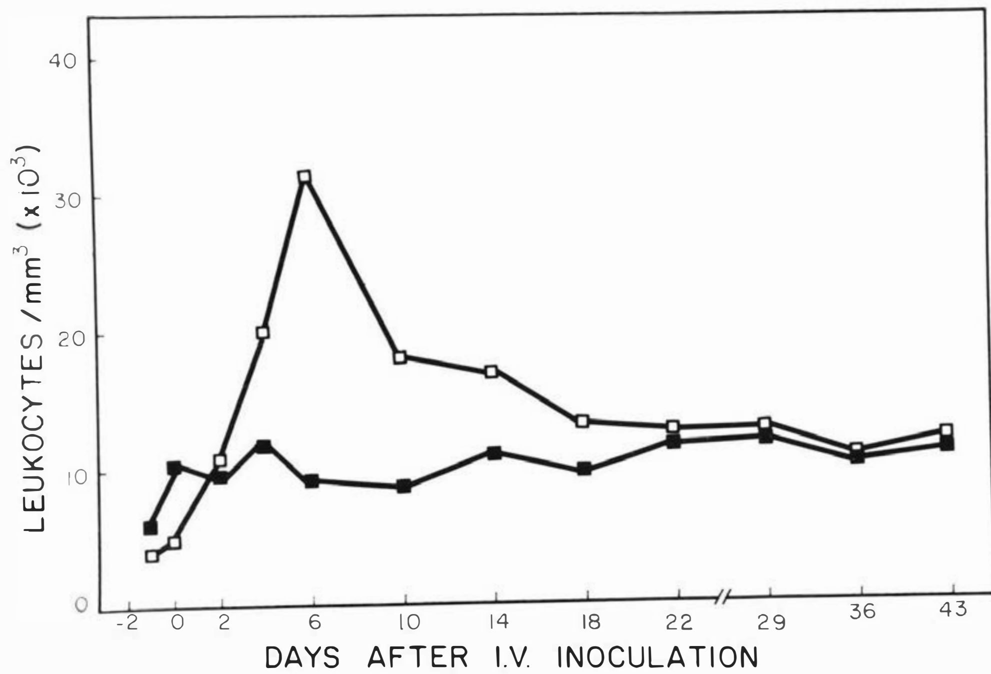

40
30
20
10
0
BODY WEIGHT (g)
-2
0
2
6
10
14
18
22
29
36
43
DAYS AFTER I.V. INOCULATION

Figure 6. Average total leukocyte counts for (□) mice infected with 2.4×10^6 *N. fowleri* and for (■) noninfected mice.



LEUKOCYTES / mm^3 ($x 10^3$)
40
30
20
10
0
-2
0
2
6
10
14
18
22
29
36
43
DAYS AFTER I.V. INOCULATION

Figure 7. Average differential leukocyte counts for mice infected with 2.4×10^6 *N. fowleri* and for noninfected mice. Lymphocytes: (○) infected mice, (●) noninfected mice; neutrophils: (△) infected mice, (▲) noninfected mice; monocytes: (□) infected mice, (■) noninfected mice.

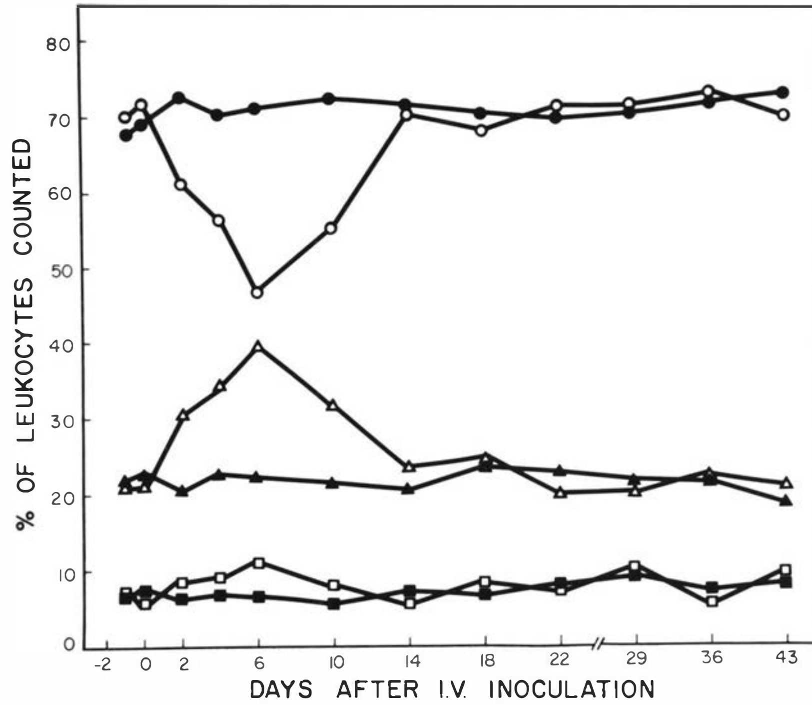

80
70
60
50
40
30
20
10
0
% OF LEUKOCYTES COUNTED
-2
0
2
6
10
14
18
22
29
36
43
DAYS AFTER I.V. INOCULATION

PLATE I

Legend

Figure 1. Portion of normal mouse liver showing a portal tract and anastomosing parenchymal cells. H&E 150X.

Figure 2. Infected 3 days. Three adjacent liver sinusoids each containing an ameba. H&E. 760X.

Figure 3. Infected 5 days. A foreign-body giant cell adjacent to a site of focal inflammation in the liver parenchyma. H&E. 760X.

Figure 4. Infected 3 days. A polymorphonuclear leukocytic inflammatory infiltrate around a hepatic portal tract. H&E. 250X.

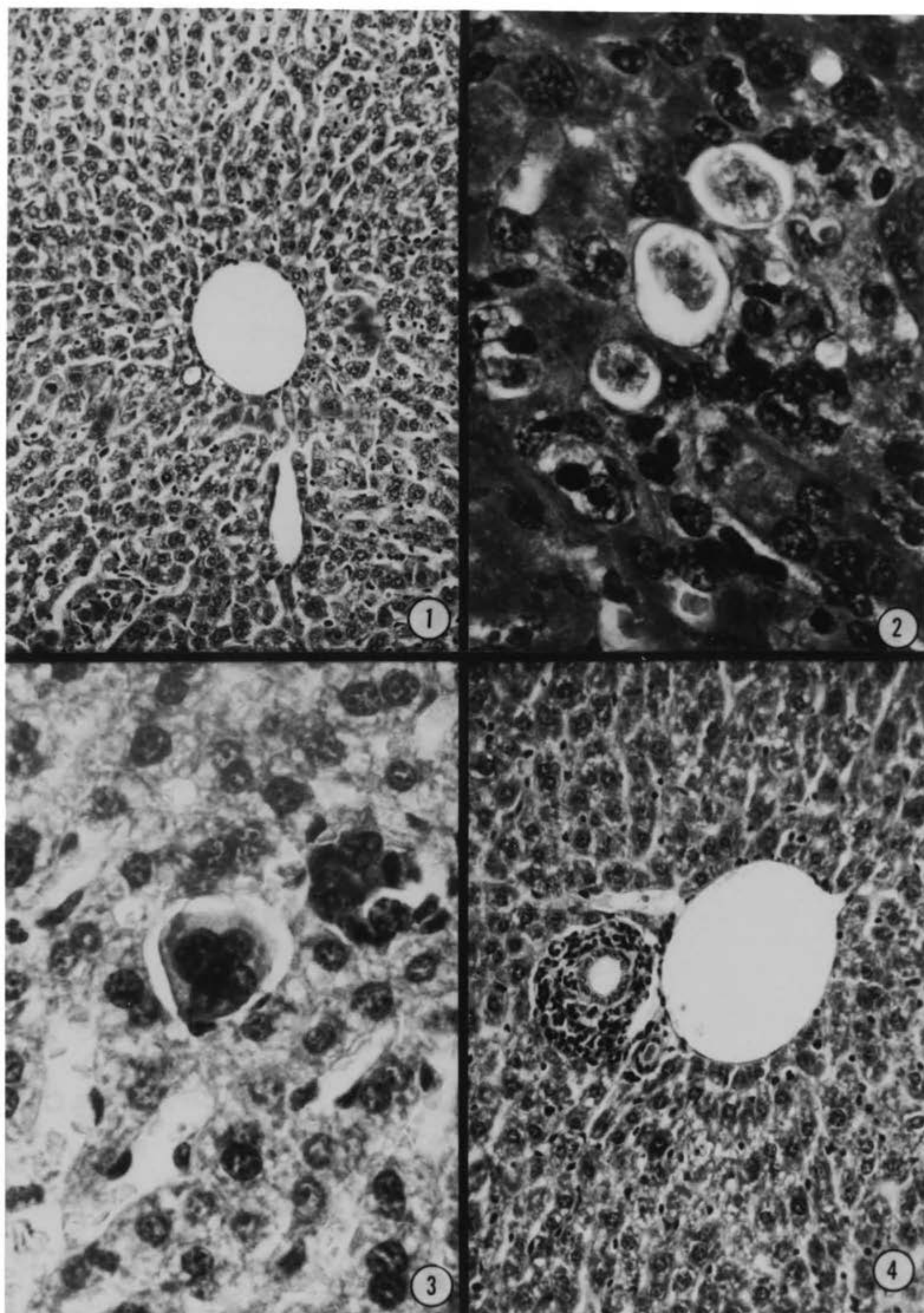

1
2
3
4

PLATE II

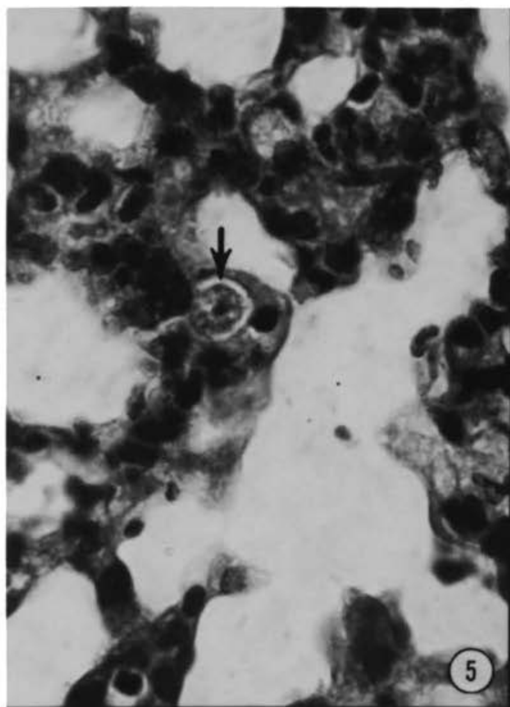
Legend

Figure 5. Infected 3 days. Ameba (arrow) in pulmonary alveolar capillary. H&E. 760X.

Figure 6. Infected 8 days. Extensive hemorrhage into interstitial spaces and alveoli of the lungs. H&E.100X.

Figure 7. Infected 3 days. Two amebae (arrows) in capillaries of a renal glomerlus that otherwise is normal. H&E.760X.

Figure 8. Infected 3 days. An ameba (arrow) in a capillary of the spleen. H&E.760X.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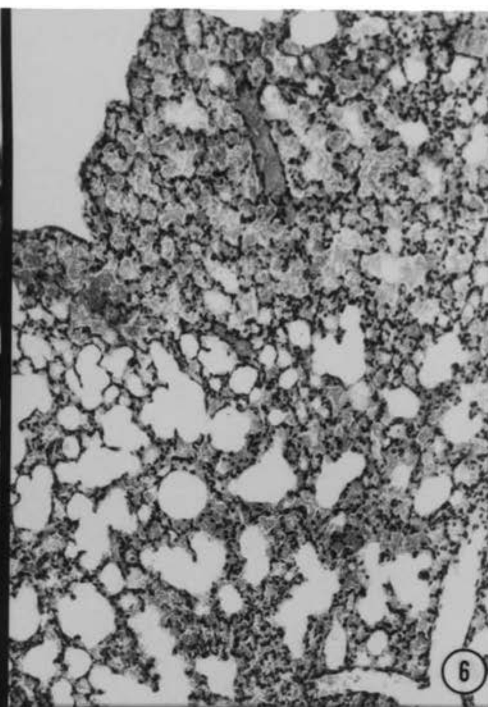
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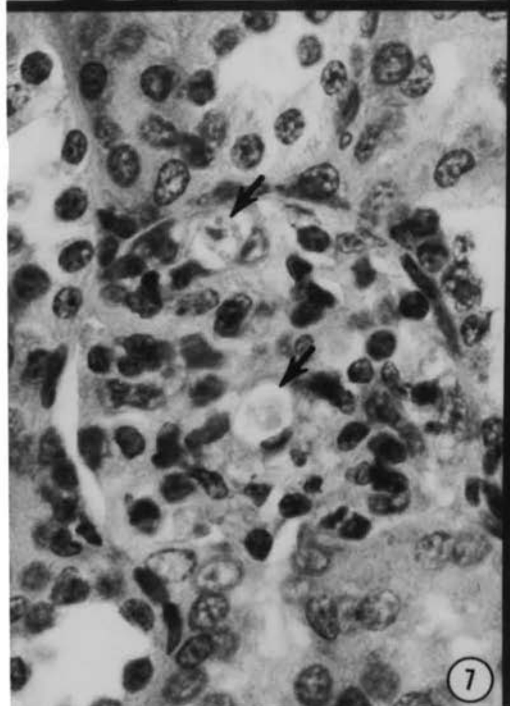
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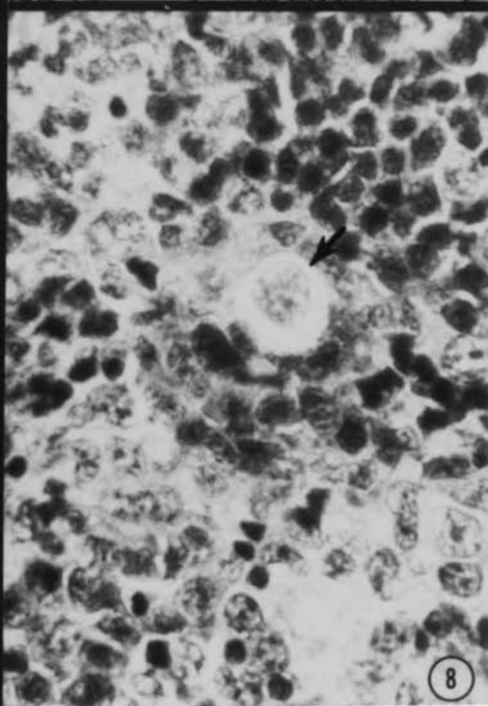
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PLATE III

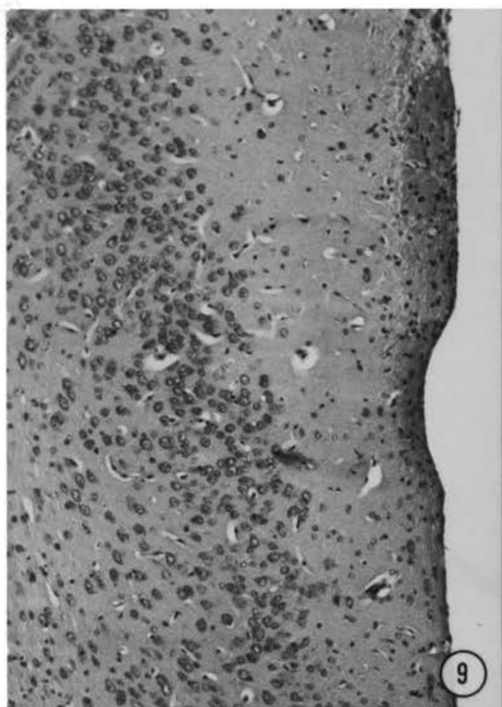
Legend

Figure 9. Noninfected. Normal cerebral cortex showing dark staining nerve cell bodies (perikarya) and intact meninges. H & E X100.

Figure 10. Noninfected. Normal cerebellum. The lighter staining outer molecular layer with scattered neurons is separated from the granular layer, composed of cells and fibers, by a single row of large Perkinje cells. An intact meninges covers the outer molecular layer. H & E X100.

Figure 11. Infected 5 days. *Naegleria fowleri* (arrow) in the cerebral cortex.Hemorrhagic necrosis is present. H & E X760.

Figure 12. Infected 5 days. Developing inflammatory infiltrate in the anterior cerebral cortex. Infiltrate consists primarily of lymphocytes. H & E X100.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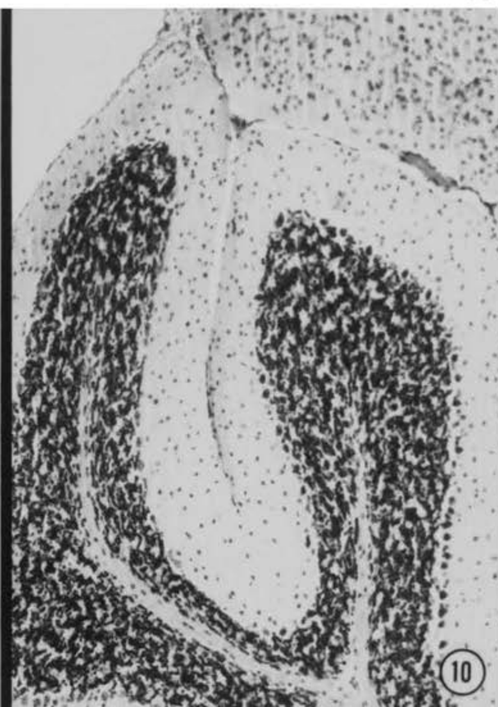
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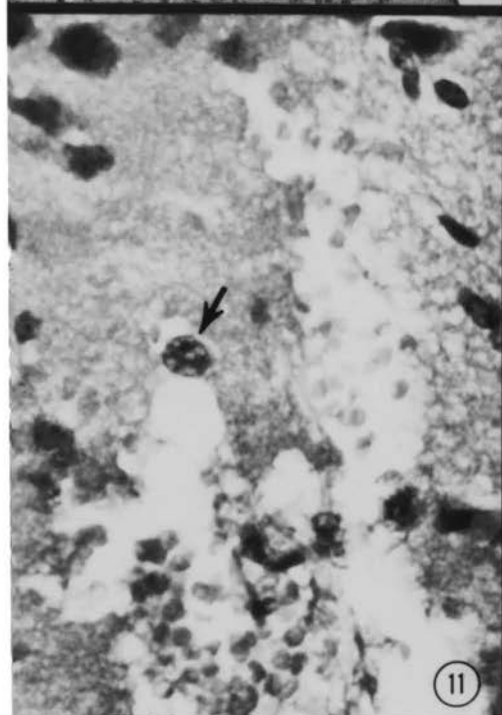
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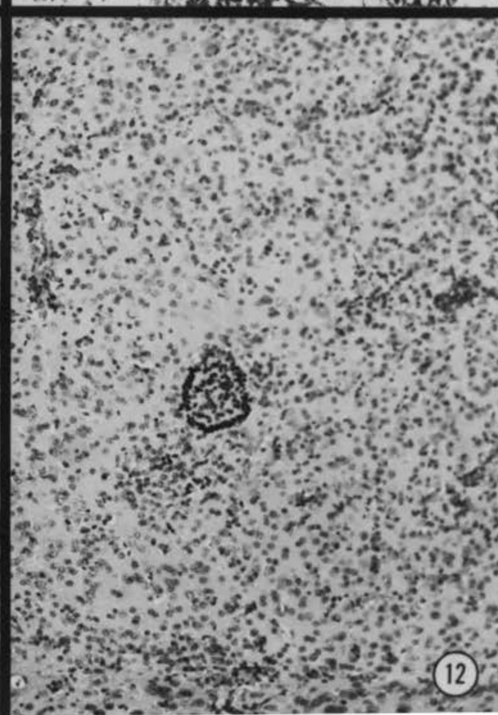
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PLATE IV

Legend

Figure 13. Infected 8 days. Meninges and cerebral cortex with acute inflammatory infiltrate comprised chiefly of mononuclear cells. H & E X100.

Figure 14. Infected 8 days. Cerebral and cerebellar meninges and cerebellar granular layer with acute inflammation, hemorrhage and moderate amebic invasion. H & E X100.

Figure 15. Infected 8 days. Dilated lateral ventricle. Demyelinated neural tissue dorsal to ventricle contains leukocytes and amebae. H & E X100.

Figure 16. Infected 8 days. Fourth ventricle with amebae (arrows) in the cerebrospinal fluid near the choroid plexus. Inflammatory cells and erythrocytes present in the dilated ventricle. Perivascular inflammation of blood vessels in cerebrum. H & E X400.

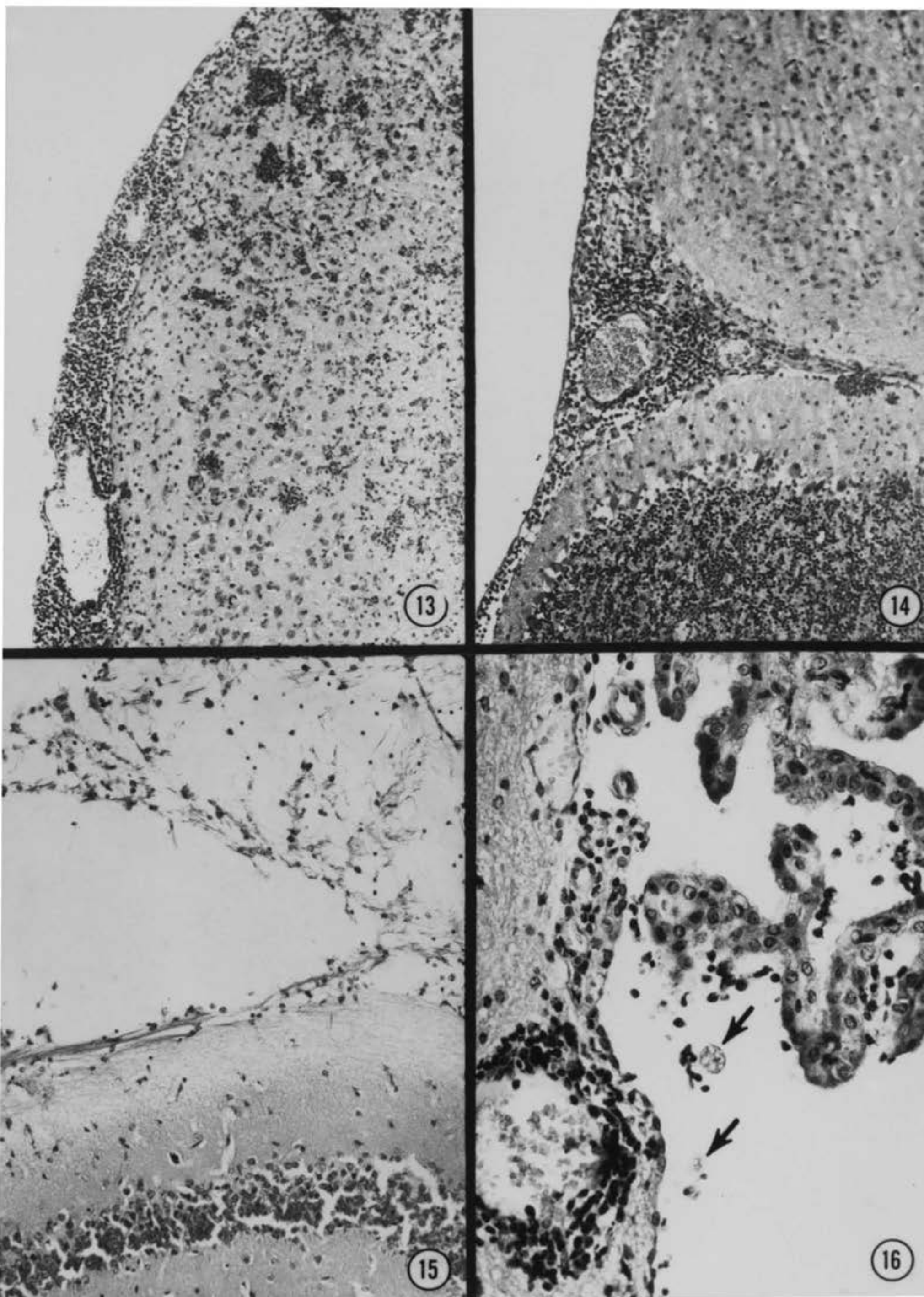

13
14
15
16

PLATE V

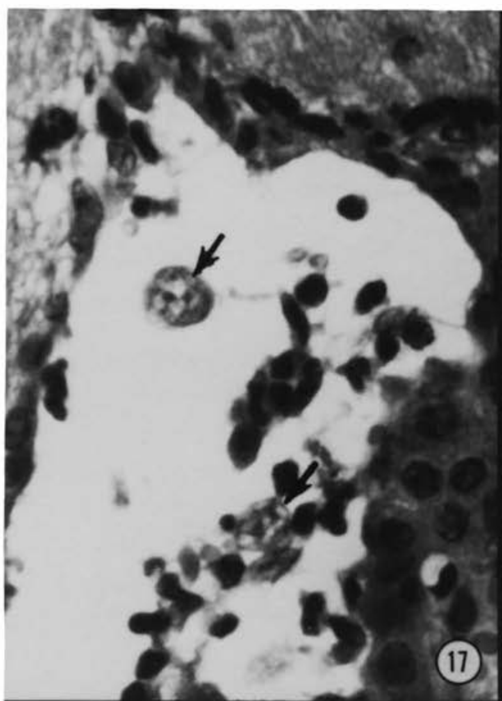
Legend

Figure 17. Infected 8 days. Amebae (arrows) in dilated lateral ventricle near choroid plexus. Inflammatory cells and erythrocytes present in cerebrospinal fluid. H&E X760.

Figure 18. Infected 8 days. Dilated fourth ventricle showing amebae (arrows); erythrocytes and inflammatory cells also present in cerebrospinal fluid. H&E X100.

Figure 19. Infected 8 days. Cerebellum with inflammation and hemorrhagic necrosis accompanying massive amebic invasion. Perivascular infiltration around cerebellar blood vessel. H&E X100.

Figure 20. Infected 8 days. Cerebellar cortex containing numerous amebae associated with inflammation and hemorrhagic necrosis. Amebae contain phagocytosed erythrocytes and cellular debris. H&E X760.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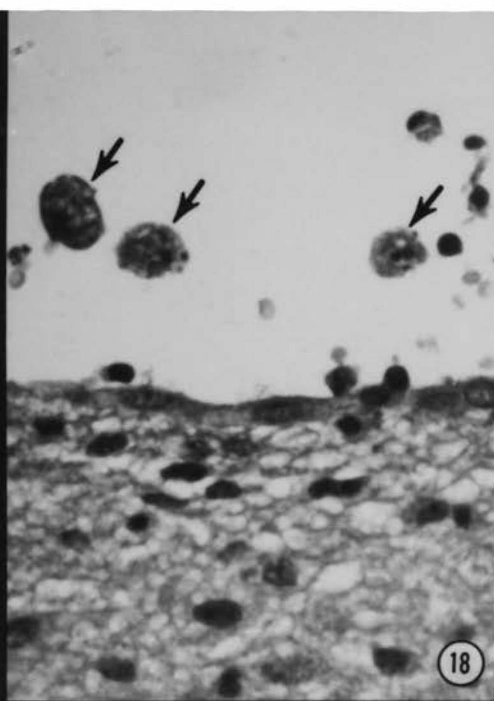
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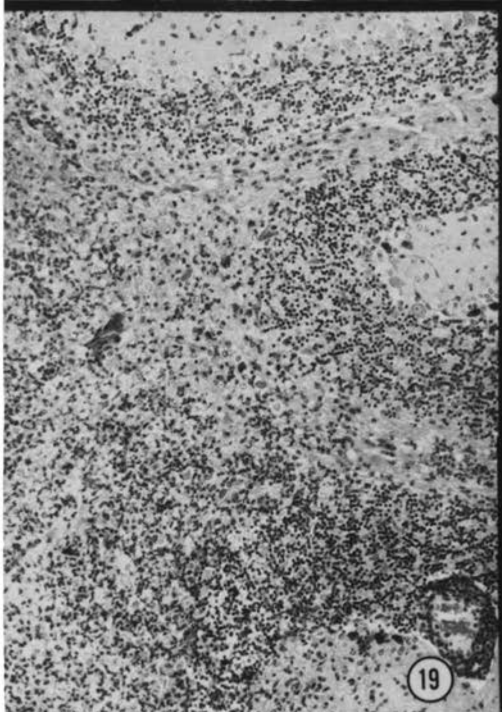
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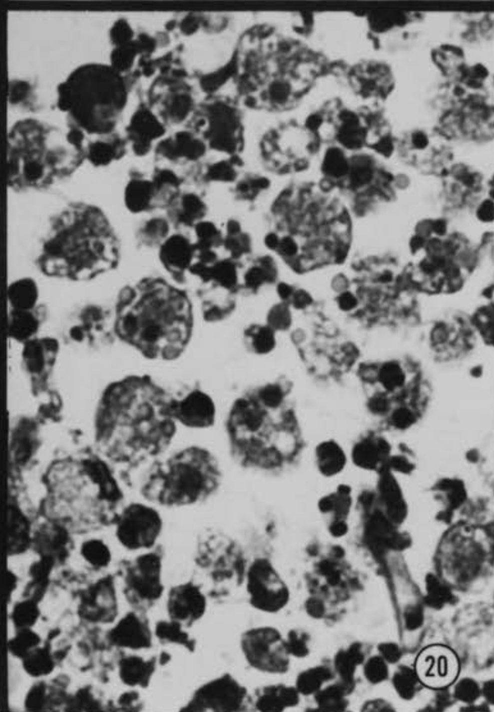
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PLATE VI

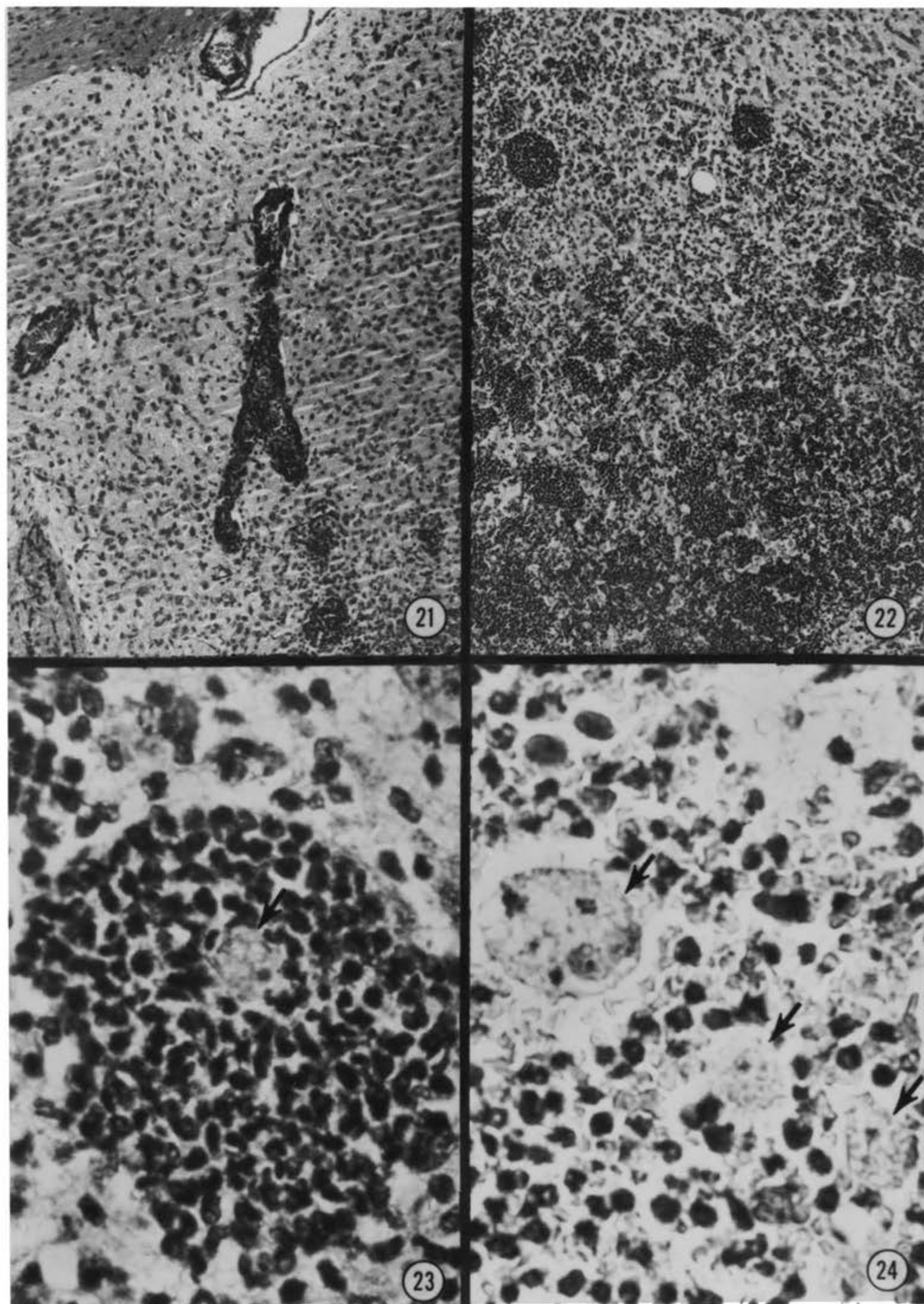
Legend

Figure 21. Infected 21 days. Cerebral cortex with focal inflammatory reaction and perivascular inflammation. Lateral ventricle is dilated. H&E X100.

Figure 22. Infected 21 days. Cerebral cortex with acute inflammation, hemorrhagic necrosis and diffuse amebic invasion. H&E X100.

Figure 23. Infected 21 days. A predominantly lymphocytic cellular response surrounding an ameba (arrow) within the cerebral cortex. H&E X760.

Figure 24. Infected 21 days. Inflammation and hemorrhagic necrosis of cerebellar cortical molecular layer. Three amebae (arrows) are present in the exudate. H&E X760.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DISCUSSION

The I.V. mouse model used in these experiments correlates both clinically and pathologically with what has been observed for I.N. infected experimental animals (Carter, 1970; Martinez et al., 1973a; Phillips, 1974; Singh and Das, 1972; Wong et al., 1975) and human primary amebic meningoencephalitis (Duma, 1978). Following I.V. inoculation of amebae in the lateral tail vein, a fatal meningoencephalitis involving cerebral cortex, cerebellum and brainstem occurred. Pathologic involvement of the liver and lungs was noted during early stages of the disease, but did not appear to contribute to the cause of death. The incubation period was approximately 4 to 5 days and the clinical course about 3 days. Occasionally mice survived longer.

Whether experimental animals are inoculated I.N., I.V., I.P. or intracerebrally with _N. fowleri_, the amebae concentrate in the brain producing various fatal pathological complications. The details that contribute to the invasivness of _N. fowleri_ are poorly understood. Investigators have speculated that either phagocytosis, secreted cytolytic enzymes or a combination of both are responsible for invasivness and virulence. Many digestive enzymes such as esterases, proteases, lipases and phospholipases (Müller, 1969) have been isolated from pathogenic free-living amebae. Chang (1976) detected phospholipases from pathogenic _Naegleria_ which produced a cytopathic effect on monkey kidney cells _in vitro_. Destruction of cells and tissue by lytic enzymes or toxins has not been confined only to _Naegleria_. Eaton et al. (1970) using _Entameba histolytica_ and Visvesvara and Balamuth (1975) using pathogenic _Acanthamoeba_ denomstrated that these amebae contained greater

concentrations of cytolytic enzymes or toxins than their nonpathogenic counterparts. The cytolytic substance responsible for the initial steps of invasion and colonization of host tissue appears to be phospholipase A. Phospholipase A, isolated from snake venom, has been shown to hydrolyze myelin (Baniks et al., 1976). Lipids, which include myelin, are found in greatest concentrations in the central nervous system. The dry weight of brain consists of approximately 50% lipids; the myelin sheath, which covers the peripheral nerves and the white matter of the brain, is composed of about 70% lipids. Some of the postulated functions of membrane lipids include sites for cell-to-cell recognition, specific cell surface antigens and, of most relevance to amebic invasion, specific receptors for toxins and other related compounds (Norton, 1976; Suzuki, 1976). With the knowledge that lipids enhance the axenic _in vitro_ growth of _N. fowleri_ (Haight, 1977) and that demyelination and encephalitis are pathological characteristics of primary amebic meningoencephalitis (Martinez et al., 1977), one can speculate as to why pathogenic _N. fowleri_ localizes and causes a fatal condition in the central nervous system following I.V. inoculation. The high concentration of lipids in the brain may exert an attractive force, thus localizing amabae in the brain. It may be that brain lipids are nutritional substrates for amebae or that lipids act as receptor sites for toxins or enzymes the amebae release.

Amebae gain access to the brain by way of hematogenous spread following I.V. inoculation. Venous flow carries amebae to the heart which passes them on to the lungs. Upon return from the lungs, the heart disseminates amebae to the rest of the body. Since 13-15% of the total resting cardiac output is being directed to the brain (Guyton, 1971;

Milnor, 1974) a large portion of the inoculum is not circulated through the liver, the major detoxifying and clearing organ of the body.

Infection was generally confined to the central nervous system following I.V. inoculation. Hematogenous dissemination of amebae involved all regions of the brain including cerebral cortex, cerebellum and brainstem; whereas after I.N. inoculation, whether naturally or experimentally acquired, involvement is primarily of the olfactory and frontal lobes. Carter (1968) described I.N. amebic invasion as centripetal, often beginning in the meningeal areas along the Virchow Robin spaces and invasion of the brain from outside inwards. Carter also contended that following I.N. inoculation, hematogenous spread of amebae to the more distant areas of the brain and body organs probably did not occur, but nonetheless should not be excluded. Dissemination of *Naegleria* has been documented in only one case of naturally acquired human primary amebic meningoencephalitis. Duma et al. (1969) reported that amebae were cultured from liver, spleen, kidney and heart blood. Carter (1968) and Duma et al. (1971) described complications which they considered unrelated to amebic invasion. These were pneumonia, interstitial pneumonitis, pulmonary edema, vascular congestion of the liver, splenitis and cloudy swelling of the renal tubular epithelial cells.

Intravenous inoculation of mice with *Naegleria* produced extensive hemorrhagic necrosis primarily of grey matter of the cerebral cortex, cerebellum and brain stem with an accompanying diffuse sanguinopurulent meningitis. As amebae invaded neural tissue, accumulations of inflammatory cells began to appear around amebae. The inflammatory infiltrate was composed primarily of mononuclear cells with many lymphocytes. Once the neural tissue surrounding the focal accumulation of inflammatory

cells became necrotic, accumulations of mononuclear cells gave way to a generalized inflammatory response consisting of polymorphonuclear leukocytes, macrophages, plasma cells and mononuclear leukocytes. Histopathological studies of human tissues invaded by *N. fowleri* (Duma, 1978) and studies with experimentally infected mice (Červa, 1971; Culbertson, 1971; Martinez et al., 1973) have noted that at the time of autopsy, the majority of cells comprising the inflammatory response were polymorphonuclear leukocytes and engulfment of amebae by these cells was a frequently observed event. A possible explanation why mononuclear cells were not observed in large numbers, as in this study, is that the histopathologic studies of humans and experimental animals were performed post mortem when polymorphonuclear leukocytes were the prominent cell type of the necrotic tissue. In this study, the lymphocytic cellular response was observed prior to the generalized hemorrhagic necrosis.

Meningitis was extensive and diffuse involving most surfaces of the cerebral cortex, cerebellum and brain stem. Mononuclear cells, primarily lymphocytes, were the predominant inflammatory infiltrate; also noted were areas of meningeal hemorrhage. Amebae were present in the meninges during the latter stages of the disease usually in conjunction with adjacent areas of hemorrhagic necrosis in neural tissue. Studies of human tissue invaded by *Naegleria* (Duma, 1978) and I.N. inoculated experimental animals (Carter, 1970; Martinez et al., 1973; Phillips, 1974; Singh and Das, 1972; Wong et al., 1975) have demonstrated meningitis, but only in the area about the base of the brain; amebae were readily observed in the subarachnoid spaces.

External and internal hydrocephalus, as evidenced by dilation of the meninges and fourth and lateral ventricles, occurred during the

latter stages of infection. The internal pressure of the cerebrospinal fluid often was so great that hydrocephalus was grossly evident by the domed cranium of some mice. Carter (1970) and Martinez (1973) also noted this with I.N. inoculated mice. Mice with extreme dilation of the ventricles at times showed degeneration of the ependyma and surrounding tissue. On several occasions, amebae and a sanguinopurulent exudate were evident near the choroid plexuses. A possible explanation for the increased cerebrospinal fluid and resulting dilation of ventricles is that the amebae, metabolic wastes from the amebae or devaculozation of leukocytes act as irritants to the choroid plexus and thus stimulate increased production of cerebrospinal fluid. Alicata and Jindrak (1970) suggested another possible mechanism for the increased secretion of cerebrospinal fluid observed in mice infected with _Angiostrongylus_ _cantonensis_. They proposed that the inflammation of the meninges narrowed the lumen of the cerebral veins, increasing intravenous pressure and thus increasing the production of cerebrospinal fluid. A third possible cause for hydrocephalus is proposed by Guyton (1971) who states that the increase in the amount of cerebrospinal fluid may result from the increased number of inflammatory cells and erythrocytes which block absorption of fluid through the arachnoid villi.

A combined lymphocytic and polymorphonuclear perivascular cuffing of blood vessels occurred throughout the course of naeglerial infection. Cuffing developed throughout all areas of the brain and was not necessarily associated with amebae in the perivascular spaces or amebic invasion of adjacent tissues. For human infections (Duma et al., 1971) and for I.N. infected mice (Martinez et al., 1973) investigators have

described amebae clustered in the perivascular spaces. Occasionally amebae were observed within the vascular lumen.

Generally, infected mice lost a certain amount of body weight during the terminal stages of a naeglerial infection. Culbertson et al. (1972) and Diffley et al., (1976) also observed a gradual loss of weight in guinea pigs following subcutaneous and I.M. inoculation of N. aerobia (N. fowleri). The weight loss can be attributed to decreased water intake (Carter, 1970) and, to a lesser extent, feed consumption.

In addition to their inability to drink and eat, mice exhibited the onset of primary amebic meningoencephalitis clinically by the appearence of bristled fur, a disinclination to move, a puffy appearance of the face with eyes kept closed and arched or hunched backs. Occasionally, they exhibited spontaneous circling movements in one direction and paralysis of the hindquarters. Death often occurred soon afterwards. These findings are similar to those described for experimental animals (Carter, 1970; Červa, 1971) using I.N. and intracerebral routes of inoculation. Other experimentors (Carter, 1970; Culbertson, 1972) using routes of inoculation less direct to the nervous system have reported no significant clinical symptoms.

Polymorphonuclear neutrophils are bone marrow derived white blood cells which play a central role in defense of the host against infection. For many infections, neutrophils act as phagocytic or killer cells through interaction with antibody, complement and chemotactic factors. In the presence of an acute infection, such as N. fowleri produces, an increase in production and release of neutrophils from bone marrow into the circulation would be expected to accompany the meningeal, cerebral and cerebellar infiltrates. This was observed. A

neutrophilia was present between days 2 and 14, reaching a maximum value of 40% by day 6. A leukocytosis which accompanied the neutrophilia reached a maximum value of 31,500 leukocytes/mm^3 blood on day 6. For noninfected mice the values on day 6 were 10,000 leukocytes/mm^3 blood with 22% neutrophils. Similar results have been described for naturally acquired human primary amebic meningoencephalitis (Butt, 1966; Callicott et al., 1968; Carter, 1968; Duma et al., 1971) and infections of monkeys (Wong et al., 1975). In all, a marked neutrophilia of approximately 2-fold with a simultaneous 3 to 4-fold rise in total leukocytes/mm^3 blood occurred.

Mononuclear cell infiltration of the meninges and brain tissue occurred in response to amebic invasion. An increase in the percentage of circulating lymphocytes and monocytes, therefore, might be expected. This however, was not observed. The percentage of lymphocytes actually decreased from a normal value of 70% at day 2 to a low of 47% on day 6. By calculating the actual numbers of cell types (total leukocytes X % of cells), we see a 6-fold increase in the number of neutrophils. The increase in neutrophils resulted in an apparent decrease in the percentage of lymphocytes whose numbers actually increased about 2-fold. There was no significant increase or decrease in the percentage of monocytes, however, the actual number of cells increased about 3-fold. Butt (1968), Callicott et al. (1968), Carter (1968), and Duma et al. (1971) all noted a similar condition in humans. Although lymphocytes were not noted in the neural tissue, the percentage of circulating lymphocytes decreased from a normal value of 27% to 7%. No changes in monocytes was noted.

Generally, the major pathological involvement in primary amebic

meningoencephalitis is limited to the central nervous system. However, complications in other tissues may occur as a result of possible toxins released by live or dying amebae. During the early stages of infection in the present study, the pulmonary capillaries were often occluded by amebae and the alveoli were congested by hemorrhage and edematous fluid. Neutrophils were the predominant infiltrating cells involved in the mild inflammatory response. Myocarditis, as described in two human cases of primary amebic meningoencephalitis (Carter, 1968; Markowitz et al., 1974), may be an important complication resulting in pulmonary edema. The lungs are particularly susceptible to edema because they are composed of large alveolar spaces lined by thin flattened cells which exert no tissue resistance against collections of fluid. Involvement of the heart can lead to pulmonary congestion and edema by causing increased pulmonary venous pressure, sodium retention and increased capillary permeability (Robbins, 1974). Amebae that are frequently seen congesting pulmonary capillaries may contribute to increased pulmonary pressure. A final possibility for pulmonary edema is, as Robbins (1974) describes it, "for obscure reasons, sudden increases in cranial pressure sometimes induces pulmonary edema." The internal and external hydrocephalus as previously described would be responsible for such an increase in intracranial pressure. Pulmonary hemorrhage, consolidation and edema have also been noted in experimental animals infected I.N. and I.V. (Chang, 1971; Culbertson et al., 1968; Culbertson et al., 1971; Diffley et al., 1976; Singh and Das, 1970).

Hepatic involvement during I.V. naeglerial infections was limited to inflammation along the portal tracts and in the parenchyma. Amebae were observed in the hepatic sinusoids without apparent inflammation.

Carter (1970) and Culbertson (1972) using mice, described focal inflammation and infiltration of foreign-body giant cells in the liver following amebic invasion and colonization. Mononuclear cell perivascular cuffing of the portal tracts was noted by the first day following infection. This was probably an initial host response to amebae colonizing around portal tracts (Culbertson, 1972).

In the present study, amebae were observed in the renal glomerular capillaries of mice without apparent pathological involvement or leukocytic infiltration. The amebae were present in the renal capillaries only during the early days of the disease. Similar findings were reported by Carter (1970) and Culbertson (1972) who observed amebae in the capillaries of kidney of mice with no apparent involvement following I.N. and subcutaneous inoculation. However, Carter (1968) observed amebae in the renal capillaries during the terminal phase of the disease and explained their presence as likely arising by hematogenous spread from an overwhelming central nervous system infection.

Amebae did not gain access to the environment through the urinary system. Due to the histological arrangement of the renal glomerulus, amebae are not able to pass through the pores of Bowman's capsule. In the kidney, an ultrafiltrate of blood plasma is formed allowing only small molecular weight substances, such as phosphates, creatinine, uric acid, urea and small amounts of albumin to filter through (Bloom and Fawcett, 1975). Larger molecular weight substances such as leukocytes, erythrocytes, blood platelets and, in the case of primary amebic meningoencephalitis, amebae are not able to pass through. If amebae did pass through the renal glomerulus and were carried into the renal tubules and eventually to the urinary bladder, they could not survive.

In the present study, when amebae are incubated *in vitro* in fresh mouse urine, they immediately round up and within 90 minutes the viability of the amebae decreased to 14% and the total cell number were reduced by one-half. Death and lysis of the amebae were most likely due to the concentrated levels of toxic waste products in the mouse urine.

Even though amebae were not recovered from cultures of spleen samples, they were observed in capillaries of H&E-stained spleen sections. No pathological involvement of the spleen tissue was observed. Carter (1970) and Diffley et al. (1976) observed in mice and guinea pigs, respectively, enlargement of the spleen following inoculation with *N. fowleri*. The enlargement of the spleen in acute inflammation, such as in primary amebic meningoencaphalitis, is usually not of significant proportions (Blaustein, 1963). The size is essentially dependent upon the degree of active hyperemia and edema and on increases in cellular elements. The cellular elements include leukocytes, erythrocytes and a proliferation of reticuloenthelial cells. The hyperplasia of reticulo-enthelial cells serve as the source of macrophages to phagocytize injured cells, microorganisms and tissue debris. In acute splenitis, one or more of the above mentioned cellular elements may be dominant, depending on the type of injuring agent.

Experiments showed that in the presence of minced spleen, growth of amebae was inhibited for about 24 hours, after which normal growth re-sumed. Homogenized spleen had the greatest effect upon amebic growth. Cell viability and number were dramatically reduced in the presence of homogenized spleen. One can speculate on why amebae did not survive in a medium designed for optimal growth following addition of minced spleen. Amebic growth was probably inhibited by released leukocytic granules

which contain digestive enzymes such as hydrolytic enzymes, myeloperoxidases, lysozymes. Also, these substances could possibly alter the pH of the medium and thus make it unsuitable for optimal growth. However, growth of amebae was examined in medium containing spleen homogenate but with pH adjusted to that for optimal growth initiation and still the amebae did not grow as well as amebae in medium without spleen homogenate (data not shown). Therefore, it appears that other components of spleen, perhaps enzymes, inhibited growth of the amebae *in vitro*.

The transfer of amebae from infected mice, whether inoculated I.N. or I.V., to other uninoculated mice appears to occur as evidenced by an increase in protection against a lethal challenge of *N. fowleri* and by the isolation of *N. fowleri* from the lungs of one uninoculated but exposed mouse. Uninoculated mice which were exposed to I.V. inoculated mice demonstrated 25% protection to a lethal challenge of *N. fowleri*. They also demonstrated a significant increase in mean time to death when compared to controls. A possible explanation for the observed protection in noninfected but exposed mice is that these animals may have acquired a subclinical infection from the infected mice, with which they were housed, via respiratory droplet exposure. A subclinical infection could afford some protection against a subsequent lethal challenge with *N. fowleri*. Amebae were frequently cultured from the lung tissue of infected mice and, as stated earlier, were abundant in areas of pulmonary hemorrhage and consolidation.

Exposure of uninoculated mice to I.N. infected mice resulted in 20% protection for the exposed mice and also an increase in mean time to death following a lethal challenge dose of *N. fowleri*. Possible

explanation for the increased protection and increased mean time to death seen in the noninfected mice following a lethal challenge would be the same as for mice inoculated I.V. Also, uninoculated mice may be exposed to N. fowleri immediately following I.N. installation of amebae in the infected animals.

Exposure of mice to N. fowleri from infected animals by means other than respiratory droplets probably does not occur. Experimentally, we have shown that amebae are highly susceptible to urine and so could not exit from the body via the urinary tract. Culbertson (1972) noted amebae surrounding portal tracts in liver sections. This could suggest a possible exit for amebae through the gall bladder, into the intestines and out with the feces. This, however, is not probable because amebae are sensitive to bile (Carter, 1970). Culbertson et al. (1972) also found intestinal lesions possibly containing amebae following subcutaneous inoculation of guinea pigs. The intestinal tract could be another possible avenue of exit and transmission except that Carter (1970) has shown that when amebae are mixed with feces they lyse. Also, N. fowleri amebae could not survive passage through the stomach with its low pH.

In order to recover at least one ameba in a 20ul sample of peripheral blood there must be at least 85 amebae in the circulation of a 20g mouse (1.7ml total blood volume; 1.5ml of blood plus 0.2ml inoculum). This would require an inoculum of 85 amebae/mouse. Therefore inocula of 10^3 (11.7 amebae/20ul), 10^6 ($1.2X10^4$ amebae/20ul) and 10^7 ($1.2X10^5$ amebae/20ul) amebae/mouse would initially supply sufficient amebae for the 20ul blood samples used.

Organisms which gain access to the circulation are generally

cleared from the blood by the fixed tissue macrophages of the mononuclear phagocyte system, especially the Kupffer cells of the liver. It is also possible that the agglutination of organisms by serum factors, perhaps nonspecific factors, serves to augment the clearance of organisms from the blood.

According to the data, the calculated LD_{50} dose of $2.4X10^6$ amebae/mouse, which was used and inoculated I.V., should have been cleared between the times required to clear inocula of 10^6 and 10^7 amebae. Thus, no amebae should have remained in the peripheral circulation after 160 minutes. Therefore, when tissues were cultured for amebae at 24 hours, only amebae established or surviving in tissues would be recovered, and not amebae still in circulation following the I.V. inoculation.

SUMMARY

These experiments show that mice infected I.V. with N. fowleri followed a similar course of infection, both clinically and pathologically, as that observed for I.N. infected experimental animals and for naturally acquired human infections. I.V. infected mice died from amebic invasion of the central nervous system and not from complications involving other organs.

Following I.V. inoculation, amebae remained in the peripheral circulation for less than 160 minutes. An incubation period of 4 to 5 days was followed by various overt clinical symptoms implicating an ongoing central nervous system infection. These included loss of weight, bristling fur, a disinclination to move, a puffy appearance of the face with eyes closed, arched or hunched back, spontaneous circling and paralysis of the hindquarters. Death shortly ensued.

Following hematogenous spread of N. fowleri, meningoencephalitis of the cerebral cortex, cerebellum and brainstem occurred. Within 3 days after inoculation, an intense meningeal mononuclear cell inflammation and hemorrhage was noted for all areas of the brain. Mononuclear cell perivascular cuffing began early and persisted throughout the remainder of the infection. Focal accumulations and a diffuse leukocytic infiltrate developed in all regions of the brain by day 5. Lymphocytes were the predominant cell type of the early inflammatory response. Later, neutrophils and lymphocytes, together with macrophages and plasma cells were present in areas of hemorrhagic necrosis. Hemorrhage occurred in areas of amebic invasion and cellular infiltration. The aggressiveness of amebae was noted by the presence of ingested red blood

cells and tissue debris.

A leukocytosis occurred between days 2 and 14 and reaching a maximum at day 6. Also, between days 2 and 14, lymphopenia and neutrophilia occurred reaching their respective minimum and maximum values by day 6. However, there was no reversal of the lymphocyte/neutrophil ratio. A slight increase in monocytes was noted by day 6. No variation of eosinophils or basophils occurred.

Lungs were edematous and hemorrhagic with amebae often seen occluding the pulmonary capillaries. A mild neutrophilic cellular response was noted. Hepatic involvement was limited to a mononuclear cell infiltration along the portal tracts, sites of focal inflammation in the parenchyma and amebae in sinusoids without apparent tissue involvement. Capillaries of the kidney and spleen contained amebae without apparent pathological involvement.

Homogenized spleen and minced spleen proved to be an inhibitor of amebic growth in both agitated and unagitated cultures. Amebae were not recovered from minced spleen in unagitated cultures even though they were observed in H&E-stained paraffin sections. Growth in agitated cultures in the presence of minced spleen showed an increased generation time during log phase growth and a reduced cell yield during stationary phase growth. Viability and cell number of amebae were also greatly reduced when homogenized spleen was added to unagitated cultures.

Fresh mouse urine was toxic to *N. fowleri*. Ameba viability decreased to 14% and total cell number was reduced by approximately one-half.

Exposure of uninoculated mice to other mice inoculated either I.V. or I.N. conferred a degree of resistance to the uninoculated but exposed

mice. Acquired resistance was determined by increased protection and mean time to death in the exposed but uninoculated mice as compared to nonexposed uninoculated control mice following a lethal challenge of *N. fowleri*.

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