PATHOGENESIS OF PATHOGENIC NAEGLERIA AMOEBA

S. L. CHANG

The Health Effects Research Laboratory, Environmental Research Center, U.S. Environmental Protection Agency, Cincinnati

Abstract. In brain sections of the Naegleria-caused cases of primary amoebic meningoencephalitis, extensive demyelination was found in the white matter, besides the severe histopathological changes and large clusters of trophozoites in the grey matter. The myelinolysis appeared to be a result of a specific phospholipolytic effect, unlike that in post-viral encephalomyelitis, which has been attributed to vascular blockade or hemorrhages. In monkey kidney cell cultures a very early cytopathic effect was observed and traced to the lytic property of the seeding culture fluid. Rat brain slices inoculated with Naegleria culture exhibited amoebic growth and demyelination in 28—52 hours incubation at 35°C. In a chemically defined medium containing sphingomyelin, casein and glucose, the Naegleria produced a limited growth paralleling the clearance of the lipid turbidity during a 72 hour incubation at 35°C. Chromatographic analysis of the turbidity-cleared cultures revealed decomposition of sphingomyelin with liberation of choline, sphingosine and fatty acids. It is, hence, concluded that the pathognecity of cytopathic effect of pathogenic Naegleria can be attributed to the latter's capacity to liberate a phospholipolytic enzyme or factor during active growth, which "makes holes" in the lipid-rich cytoplasmic membrane of cells as well as demyelinizes nerve tissue.

The primary amoebic meningoencephalitis (PAM) caused by pathogenic Naegleria is unique in that the cases, with a few exceptions, were associated with swimming (Caster 1972, Duma 1972, Chang 1974a) and the causative amoeba literally eats its way from the nasal, into the cranial cavity. This direct route of cranial invasion through the olfactory mucosal epithelium and cribiform plate along the olfactory nerve plexus has been indicated in case autopsy findings (Calicott et al. 1968, Duma et al. 1971, Carter 1972, Hermanne et al. 1974) and demonstrated in animal experimentation (Culbertson et al. 1968, Martinez et al. 1973).

The most remarkable pathologic changes in the central nervous system (CNS) reported of PAM cases have been the location of the severest lesions in the olfactory lobes and the aggregation of trophozoites in the necrotized cortex and perivascular spaces adjacent to the acutely inflamed cortical areas. Attention, however, was called in one report (Duma et al. 1971) to the manifestation of myelinolysis in the white matter of both the brain and spinal cord without cellular infiltration or vascular damage.

In primary monkey kidney (PMK) cell cultures all the strains of pathogenic Naegleria studied were found to exhibit an early CPE leading to a rapid cytolysis, and a cytotoxic or cytolytic factor was demonstrated in the culture fluids, (Chang 1971, 1974 b). Although the highly unstable nature of the factor made it difficult, if not impossible, to study its characteristics, the fact that the CPE was most apparent of fluids from cultures at the peak of amoebic growth and least apparent from old cultures clearly indicates that the factor was liberated during active growth of the trophozoites, (Chang 1971, 1974 b).

These pathologic and cytopathic findings led to the belief that the pathogenesis of pathogenic Naegleria is related to the amoeba's ability to liberate a phospholipolytic enzyme or enzymelike substance which damages the cytoplasmic membrane thus resulting in cytolysis. This activity is believed essential to facilitate tissue invasion or obtain cell debris for food as well as to save the amoeba from being annihilated by phagocytosis (Chang 1971). The study presented herein was intended to validate this belief.
MATERIALS AND METHODS

Brain sections from 14 PAM case autopsies were examined for demyelination; they were kindly supplied by the following persons: Dr. R. F. Carter, Adelaide Children's Hospital, Adelade, South Australia — sections from cases 61, 65, 66, 69 and 71, (Carter 1972); Dr. J. B. Jadin and Dr. E. Willaert, Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium — sections from cases VB (Jadin et al. 1971) and Haas (Hermanne et al. 1974); Dr. R. J. Duma, Virginia Medical College, Richmond — sections from cases CJ, LL, WM, and TY (Duma 1972); the late Dr. C. G. Butt, Orange County Memorial Hospital, Orlando, Florida — sections from case HB2 (Butt et al. 1968); Dr. E. Mezger, Lakeland General Hospital, Lakeland, Florida — sections from case GJ (Chang and Mezger, in preparation). All sections were stained with the hematoxylin-eosin stain or Luxol fast blue-periodic acid-Schiff stain.

The strains of pathogenic *Naegleria* used in the study were gratefully received from the same person who supplied the brain sections. The Florida and Virginia strains were given the same designations as the cases from whom they were isolated. The Australian A 1 and A 2 strains were originated from cases 66 and 69, respectively, and the Belgian 359 and 838 strains, from cases VB and Haas, respectively. They were all isolated from the cerebrospinal fluids (CSF) of the respective cases and grown in both the PMK cell cultures and axenically the calf serum — casein — glucose — yeast extract (CSCGYE medium), (Chang 1974a).

The primary monkey kidney cells were purchased from a commercial source and prepared as tube cultures. The growth medium contained medium 190 plus 0.5% albumin hydrolysat and 5% fetal calf serum and the maintenance medium, Earle's balanced salt solution plus 2% fetal calf serum. Fifty ng/ml each of penicillin and streptomycin were added to each medium prior to dispensing. All tube cultures were incubated at 35°C in a stationary phase.

The CSCGYE medium consisted of an autoclaved base fluid containing (w/v) 0.1% isoelectric casein, 0.25% glucose, 0.15% Na$_2$HPO$_4$ and 0.08% KH$_2$PO$_4$ in distilled water, to 88 parts of which were added 10 parts of fetal calf serum and 2 parts of fresh yeast extract (commercially available). The medium was dispensed, with or without the two antibiotics, in 5-ml amounts in screw-capped, 15 x 150 mm culture tubes.

The sphingomyelin-incorporated, chemically defined medium was prepared by adding slowly a 2% sphingomyelin in absolute ethanol to the base fluid of the CSCGYE medium until 150/µg/ml of the lipid was reached. At this concentration a distinct turbidity was observed. The approximately 5% ethanol in the medium was found to exert no detrimental effect on the amoeba.

The amoebic growth and CPE in the PMK cell cultures were ascertained by direct microscopic examination under a 10 x objective. Photomicrographs of cultures and brain sections were taken with a Leitz Orthomat camera and those under a phase-contrast, with a Zeiss camera-attached microscope. Trophozoites grown in the CSCGYE medium were used to seed the sphingomyelin-incorporated medium to avoid introduction of cell debris present in cell cultures. A CHCl$_3$; CH$_2$OH : H$_2$O Gel G system was used in the chromatographic analysis of sphingomyelin breakdown in the turbidity-cleared cultures.

RESULTS

Observations on CPE in the PMK cell cultures can be grouped into two major categories: namely, a very early CPE and rapid cell destruction by the American, European and New Zealand strains and a somewhat slower CPE and cell destruction by the Australian strains. The strains isolated from waters appeared to have about the same CPE as the case strains in respective geographic areas. Results of the American-European-New Zealand strains were represented by the Florida GJ strain and are illustrated in Plate I and those of the Australian strains were represented by the A2 strain and are shown in Plate II.

Plate I demonstrates that as early as 15 minutes after the medium being replaced with the culture fluid, the kidney cells exhibited a slight shrinkage and increased demarcation (Fig. 2). After another 15 minutes these changes became more apparent, and there was focal cell fusion (Fig. 3). The presence of very few trophozoites in the inoculum was evidenced by their absence in these illustrations. (Figs. 1—3). After 12 hours incubation the spindle cells became filamentous and cuboidal cells, rounded and granular in appearance (Fig. 4). Twenty-four hours after seeding most of cells
were replaced by trophozoites, leaving a few islands of degenerated, fused cells (Fig. 5). In the cultures receiving the washed trophozoites, no apparent CPE was observed 2 hours after seeding in spite of the large number of trophozoites present (Fig. 6). These results clearly indicate that the early CPE was unrelated to the seeing trophozoites but resulted from the effect of some cytolysis factor carried in the seeding culture fluid.

In Plate II we see that, in spite of the large number of trophozoites carried in the seeding fluid (Fig. 1), there was only a slight increase in cell demarcation after 2 hours incubation (Fig. 2). The shrinkage and increased demarcation of cells did not appear until 4 hours after seeding (Fig. 3). The considerable cell degeneration after 12 hours incubation (Fig. 4) can be attributed to the cytolysis factor liberated during the growth of the large number of seeded trophozoites, since there was, at least, a 3-fold increase in amoebic population. A rapid cell degeneration took place during the 24- to 48-hours period (Figs. 5, 6), which was also attributable to the growth of the seeded trophozoites.

Interestingly, pathologic changes in the white matter were also less severe in the Australian PAM cases than those in cases in other geographic areas. Photomicrographs of brain sections of cases CJ, WM, HB2, GJ, and Haas were employed to represent the cases in latter areas and are shown in Plate III. Similar photomicrographs of cases 66 and 69 were used to represent the Australian cases and are illustrated in Plate IV.

We see in Plate III quite marked demyelination and eosinophilic edema in the white matter of all 5 brains section (Figs. 1—5). Of interest was the absence of trophozoites, inflammatory cell infiltration, hemorrhages or vascular blockage and the presence of rod cells (pointed by arrows in Figs. 2, 3). The naked axons and microglia cells are shown in high magnification in another brain section from case WM (Fig. 6).

The demyelination and edema in the white matter were less severe and more focalized in the Australian cases (Plate IV, Figs. 1-3). There was also absence of trophozoites, cellular infiltration or vacuolar blockage.

In the rat brain slices inoculated with American, European, and New Zealand strains, naked axons were observed when the slices were crushed with pressure exerted on the cover-slips 28 hours after seeding and stained with hematoxylin, as illustrated in Plate IV, Fig. 4. In another similar preparation examined under phase-contrast 52 hours after seeding, demyelinated and fragmented axons were observed (Plate IV, Fig. 5). The Australian strains exhibited similar effects on the white matter but after 24 hours longer incubation.

To demonstrate that pathogenic *Naegleria* liberates a phospholipolytic enzyme or enzyme-like factor during its growth activity, all strains employed in the study were tested for their growth pattern and capacity to decompose sphingomyelin in the lipid-containing medium. Results of these tests were represented in those obtained with GJ, 838, Taupo and A2 strains and are illustrated in text Fig. 1, media 1—4, respectively.

In Fig. 1 we see a partial clearance of the lipid turbidity 24 hours after seeding (B); the GJ strain appeared to produce the greatest amount of clearance while the A2 strain, the least. In 48 hours incubation the turbidity in the GJ culture was cleared almost to the same degree as the other three in 72 hours. (C). After 3 days, the GJ culture was almost completely cleared and the A2, somewhat slightly more turbid than the 838 and Taupo cultures (D). No further decrease in turbidity was observed in subsequent days. The unseeded medium (5) and the seeded medium without the lipid (6) remained uncharged.

Microscopic examination revealed comparable, moderate growths in all 4 cultures during the first 3 days, with about 3-fold increase in trophozoite population. From the 4th day on, there were fewer and fewer trophozoites until the 7th—8th day when all trophozoites vanished, due to apparently the lack of some essential growth-supporting factor(s).
Chromatographic analysis of culture fluids revealed a breakdown of sphingomyelin with liberation of choline, sphingosine and fatty acids.

Fig. 1. A — sphingomyelin-incorporated medium soon after seeding with the GJ (1), 838 (2), Taupo (3), and A2 (4) strains of *Naegleria*; (5), medium seeding; (6) seeded medium without sphingomyelin. B, C, D, same cultures after 24, 48, and 72 hours incubation. Notice the partial clearance of the lipid turbidity after 24 hours and the faster clearance in (1) and slower clearance in (4). A slight residual turbidity persisted after 72 hours incubation. Control tubes remained unchanged.

**DISCUSSION**

Extensive demyelination in acute infectious encephalitis has only been reported of post-viral (measles, vaccinia, herpes, varicella, mumps or influenza) and rabies (Geschickter and Cannon 1963, Rhodes and Van Rooyen 1968, Fenner and White 1976) in which there is extensive perivascular demyelination associated with hemorrhagic, engorged or thrombotic blood vessels, suggesting vascular blockage as the cause of myelin loss. In the *Naegleria*-caused PAM, demyelination occurs in the white matter adjacent to the acutely inflamed cortex and more widely scattered than confined to the perivascular area and is not associated with vascular blockage. It can be best explained on the effect of a specific phospholipolytic enzyme or factor liberated by the trophozoites flourishing in the grey matter.
The extremely early CPE exerted on the monkey kidney cells by fluids from cultures at the peak of amoebic growth and the decomposition of sphingomyelin coincided with the growth of trophozoite population lend support to belief that the pathogenesis of pathogenic *Naegleria* is related to its capacity to liberate a cytolytic factor, probably a phospholipolytic enzyme, during the active growing phase. This pathogenic feature adds another dimension to the disease process in that the mere presence of pathogenic *Naegleria* in the nasal cavity by no means ensues the cranial invasion but the existence of an intranasal environment favourable for the amoebic growth to facilitate the liberation of the cytolytic factor is apparently a prerequisite to the cranial invasion. In a preceeding communication, it has been shown, for instance, that the nasal discharge of allergic rhinitis supported a limited multiplication of *Naegleria* trophozoits associated with CPE on epithelial cells (Chang 1974 a). This may explain why there have been no PAM cases in the area during the past two summers in spite of the extensive existence of pathogenic *Naegleria* in the lakes and undiminished swimming activities (Wellings, personal communication).

It is also interesting to note that the Australian PAM cases were found to show less severe inflammatory changes in the grey matter and meninges than the cases in other geographic areas (Chang 1974 a). The present findings were, therefore, in agreement and support the contention that the Australian strains of pathogenic *Naegleria* are inherently less pathogenic and less cytopathic than the other geographic strains. What this difference may indicate in understanding the epidemiology of PAM remains to be ascertained.

**ПАТОГЕНЕЗ ПАТОГЕННОЙ АМЕБЫ NAEGLERIA**

С. Л. Ченг

Резюме. В срезах мозга больных первичным амебным менингоэнцефалитом найдена экстенсивная демиелинизация в белом веществе, кроме выражительных гистопатологических изменений и больших пучков трофозоитов в сером веществе. Демиелинизация, по-видимому, причинена специфическим фосфолиполитическим действием, тогда как у послевирусного энцефаломиелита она приписывается блокаде сосудов или геморрагиями. В культурах клеток почек обезьяны наблюдали преждевременное цитопатологическое действие и изучали цитолитическое свойство жидкости посевной культуры. В ломтиках крыс головного мозга, зараженных культурой *Naegleria* наблюдали амебный рост и демиелинизацию в течение 28—52 часов после заражения при температуре 35 °С. В химически определенной среде, содержащей сфингомезелин, казеин и глюкозу, рост *Naegleria* был ограниченный и проходил параллельно с очисткой мутных лиофидов в течение 72 часов инкубации при 35 °С. При хроматографическом анализе очищенных культур обнаружили распад сфингомезелина и освобождение холина, сфингозина и жирных кислот. Заключается, что патогенность цитопатического действия патогенной амебы *Naegleria* можно приписывать ее способности освобождать, в течение активного роста, фосфолиполитические ферменты или фактор „делящий дыры“ в мембране цитоплазмы клеток, богатой лиофидами и причиняет демиелинизацию нервной ткани.
REFERENCES


S. L. Ch., 1035 Juanita Drive, Walnut Creek, 94595 California, U.S.A.
Fig. 1. PMK cell cultures seeded with GJ strain. A, B, C, D, and E, O, 1/4, 1/2, 12, and 24 hours after medium replaced with culture fluid at peak of amoebic growth, with very few trophozoites; F, cell culture seeded with washed trophozoites. B and C demonstrate early CPE; D and E, rapid cell destruction; F, no apparent CPE 2 hours after seeding (×93).

Note: Sequence of Figures from top to bottom: left side: Figs. 1—3, right side: Figs. 4—6. The same sequence in Plates II—IV.
Fig. 1. PMK cell culture seeded with A2 strain. A, B, C, D, E, and F, 0, 1, 4, 12, 24, and 48 hours, respectively, after fluid replaced in similar manner as in Plate I, Fig. 1 but with many trophozoites. The CPE and cell destruction were much slower than those with GJ strain (× 96).
Fig. 1. American and Belgian PAM brain sections showing white matter. Extensive demyelination and eosinophilic edema in cases CJ (A), WM (B), HB2 (C), GJ (D), and Haas (E), A, D, E, (×100). B, C, (×200). F, an area in WM brain section showing naked axons (hematoxylin stain). (×865).
Fig. 1. A, B, and C. Australian PAM brain sections showing white matter. Demyelination and edema in cases 66 (A), 69 (B), and 71 (C) less severe those in Fig. 3. (×87). D. Demyelinated nerve fibers in crushed rat brain slice 28 hours after seeding with GJ strain. (×865). E. Naked and fragmented axons in a similar preparation as in (D) 52 hours after seeding (phase-contrast). (×350).