OBSERVATIONS BY LIGHT MICROSCOPY ON THE CYTOPATHOGENICITY OF *NAEGLERIA FOWLERI* IN MOUSE EMBRYO-CELL CULTURES

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PLATES XVII–XXIII

NAEGLERIA FOWLERI is the causative agent of acute primary-amoebic meningoencephalitis (PAME), a rare and usually fatal disease of young persons, associated with swimming in fresh waters (Carter, 1972; Duma, 1972; Chang, 1974). Pathological findings suggest that water-borne amoebae become established initially in the nasal mucosa, then migrate along olfactory-nerve filaments to the brain (Carter, 1968 and 1972; Duma *et al.*, 1971). This migration is associated with progressive host-tissue destruction culminating in severe haemorrhagic necrosis of the olfactory lobes and superficial-cerebral grey matter, together with a purulent meningitis (Butt, Baro and Knorr, 1968; Carter, 1968 and 1972; Duma *et al.*, 1971). The precise mechanism of cell and tissue damage by the amoebae is largely unknown. Electron microscope studies of experimentally induced PAME in mice, which follows an almost identical course to that in man (Carter, 1970), have revealed signs of phagocytic activity by trophozoites (Martinez *et al.*, 1971 and 1973; Maitra *et al.*, 1974; Visvesvara and Callaway, 1974), but many authors agree that cytolytic enzymes released by amoebae may also participate in host-tissue disruption (Duma *et al.*, 1971; Martinez *et al.*, 1973; Chang, 1974; Maitra *et al.*, 1974; Visvesvara and Callaway, 1974).

N. fowleri causes a destructive cytopathic effect (CPE) in mammalian-cell cultures (Culbertson, Ensminger and Overton, 1968; Carter, 1970; Culbertson, 1970 and 1971; Fulton, 1970) and it is generally supposed that this cytopathic activity is associated with trophozoite pathogenicity (Culbertson, 1971; Newton, 1972; Chang, 1973). Opinion is divided on how cell damage occurs *in vitro*. Chang (1971 and 1974) reported that supernatant medium from naegleria-infected cultures of mammalian cells induced cell degeneration when fresh cultures were inoculated with it, suggesting that amoebae secrete cytolytic or cytotoxic enzyme-like substances. On the other hand, while recognising that amoebae may well release cytolytic enzymes, Visvesvara and Callaway (1974) showed that the organisms could destroy cultured cells directly by phagocytosis of host-cell cytoplasm. Naegleria-induced CPE has also been attributed to the transmission of infectious cytopathic agents from trophozoites to susceptible mammalian cells (Dunnebacke and Schuster, 1971 and 1974).

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In order to resolve these conflicting views and to determine more precisely the mechanisms of naegleria cytopathogenicity, the interaction of amoebae with cultured mouse-embryo cells has been studied. Observations of trophozoite behaviour in cell cultures are reported with particular reference to the possible role of extracellular cytotoxic substances in the production of a CPE. In addition, the process of mammalian-cell destruction by amoebae, as recorded by sequential photomicrography and time-lapse cinemicrography, is described in detail for the first time.

MATERIALS AND METHODS

Amoeba strain. *N. fowleri* strain HB-1 was kindly supplied by Mr F. C. Belton of the Microbiological Research Establishment, Porton, and maintained axenically at 37°C in Fulton's medium A (Fulton, 1970) containing penicillin (100 units per ml) and streptomycin (100 µg per ml). Trophozoites for inoculation of cell cultures were harvested from 24–48 h axenic cultures and washed by centrifugation at 200 *g* for 10 min. in three changes of amoeba saline (Page, 1967*a*). Amoeba concentrations in final suspensions were determined by haemocytometer counts.

Cell cultures. Secondary mouse-embryo (ME) cell cultures were chosen to complement the use of living mice in studies of naegleria pathogenicity (Duma, 1972). Three-day-old primary ME-cell cultures, prepared from 18-day embryos, were trypsinised and the cells suspended at a concentration of 2×10^5 per ml in Eagle's minimal essential medium (MEM) plus 10% calf serum (CS), 10% tryptose-phosphate broth (TPB), penicillin (100 units per ml) and streptomycin (100 µg per ml). Tissue-culture tubes, 100 mm × 12 mm, and Leighton coverslip-tubes were seeded with 0.5 ml and 2.0 ml of ME-cell suspension respectively. Secondary monolayers for cytotoxicity assays were prepared by adding 0.2 ml ME-cell suspension to each of the 96 flat-bottomed wells of a disposable tissue-culture tray (Linbro; Gibco Bio-cult Ltd, Paisley, Scotland), that was then sealed with adhesive covers. All cultures were incubated for 2 days at 37°C and the medium was then changed to maintenance medium (MM) consisting of Eagle's MEM supplemented with 2% CS and 3% TPB with added antibiotics. For continuous observation of naegleria cytopathogenicity in living cultures, disposable tissue-culture chambers (Sterilin Ltd, Richmond, Surrey) complete with 32-mm² no. 1½ coverslips attached by molten beeswax (fig. 1) were each seeded with approximately 0.18 ml ME-cell suspension by means of a 1-ml syringe and 25-gauge 1.6-cm needle. Inverted chambers were incubated at 37°C for 2 days in an atmosphere of 5% CO₂ in air.

Cytopathogenicity studies. The susceptibility of ME cells to naegleria cytopathogenicity was assessed by a simple tube-assay procedure. Serial 10-fold dilutions, in amoeba saline, of trophozoite suspensions of known concentration were inoculated in 0.1-ml volumes into ME-cell cultures (four tubes per dilution). The cultures were incubated at 37°C for 5 days and examined for the presence of CPE. The titres of the original amoeba suspensions, in terms of mean tissue-culture infective dose (TCD₅₀) per ml, were calculated by the method of Reed and Muench (1938).

FIG. 1.—Diagrammatic cross-section of tissue culture chamber. a = Coverglass forming upper surface of chamber with mouse-embryo (ME) cell monolayer attached (— — —). b = Lower surface of chamber. c = Inoculation duct (one each side). × 3.

Tissue-culture chambers were inoculated with naegleria trophozoites by replacing the growth medium with MM containing 5×10^4 amoebae. The inoculation ducts were sealed with molten beeswax and the chambers were inverted for 5 min. to allow attachment of the amoebae to the monolayers. The chambers were then clamped upright to a Biotherm biological warm (37°C) stage attached to a Zetopan research microscope (Reichert-Jung UK, Slough). The cultures were observed by phase-contrast optics with a long-focus condenser. Leighton-tube cultures were inoculated with 5×10^3 to 1×10^5 amoebae, incubated for up to 24 h at 37°C and examined periodically for CPE.

Staining procedure. Coverslip cultures showing advanced CPE were removed from Leighton tubes, rinsed gently in phosphate-buffered saline, pH 7.2, and immersed for 1 h in Carnoy's fixative. The preparations were stained with 4% Giemsa R-66 (Duguid, 1968) and examined microscopically by bright-field illumination.

Photomicrography and cinemicrography. Photomicrographs were taken on Ilford FP-4 35-mm film with a green interference filter (Wild Heerbrugg U.K. Ltd, Maidstone, Kent) inserted in the light path to improve contrast. Time-lapse cinemicrography was carried out with a Paillard Bolex H-16 16-mm camera fitted with a single-frame actuator operated at 2-s intervals by means of an electromechanical pulse generator. The exposed cine-film (Kodak Tri-X) was processed by Filmatic Laboratories, London, W11.

Cytotoxicity assays. Two methods were used to detect cell-free cytolytic or cytotoxic activity in naegleria-infected ME-cell cultures.

In the first method, 1-litre flat bottles containing secondary ME-cell monolayers (c. 1.5×10^7 cells) were inoculated with 50 ml of MM containing 10^4 naegleria trophozoites per ml. The cultures were incubated at 37°C and 3-ml samples of culture medium were removed daily for up to 3 days, when CPE was complete. Each sample was centrifuged at 2000 *g* for 10 min. to deposit amoebae and the supernates were stored at -70°C to await simultaneous assay for cytotoxic activity. After withdrawing the final samples, the trophozoites in the remaining culture medium (c. 3×10^5 amoebae per ml) were disrupted ultrasonically with a Mullard 50-watt probe (Measuring and Scientific Equipment Ltd, Crawley, Sussex). The sonicated extracts were centrifuged as above and stored at -70°C. Trophozoites suspended in MM alone (10^6 amoebae per ml) and uninoculated control ME-cell cultures were processed in the same way. All harvests were thawed quickly and serial two-fold dilutions were inoculated on to fresh (indicator) ME-cell monolayers in tissue-culture trays. The trays were sealed, incubated at 37°C, and examined daily by means of an inverted microscope for the presence of cytolytic or cytotoxic effect (CTE).

In the second method, tissue-culture chambers were seeded twice, on the lower and upper surfaces (see fig. 1), to produce confluent and semi-confluent monolayers respectively of secondary-ME cells separated by a distance of 0.7-0.8 mm. The chambers were then inoculated with 10^4 naegleria trophozoites in MM, sealed and placed upright at 4°C for 15 min. This ensured that the amoebae became established only on the lower ME-cell monolayer, the cells of the upper layer being used as indicators of soluble cytotoxins. The chambers were then transferred gently to an incubator (37°C) and placed upright. Uninoculated cultures were used as controls and all cultures were examined daily by phase-contrast microscopy.

RESULTS

Appearance of naegleria-induced CPE in ME-cell cultures

The trophozoites of *N. fowleri* strain HB-1 caused a distinctive CPE in ME-cell monolayers in chamber cultures. Mammalian-cell damage was first detected within 30 min. of inoculation of amoebae, and by 24 h, the monolayers were almost completely destroyed (figs. 2 and 3). Cytopathic activity appeared to be directed against ME-cell cytoplasm; the cytoplasm was characteristically reduced to long often-branched filaments, and isolated nuclei became increasingly prominent as the CPE progressed. This process of cytoplasmic disruption

TABLE I

Relationship between trophozoite concentration and ME-cell culture infectivity of suspensions of Naegleria fowleri strain HB-1

Trophozoite concentration* of suspension (per ml)	ME-cell infectivity of suspension (TCD50 per ml)	Ratio of trophozoite concentration to ME-cell infectivity
2.27×10^6	3.16×10^6	0.72
1.12×10^6	5.62×10^5	1.99
2.40×10^6	1.00×10^6	2.40
4.50×10^5	5.62×10^5	0.80
1.25×10^5	1.78×10^5	0.70
4.50×10^4	5.62×10^4	0.80

Mean
1.24

* Haemocytometer count.

ME = Mouse embryo.

was confirmed in Giemsa-stained cultures (fig. 4). ME-cell nuclei became rounded and pyknotic, and trophozoites were found closely applied to damaged cells or attached to them by strands of cytoplasm. The apparently intracellular location of a few amoebae was a constant finding.

Titration experiments suggested that ME cells were highly susceptible to naegleria cytopathic activity (table I). There was a fairly close correlation, with a ratio approaching unity, between the haemocytometer count of each trophozoite suspension and its cell-culture infectivity in terms of TCD50. From these results, it appeared that a CPE could occur in ME-cell cultures after inoculation with a single amoeba.

Search for cell-free cytotoxins

The results of medium-transfer experiments are summarised in table II. Supernatant medium from strain HB-1 suspensions in MM showed no cytotoxic activity; the morphology of the indicator ME cells remained quite normal, regardless of the inoculum dilution or time of harvesting, for about 7 days. Thereafter, all indicator monolayers, including uninoculated controls, began to degenerate. By contrast, sonicated extracts of the suspensions in MM caused cytotoxic changes in ME cells within 24 h of inoculation. These changes, induced only by undiluted extracts, were quite unlike those observed in ME-cell monolayers inoculated with intact trophozoites. Affected indicator cells were grossly elongated or "stringy", and possessed a brownish, granular cytoplasm. Aggregates of rounded cells and occasional syncytia containing three or four nuclei were also seen. This appearance was transient, and the ME cells gradually recovered on further incubation, only to degenerate again after the 7th day.

Samples of medium from amoeba-infected ME-cell cultures induced cytotoxic changes in indicator cells with a speed and severity that increased as the cultures grew older and developed a greater degree of CPE. However, the effect could not be related specifically to trophozoite activity, as medium from uninoculated ME-cell cultures possessed similar cytotoxicity. ME-cell degeneration occurred only with undiluted inocula and resembled that appearing later in

TABLE II

ME-cell cytotoxicity of supernatant medium and sonicated extracts from suspensions of N. fowleri strain HB-1, ME-cell cultures infected with strain HB-1, and control ME-cell cultures

Culture system	Material harvested from culture	Interval (days) between inoculation and harvest of culture	CPE in harvested culture	CTE of undiluted harvest on ME cells after 7 days' incubation
Trophozoites suspended in MM	Medium	0	...	—
	Medium	1	...	—
	Medium	2	...	—
	Medium	3	...	—
	Extract	3	...	+ (1)*
Trophozoites in ME-cell culture	Medium	0	—	—
	Medium	1	±	+ (6)
	Medium	2	++	++ (4)
	Medium	3	+++	+++ (3)
	Extract	3	+++	+++ (1)
Control ME-cell culture	Medium	0	—	—
	Medium	1	—	+ (4)
	Medium	2	—	+++ (4)
	Medium	3	—	+++ (3)
	Extract	3	—	+++ (1)
Indicator ME-cell control		...	...	—

MM = Maintenance medium; ME = mouse embryo; CPE = cytopathic effect; CTE = cytotoxic effect.

* Day on which CTE first appeared is given in parenthesis.

Degrees of CPE or CTE are indicated as follows: — = none; ± = 25%; + = 50%; ++ = 75%; +++ = 100%.

uninoculated-control indicator cells. Undiluted sonicated extracts of naegleria-infected and control ME-cell cultures were also highly cytotoxic and caused, in indicator monolayers within 24 h, CTEs that were identical. The CTE was similar in appearance to that induced by sonicated trophozoite extracts, but was more severe; the indicator cells failed to recover and began to disintegrate after 2 or 3 days' incubation. The cytotoxic factor or factors present in all sonicated extracts could not be serially passaged. Medium from indicator cultures showing an advanced CTE caused no further cytotoxic changes of a similar nature when 1 in 2 dilutions were transferred to fresh ME-cell monolayers.

Observations of naegleria cytopathogenicity in tissue-culture chambers containing two ME-cell monolayers failed to reveal any cell-free cytotoxic or cytolytic activity. Trophozoites were confined initially to the lower cell sheet, which showed complete CPE within 24 h (fig. 5a). The indicator cells of the upper monolayer remained quite normal in appearance and showed no cytotoxic changes (fig. 5b). After 48 h, amoebae gained access to the upper monolayer, apparently via the meniscus of the culture medium. The organisms first became established at the periphery of the monolayer, then migrated gradually and

uniformly towards the centre, destroying the ME cells as they advanced. Cells immediately in front of the moving line of trophozoites remained undamaged until overtaken. These observations suggested that the CPE was induced by direct contact between active amoebae and the mammalian cells rather than by the action of released cytotoxins.

Observations on the cytopathic activity of Naegleria fowleri by time-lapse cinemicrography and photomicrography

Time-lapse cinemicrography revealed dramatically the violent and often rapid destruction of ME cells by naegleria trophozoites. Amoebae inoculated into culture chambers were initially quite inactive, but quickly resumed normal motility and began to wander randomly over the field, pausing only when contact was made with mammalian cells. The trophozoites then showed increased pseudopodial activity and began to move vigorously over and around the ME cells. At the same time, the mammalian-cell cytoplasm gradually contracted and was eventually reduced to filamentous remnants attached to the nuclei. Adjacent cells remained unaffected until they themselves were physically attacked. Individual trophozoites did not always remain attached to a susceptible ME cell until its destruction was complete, but frequently moved to neighbouring cells. Disruption of the original cell then ceased, although brief, sporadic eruptions of cytoplasm previously in contact with the departed amoeba were sometimes seen. Most ME cells were fibroblast-like and possessed two or more cytoplasmic processes that were usually the sites of initial damage. These cells were destroyed quite quickly, often within a few minutes, particularly when attacked by several trophozoites simultaneously. The few epithelial cells that were always present in the monolayers were clearly less susceptible, and their disruption was delayed, despite repeated contact with amoebae.

FIG. 15.—Process of engulfment by trophozoites of *Naegleria fowleri* strain HB-1. Drawings based on observations of living amoebae in ME cell cultures. (a) Two blunt, hyaline pseudopodia are formed and curve inwards. (b) Membranous structures appear above and below the pseudopodia and move towards their distal ends. (c) The tips of the pseudopodia and the membranous structures fuse, enclosing a vacuole.

Although the mechanism of naegleria cytopathogenicity could not be resolved clearly, single-frame analysis of the film suggested that the amoebae were engaged in phagocytic activity. This impression was enhanced when the destruction of individual ME cells was observed continuously by high-power microscopy with a $\times 100$ objective, and recorded by serial photomicrography. A representative sequence, timed from when observation began, is illustrated and described in figs. 6–14. ME cells again showed cytoplasmic disruption only at the point of contact with a trophozoite, and the apparent engulfment of mammalian-cell cytoplasm by amoebae was seen frequently (fig. 12). This ingestion process, illustrated diagrammatically in fig. 15, began with the formation by the amoebae of two, clear, hyaline pseudopodia that curved towards each other. Membranous structures appeared above and below the pseudopodia and moved towards their distal ends which then fused, enclosing a vacuole. Frequently, the initial departure of a trophozoite from the immediate area of cytoplasmic disturbance was followed by partial recovery of the ME cells (figs. 7, 8, 10 and 11), although continued trophozoite activity eventually resulted in overwhelming cytoplasmic loss and in death of the mammalian cells.

DISCUSSION

According to Chang (1971 and 1974), *N. fowleri* trophozoites secrete cytotoxic substances that cause disintegration of mammalian cells *in vitro*, and the amoebae then feed on the resulting cell debris, but the evidence presented in support of this hypothesis is open to an alternative interpretation. Inoculation of mammalian-cell cultures with cytopathogenic amoebae initiates a complex series of events involving not only an increase in the trophozoite population, causing progressive CPE, but also a decline in the population of intact mammalian cells. Surviving cells may cause gradual deterioration of the medium as a result of the accumulation of toxic products, including hydrogen ions, or of the depletion of essential nutrients (Paul, 1970). Thus the appearance of "cytotoxic activity" in the culture medium, as expressed by the induction of premature morphological changes in an appropriate indicator-cell system, may not be attributable exclusively to the presence of amoebae.

The present observations do not support the concept that *N. fowleri* trophozoites secrete cytotoxic substances *in vitro*, although the possible effect of concentration procedures was not assessed. There was no evidence that heavy concentrations of amoebae led to cytotoxic activity of the medium in which the organisms were suspended, despite examination by means of indicator-cell cultures known to be highly sensitive to naegleria cytopathogenicity. The eventual degeneration of both inoculated and control indicator cells was almost certainly due to gradual deterioration or exhaustion of the culture medium, which clearly could not be replaced without frustrating the experimental objectives. Similarly, the increasing cytotoxicity shown by serial harvests of supernate from naegleria-infected ME-cell cultures was consistent with depletion of the medium by the surviving mammalian cells, as medium from control cultures caused a similar CTE. Thus, the rate of indicator ME-cell degeneration

merely reflected the degree of exhaustion of the medium at the time of sampling, rather than the accumulation of naegleria-associated cytotoxins.

On the other hand, sonicated extracts of amoeba-infected ME-cell cultures caused rapid indicator-cell degeneration resembling that ascribed by Chang (1971 and 1974) to naegleria cytotoxic activity. Certainly, this ME-cell degeneration could not have been due solely to exhaustion of the medium, as no immediate cytotoxicity was shown by supernatant medium withdrawn from the same cultures shortly before the extracts were prepared. This suggested that the cytotoxic factor was normally cell-bound, and possibly represented intracellular lysosomal enzymes released by sonic disruption of the amoebae remaining in the cultures. However, the CTE observed was not associated specifically with naegleria cytopathogenicity; all extracts, including those of uninoculated ME-cell cultures, caused identical cytotoxic changes. CTE and CPE were characterised by quite different morphological changes in affected ME cells, and the former was never seen in cultures inoculated with living amoebae.

Trophozoite activity in tissue-culture chambers containing two ME-cell monolayers was particularly significant. If released cytotoxic substances were responsible for the rapid destruction of the lower monolayer, similar cytopathic changes might have been anticipated in the nearby indicator-ME cells. No such changes were seen, although the distance between the cell sheets was very small, and there was no physical barrier preventing the free diffusion of any cytotoxin. Moreover, the small volume of culture medium in the chambers reduced the likelihood that any cytotoxic agent might be diluted out before its effects could become apparent. Only when amoebae migrated to the upper monolayer and came into direct contact with the indicator-ME cells did these show signs of damage.

The distinctive CPE caused by trophozoites of *N. fowleri* strain HB-1 in ME cells generally conformed to previous brief descriptions of changes in other cell lines (Carter, 1970; Culbertson, 1971; Visvesvara and Callaway, 1974). The appearance of intracellular amoebae, particularly in fixed and stained preparations, was noted also by Chang (1974) and Visvesvara and Callaway (1974) although their suggestion that the organisms were engulfed by the cultured cells seems most improbable in the light of the present observations; time-lapse cinemicrography indicated that the vigorously motile trophozoites could easily evade capture and ingestion by the relatively-static mammalian cells. A more likely explanation is that an amoeba occasionally becomes superimposed on a mammalian cell and creates a hole or depression by disruption of the underlying cell cytoplasm. Such an organism might well appear to be intracellular, especially in a fixed and stained preparation. The trophozoite illustrated in figs. 11 and 12, for example, if seen in such a preparation, would almost certainly seem to lie within the ME cell.

Continuous observation of CPE development, both directly and by time-lapse cinemicrography confirmed that ME cells were damaged only during contact with amoebae. There was no evidence that trophozoites merely ingested debris resulting from earlier cytotoxic disintegration of the mammalian cells (Chang, 1971 and 1974; Chang *et al.*, 1975). Indeed, cell disintegration was

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FIG. 2.—Normal (uninoculated) ME-cell culture. Phase contrast (PC). $\times 240$.

FIG. 3.—ME-cell culture showing characteristic cytopathic effect 24 h after inoculation with trophozoites of *Naegleria fowleri* strain HB-1. PC. $\times 240$.

FIG. 4.—ME-cell culture 5 h after inoculation with trophozoites. Two amoebae (T) are shown, one apparently lying within a mammalian cell. Giemsa. $\times 670$.

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FIG. 5.—Tissue culture chamber containing two ME-cell monolayers 24 h after inoculation with trophozoites. (a) Lower surface of chamber showing complete destruction of the ME cells. (b) Upper monolayer of the same preparation. The indicator cells show no cytotoxic changes. PC. $\times 200$.

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FIG. 6.—This and figs. 7–14 illustrate the cytopathic activity of *N. fowleri* strain HB-1 as shown by serial photomicrography. (0 min.). Trophozoite (T) attached to single ME cell. The amoeba remained in this position for about 20 min. PC. $\times 900$.

FIG. 7.—(24.5 min.). The trophozoite moves slowly towards the centre of the ME cell leaving strands of disrupted mammalian-cell cytoplasm behind (arrow). PC. $\times 1450$.

CYTOPATHOGENICITY OF *NAEGLERIA FOWLERI*

FIG. 8.—(45 min.). The damaged ME-cell membrane and cytoplasm in the region previously occupied by the amoeba shows partial recovery. PC. $\times 1450$.

FIG. 9.—(57 min.). Increased pseudopodial activity by the trophozoite results in displacement and distortion of the ME-cell nucleus (cf. fig. 6). PC. $\times 1450$.

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FIG. 10.—(58 min.). As the amoeba moves over the ME cell, large cytoplasmic vacuoles or holes are revealed (arrows). PC. $\times 1450$.

FIG. 11.—(68 min.). The holes disappear as the ME-cell cytoplasm contracts, but further cytoplasmic disruption is seen adjacent to the vigorously active trophozoite (arrow). PC. $\times 1450$.

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FIG. 12.—(69.5 min.). Large holes separated by thin filaments develop rapidly in the ME-cell cytoplasm. Note the distinctive trophozoite pseudopodia that have fused to enclose a vacuole (arrow). PC. $\times 1450$.

FIG. 13.—(78 min.). The ME-cell cytoplasm is quickly reduced to a network of thin filaments attached to cytoplasmic "nodules". PC. $\times 1450$.

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FIG. 14—(130 min.). ME cell showing further nuclear displacement (arrow) by the attached trophozoite and almost complete loss of cytoplasm. Observation was terminated at this point. PC. $\times 1450$.

rarely seen, CPE being characterised by gradual shrinkage of ME-cell cytoplasm in the presence of attached trophozoites. Cytopathic changes in individual ME cells ceased when amoebae moved away to attack neighbouring cells. This constant wandering of trophozoites from cell to cell was probably the cause of widespread CPE by relatively few organisms, a feature thought by Chang *et al.* (1975) to indicate the action of soluble cytotoxins released by the amoebae.

The precise mechanism of ME-cell destruction was not clearly discerned. Brief consideration was given to the possible transmission of cytopathic agents from trophozoites to susceptible mammalian cells, as was suggested by Dunnebacke and Schuster (1971 and 1974). However, the role of any amoeba-associated agent with a virus-like mode of replication was discounted, as individual ME cells frequently showed signs of damage immediately they came into contact with trophozoites, and often were completely destroyed within a few minutes. This violent disruption of ME cells, characterized by loss of cytoplasm, suggested that the amoebae were engaged in phagocytic activity, by means of which they presumably engulfed portions of mammalian-cell cytoplasm. In particular, the formation of distinctive ingesting pseudopodia by trophozoites attached to ME cells closely resembled that of "food cups" in species of *Acanthamoeba* as described by Page (1967*b*). Moreover, initial ME-cell damage by amoebae was usually confined to cytoplasmic processes, possibly because these were easily surrounded and engulfed by pseudopodia, so providing suitable starting points for trophozoite attack. Certainly, cells of fibroblastic morphology were particularly prone to disruption, whereas epithelial cells lacking pronounced cytoplasmic protruberances were more resistant and succumbed only after prolonged contact with amoebae.

Despite these observations, it was by no means certain that phagocytosis alone was responsible for mammalian-cell damage, although such a mechanism would be quite consistent with the phagotrophic behaviour of amoebae in general. Similar conclusions were reached by Visvesvara and Callaway (1974) who demonstrated phagocytic activity by *N. fowleri* trophozoites *in vitro*, but thought that cytolytic enzymes released by the amoebae might also participate in CPE development. While the present studies cast doubt on the latter suggestion, they do not exclude the possibility that trophozoites possess membrane-associated enzymes that are activated by contact with a host cell, as was proposed by Eaton, Meerovitch and Costerton (1969 and 1970) to explain the *in-vitro* cytopathogenicity of *Entamoeba histolytica*. The results of further studies designed to resolve this problem will be reported elsewhere.

SUMMARY

Naegleria fowleri, strain HB-1, caused a destructive cytopathic effect (CPE) in secondary mouse-embryo (ME) cells. No evidence was found to suggest that cell-free cytotoxic factors secreted by the amoebae play a part in ME-cell destruction. In culture systems designed for the study of cytopathic factors, mammalian-cell damage seemed to occur only as a result of direct contact with active amoebae. This was confirmed when the progressive destruction of

individual ME cells was observed continuously by direct microscopy and time-lapse cinemicrography. The cytoplasmic shrinkage characteristic of naegleria-induced CPE appeared to be associated with phagocytic activity of trophozoites. Adjacent ME cells remained undamaged until they themselves were physically attacked. The apparently intracellular location of amoebae seen in fixed and stained preparations was considered to be an artefact created when trophozoites and ME cells were superimposed.

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