INHIBITION BY AMOEBA-SPECIFIC ANTISERUM AND BY CYTOCHALASIN B OF THE CYTOPATHOGENICITY OF NAEGLERIA FOWLERI IN MOUSE EMBRYO-CELL CULTURES

T. BROWN
Department of Bacteriology, University of Aberdeen, Foresterhill, Aberdeen AB9 2ZD

NAEGLERIA FOWLERI, the causative agent of acute primary meningoencephalitis, has been described as one of the most lethal of micro-organisms (Phillips, 1974). In man and in experimentally infected laboratory animals, amoebae proliferate rapidly and cause extensive destruction of the olfactory and brain tissues (Carter, 1968, 1970, 1972; Duma, 1972). In cell cultures too, amoebae exhibit a pronounced cytopathic activity that is thought to correlate with pathogenicity in vivo (Culbertson, 1971; Newton, 1972; Chang, 1973). Secondary mouse-embryo (ME) cells were recently shown to provide a sensitive in-vitro host system for the study of mechanisms of naegleria-induced mammalian-cell damage by techniques that are precluded in the intact animal (Brown, 1978). Observations of trophozoite behaviour in these cultures suggested that amoebae destroy mammalian cells only while in direct contact with them, rather than by secreting cytotoxic or cytolytic substances into the culture medium. While revealing some evidence for phagocytic activity by trophozoites, these studies did not exclude the possibility that amoebae possess membrane-associated cytotoxic enzymes that are activated by contact with a susceptible host cell and participate in its destruction. Eaton, Meerovitch and Costerton (1969, 1970) have proposed such a mechanism for the in-vitro cytopathogenicity of Entamoeba histolytica, which also appears to kill cells on contact (Jarumilinta and Kradolfer, 1964), and certain features of tissue damage by N. fowleri in mice have likewise been interpreted in terms of enzyme-mediated cytolysis (Martinez et al., 1973; Maitra et al., 1974, 1976; Visvesvara and Callaway, 1974). It was decided, therefore, to investigate the possible existence of amoeba-associated cytotoxins by observing whether the cytopathogenicity of Naegleria in ME-cell cultures could still be expressed in the presence of non-lethal inhibitors of trophozoite motility and phagocytosis.

MATERIALS AND METHODS

Cultures. Procedures for the maintenance of N. fowleri strain HB-1 in axenic culture, the

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processing of trophozoites for inoculation on to mammalian-cell monolayers and the preparation of secondary ME-cell cultures and associated media were as reported previously (Brown, 1978). Monolayers were established in 100 x 12-mm tissue-culture tubes and, for photomicrography, in disposable tissue-culture chambers (Sterilin Ltd, Richmond, Surrey; see Brown, 1978).

**Antiserum preparation.** Procedures were based on those described by Anderson and Jamieson (1972). Trophozoites from three-day axenic cultures were washed by centrifugation at 200 g for 10 min. in three changes of amoeba saline (Page, 1967) and suspended at a concentration of 1 x 10^6/ml in 0·85% saline solution containing 0·1% of formalin. The suspensions were stored at 4°C for up to 18 days while awaiting inoculation into animals.

New Zealand White rabbits (3-4 kg) were inoculated intravenously on days 0, 3, 7, 12, and 18 with, respectively, 0·5 ml, 0·5 ml, 1·0 ml, 2·0 ml and 1·0 ml of warmed (37°C) formalised amoeba suspensions. Sera from pre-inoculation and post-inoculation bleeds, collected on days 0, 24 and 34, were stored in 2·0-ml volumes at —20°C.

**Antiserum assay.** *Naegleria* strain HB-1 antiserum titres were determined by immobilisation (TI) and agglutination (TA) tests in which the antigens were, respectively, living trophozoites suspended in ME-cell maintenance medium (MM) and formalised amoebae at a concentration of 1-2 x 10^6/ml (Singh and Das, 1970; Anderson and Jamieson, 1972); 0·1 ml of the appropriate antigen was added to 0·1 ml of doubling dilutions of non-inactivated sera in amoeba saline (TA tests) or ME-cell MM (TI tests), prepared in the wells of disposable flat-bottomed tissue-culture trays (Linbro; Gibco-Biocult Ltd, Paisley, Scotland). The wells were sealed with adhesive covers and the plates were incubated at 37°C for 2 h. Normal (non-immune) rabbit serum and diluent controls were included in each test and the results were read with a Nikon stereo-microscope. Antiserum titres by TA and TI tests, recorded as the highest final dilutions causing complete trophozoite agglutination or complete inhibition of translational movement, were 512 and 32 respectively. Normal rabbit serum at a minimum dilution of 1 in 4 failed to agglutinate or immobilise amoebae.

**Inhibition of naegleria cytopathogenicity by specific antiserum.** Washed trophozoites were suspended at a concentration of 10^5 or 10^4/ml in ME-cell MM containing four immobilising units (4 x TI titre) of *N. fowleri*-specific rabbit antiserum. Normal rabbit serum was used in controls and the amoeba-serum mixtures were held at room temperature (c. 22°C) for 30 min., then inoculated in 1-0-ml volumes into duplicate culture tubes containing confluent ME-cell monolayers. The tubes were incubated for up to 7 days at 37°C and inspected daily for the appearance of cytopathic effect (CPE).

**Cytochalasin B** (Aldrich Chemical Co. Ltd, Dorset) was dissolved in dimethylsulphoxide (DMSO) to give a stock concentration of 10^4 µg/ml and stored at 4°C. The drug was shown to inhibit phagocytosis by *Naegleria* trophozoites by suspending amoebae (1·0 x 10^5/ml) in ME-cell MM containing cytochalasin B at concentrations ranging from 1 µg/ml to 10 µg/ml and, after incubation for 30 min., adding 0·1 ml of a suspension (c. 0·16%, based on packed-cell volume) of day-old-chick erythrocytes in MM. Cytochalasin B-free controls were included and the suspensions were incubated at 37°C for up to 24 h. Wet preparations were examined microscopically (x 10 objective) at appropriate intervals and the proportion of amoebae containing ingested erythrocytes, expressed as a percentage of the total number of trophozoites present in three low-power fields, was noted.

**Inhibition of the cytopathogenicity of Naegleria** was studied by the inoculation of amoebae that had been treated with 10 µg/ml (2·1 x 10^-2M) cytochalasin B for 30 min. at room temperature into tube cultures of ME cells to give cytochalasin B and trophozoite concentrations of 10 µg/ml and 5 x 10^3/ml respectively. Uninoculated ME-cell monolayers and cell cultures inoculated with cytochalasin B or amoebae only were used as controls. The medium in all cultures, including controls, contained 0·1% DMSO, used as the solvent for cytochalasin B. The cultures were incubated at 37°C for up to 8 days and examined daily by low-power microscopy.

**Colchicine.** The above methods were also used to investigate the effect of colchicine (BDH Chemicals Ltd, Poole, Dorset) at concentrations of 10^-6M-10^-8M on the phagocytic activity and cytopathogenicity of *Naegleria*. 
RESULTS

Inhibition of N. fowleri cytopathogenicity by amoeba antiserum

The cytopathic activity of Naegleria in ME-cell cultures was inhibited by amoeba antiserum (Table I), and the trophozoites were immobilised and agglutinated. Clumps of amoebae were seen scattered over intact ME-cell monolayers 24 h after inoculation (fig. 1a), and individual ME cells immediately beneath the clumps appeared quite normal when the organisms were dislodged by gentle shaking of the culture tubes. After incubation for another 24 h, the initially aggregated organisms in cultures receiving \(10^5\) trophozoites began to move very slowly in ever-expanding circles, destroying the ME cells as they advanced, to produce clear plaques in the monolayers (fig. 1b). However, complete CPE occurred only after 5 days, whereas the same number of amoebae in the presence of normal rabbit serum caused total destruction of the ME cells within 24 h. In cultures inoculated with \(10^4\) trophozoites, the amoebae remained agglutinated in the presence of specific antiserum throughout the period of observation, although they continued to show signs of life in the form of spasmodic cytoplasmic eruptions. CPE was not observed in the ME-cell monolayers in the immediate vicinity of the agglutinated organisms.

**Effect of cytochalasin B and colchicine on phagocytic and cytopathic activity of Naegleria**

The results of preliminary experiments with cytochalasin B, in which avian erythrocytes were used as indicators of phagocytosis by amoebae, are presented in table II. In control suspensions, erythrocytes were quickly engulfed by *Naegleria* trophozoites and numerous ingested red cells were clearly visible within the cytoplasm of the amoebae (fig. 2). By contrast, cytochalasin B at
TABLE II

Effect of cytochalasin B on the ingestion of day-old chick erythrocytes by trophozoites of Naegleria strain HB-1

<table>
<thead>
<tr>
<th>Time (hours) after addition of erythrocytes</th>
<th>Percentage* of amoebae containing ingested erythrocytes in the presence of cytochalasin B at concentrations (µg/ml) of</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>7-5</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
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<td>0</td>
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<tr>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
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</table>

* Approximate assessment.

concentrations exceeding 5 µg/ml totally inhibited erythrocyte ingestion (fig. 3), although the amoebae remained active and continued to show random motility after 24 h.

The use of DMSO as the solvent for cytochalasin B did not influence cell-culture findings. The morphology of control ME cells appeared normal when DMSO alone was incorporated in the culture medium (fig. 4). The cytopathogenicity of Naegleria was also unaffected by DMSO, and ME-cell cultures inoculated with amoebae in the absence of cytochalasin B were completely destroyed within 24 h (fig. 5). Cytochalasin B itself, however, induced unusual morphological changes in the ME cells, notably the formation of distinctive dendritic processes (fig. 6); the nuclei were often displaced to the periphery of the cells and occasionally were completely extruded. These changes occurred within 24 h from the addition of cytochalasin B, but progressed no further over the next seven days. If cytochalasin B was removed by changing the medium after the third day, the ME cells gradually resumed their normal morphology.

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Fig. 1.—Mouse-embryo (ME) cell culture inoculated with trophozoites of Naegleria strain HB-1 in the presence of amoeba-specific antiserum. (a) 24 h after inoculation, showing immobilised and agglutinated amoebae. Adjacent ME cells appear undamaged. Phase contrast (PC). x 125. (b) 48 h later, the trophozoites have resumed motility, and destruction of ME-cells by the expanding circle of amoebae results in the formation of a well-defined plaque in the monolayer. PC. x 125.

Fig. 2.—Trophozoites of Naegleria strain HB-1 3 h after the addition of chick erythrocytes (control suspension). The amoebae (arrows) contain numerous ingested red cells. PC. x 490.

Fig. 3.—Trophozoites of Naegleria strain HB-1 3 h after suspension in medium containing chick erythrocytes and cytochalasin B (10 µg/ml). Phagocytosis of erythrocytes by amoebae (arrows) is inhibited. PC. x 490.

Fig. 4.—Normal (uninoculated) ME-cell culture. The medium contained 0-1% DMSO. PC. x 125.

Fig. 5.—ME-cell culture 24 h after inoculation with 5 x 10³ trophozoites of Naegleria strain HB-1 in the presence of 0-1% DMSO alone. The monolayer is completely destroyed. PC. x 125.

Fig. 6.—ME-cell culture 24 h after the addition of cytochalasin B (10 µg/ml), showing characteristic morphological changes induced by the drug. PC. x 125.

Fig. 7.—ME-cell culture 24 h after inoculation with 5 x 10³ trophozoites of Naegleria strain HB-1 in the presence of cytochalasin B (10 µg/ml). The mammalian cells show only those morphological changes associated with cytochalasin B (compare with figs. 5 and 6). PC. x 125.
INHIBITION OF NAEGLERIA CYTOPATHOGENICITY

[Plate XXIII]

(facing page 358)
ME cells inoculated with Naegleria trophozoites in the presence of cytochalasin B showed only the morphological changes characteristic of the drug alone (fig. 7). There was no evidence for disruption of mammalian-cell cytoplasm despite repeated contact with amoebae that remained alive and active, although motility was somewhat sluggish and erratic. The organisms contained few cytoplasmic vacuoles and became gradually smaller as incubation continued. By the fifth day, the trophozoites usually were no longer viable, but if the culture medium was changed beforehand they recovered and showed normal cytopathic activity.

Colchicine at concentrations of $10^{-4}$–$10^{-6}$M failed to prevent engulfment of erythrocytes by amoebae. Similar colchicine concentrations in naegleria-infected ME-cell cultures also caused no apparent inhibition of cytopathogenicity; the CPE progressed at the same rate as in colchicine-free control cultures and was complete 24 h after inoculation.

**DISCUSSION**

These results suggest that the cytopathogenicity of *N. fowleri* in ME cells does not involve amoeba-associated cytotoxic activity, but depends on normal pseudopodium function. Trophozoites that were immobilised, and agglutinated, by specific antiserum were unable to cause CPE; no detectable damage of ME cells occurred even in the region of large aggregates of amoebae. As pseudopodia may be formed by amoebae to effect ingestion as well as movement, the antiserum probably inhibited both activities, particularly in cultures inoculated with $10^4$ trophozoites, in which the organisms remained immobilised and non-cytopathogenic long after control cultures had been completely destroyed. The slowly progressing CPE in heavily inoculated cultures appeared only when the trophozoites began to recover from the inhibitory effects of the antiserum and presumably resumed phagocytosis in addition to motility. It was not clear how this recovery occurred, but Biagi and Beltrán (1969) have shown that the ability of *E. histolytica* to regain mobility in the presence of specific antiserum is associated with endocytosis and digestion of surface-bound antibodies. The specific immobilisation of *Acanthamoeba castellanii* may also be a temporary phenomenon (Krantz, 1969).

The total inhibition of naegleria cytopathogenicity in the presence of cytochalasin B was particularly significant. This was not thought to be due to increased resistance of the ME cells, although they were clearly affected by the drug and showed changes in morphology similar to those observed in mouse L-cells by Carter (1967). If, as previously suggested (Brown, 1978), the cytoplasmic processes of fibroblast-like cells are prone to attack by *Naegleria* trophozoites, the exaggerated protuberances of ME cells induced by cytochalasin B should have been especially susceptible to damage. Instead, the drug appeared to inhibit the cytopathogenicity of *Naegleria* by influencing trophozoite behaviour. A metabolite of *Helminthosporium dematioides* (Carter, 1967), cytochalasin B, is known to induce a wide range of dose-dependent changes in cellular-transport and motility processes (Wessells *et al.*, 1971;
Pollard and Weihing, 1974). At relatively high concentrations (c. 10^{-5}M), the drug inhibits phagocytosis by mammalian macrophages and polymorphonuclear leucocytes, possibly by disrupting contractile microfilament systems (Allison, Davies and de Petris, 1971; Axline and Reaven, 1972; Davies et al., 1973; Hartwig and Stossel, 1976; Weihing, 1976). That cytochalasin B also prevents phagocytosis by *Naegleria* trophozoites was demonstrated during the present studies by the inability of treated amoebae to engulf chick erythrocytes. By implication, the failure of amoebae to cause CPE in ME-cell cultures in the presence of cytochalasin B was likewise associated with the suppression of the feeding activity of trophozoites. Certainly, the amoebae showed signs of starvation, including lack of cytoplasmic vacuoles, gradually decreasing size and failure to thrive, while the random, uncoordinated motion of the organisms was consistent with pseudopodium dysfunction. On the other hand, the inability of colchicine to prevent ingestion of erythrocytes and destruction of ME-cells by *Naegleria* trophozoites was not entirely unexpected. Although colchicine disrupts microtubules (Novikoff and Holtzman, 1970) which, with microfilaments, may participate in cell movement and possibly endocytosis (Allison et al., 1971; Reaven and Axline, 1973), phagocytosis by macrophages has been shown to proceed normally in the presence of the drug (Allison et al., 1971; Bhisey and Freed, 1971).

A notable feature of these studies was the inhibition of *Naegleria* cytopathogenicity by two quite different agents without directly affecting the viability of the organisms. Trophozoites in the presence of specific antiserum or cytochalasin B were no longer cytopathogenic, though still alive and in constant contact with the ME cells. In these circumstances, there was no evidence for any cytotoxic mechanism of mammalian-cell damage by the amoebae. It is possible, of course, that the immunological and chemical inhibitors of trophozoite movement and phagocytosis also blocked the release or action of amoeba-associated cytotoxins. However, the demonstrable association between trophozoite feeding activity and cytopathogenicity, seen particularly in studies with cytochalasin B, is in keeping with previous observations (Brown, 1978) and suggests that the amoebae destroy cultured mammalian cells by phagocytosis alone.

**Summary**

Inhibitors of trophozoite motility and phagocytosis were used to investigate the mechanism of *Naegleria fowleri* cytopathogenicity in mouse-embryo (ME)-cell cultures. Amoebae that were immobilised and agglutinated by specific antiserum exhibited no cytopathic activity, although they remained alive and were in constant contact with the ME cells. Mammalian-cell damage occurred only when the organisms recovered pseudopodium function and began to migrate over the monolayers as they overcame the inhibitory effects of the antiserum. Cytochalasin B at a concentration of 10 µg/ml, shown to prevent the engulfment of chick erythrocytes by amoebae, also inhibited the cytopathogenicity of *Naegleria* when incorporated in ME-cell culture medium. Despite
repeated contact with active trophozoites, the ME cells showed only those morphological changes characteristically induced by cytochalasin B itself. The amoebae in turn showed signs of starvation after 3 or 4 days’ incubation, suggesting that the feeding activity of trophozoites was suppressed. Colchicine, on the other hand, inhibited neither the ingestion of erythrocytes nor the destruction of ME cells by amoebae. It was concluded that the cytopathogenicity of N. fowleri in ME-cell cultures was due to physical rather than biochemical or cytotoxic mechanisms and was associated with the phagocytic activity of trophozoites.

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