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Activation of the alternative pathway of complement by *Acanthamoeba culbertsoni*

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SUMMARY

Normal human serum (NHS) contained an amoebicidal property for *Acanthamoeba culbertsoni.* Killing was quantitated by measuring the ability of the amoebae to undergo cell division subsequent to exposure to NHS, and also by microscopical examination. Plasma membrane disruption and extrusion of intracellular components occurred within 5–10 min following exposure to NHS. Adsorption of specific antibody did not remove the amoebicidal activity while heating serum at 56°C/30 min or treatment with zymosan prevented the killing of *A. culbertsoni.* Haemolytic complement was consumed and C3 conversion occurred during the incubation of NHS with amoebae. Killing required the presence of the late complement components. The findings that (a) amoebae were killed in C2 deficient human serum and ethylene glycol tetra-acetic acid (EGTA), but not ethylenediamine tetra-acetic acid (EDTA) treated NHS; (b) haemolytic complement consumption, which occurred by incubating NHS with the amoebae, could be prevented by addition of EDTA, but not EGTA and (c) conversion of C3 occurred in the presence of EGTA, but not EDTA, indicated that activation of the alternative pathway of complement was involved. This may be of importance as a natural defence mechanism in humans against *A. culbertsoni* infections.

Keywords acanthamoeba lysis complement alternative pathway

INTRODUCTION

Meningoencephalitis caused by free living amoebae may be divided into two main types; firstly that resembling fulminating bacterial meningitis, described as a well defined, acutely fatal disease and caused by a single species, *Naegleria fowleri* (Carter, 1972; Thong, 1980) and, secondly, that characterized by focal granulomatous brain lesions. This is a subacute or chronic infection, and the organisms responsible are believed to be mostly *Acanthamoeba* species (Martinez, 1981).

Acanthamoeba meningoencephalitis has been shown to occur in chronically ill and debilitated individuals, or those undergoing immunosuppressive therapy (Martinez, 1980). These clinical findings have suggested that acanthamoeba is predominantly an 'opportunistic' pathogen. At least one of the reasons why opportunistic pathogens fail to infect normal, healthy individuals is because of their capacity to activate the alternative complement pathway. It is quite probable that this primordial defence mechanism may be responsible for natural resistance to free living amoebae. Results from the present study demonstrated that acanthamoeba was killed by normal human serum (NHS), an event which involved complement activation by the alternative pathway.

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MATERIALS AND METHODS

Amoebae. The amoebae used in these experiments were purchased from the American Type Culture Collection (Rockville, Maryland, USA) as *Acanthamoeba culbertsoni* strains A-1, A-5, and HN-3. Strain HN-3 is now believed to be *A. rhysodes* (Culbertson, 1971; Daggett *et al.*, 1980). The amoebae were maintained axenically in trypticase soy broth in LUX 75 cm^2, 250 ml tissue culture flasks at 25–27°C. When required for experimental purposes, the amoebae were prepared from cultures by agitating the flasks, centrifuging the cell containing medium (200*g*/5 min) and washing the amoebae in trypticase soy broth. The cells were resuspended to the required concentration in Hanks' balanced salt solution (HBSS) supplemented with 0·5 mM $MgCl_2$ and 0·15 mM $CaCl_2$.

Sera. Normal human serum (NHS) was prepared from healthy volunteers and stored at −70°C for the duration of the experiments. Serum completely lacking C2 was obtained from patients genetically deficient in this component of complement (Thong & Ferrante, 1978; Ferrante & Thong, 1979; Thong, Simpson & Müller-Eberhard, 1980). C6 deficient serum was prepared from rabbits genetically deficient in C6, obtained from the Institute of Medical and Veterinary Science, Adelaide, South Australia.

Adsorption of serum with zymosan or amoebae. NHS was incubated with zymosan (2 mg/ml) at 37°C for 30 min. Zymosan was removed from the serum by centrifugation at 300*g* for 15 min and millipore filtration (0·22 μm).

To remove specific antibody, NHS was repeatedly adsorbed with amoebae until no more specific antibody could be detected using the indirect immunofluorescence technique. Each adsorption step was carried out at 4°C for 45 min. The amoebae were removed as described for zymosan. Haemolytic complement activity was retained following adsorption of specific antibody.

Amoebicidal assay. The amoebicidal reaction was carried out in screw-capped culture tubes (16 × 125 mm, LUX Scientific Corporation, California, USA). To a total of 1×10^5 amoebae was added the required serum concentration in a total volume of 1 ml. The suspending medium was HBSS (with $MgCl_2/CaCl_2$). The tubes were incubated with rotation at 37°C/45 min unless stated otherwise. At the end of the incubation, 5 ml of trypticase soy broth was added to each tube, the amoebae pelleted by centrifugation (400*g* for 5 min), the supernatant decanted, the procedure repeated and finally fresh trypticase soy broth (5 ml) added to the amoebae. Following resuspension, the amoebae were re-incubated with rotation at 37°C/48 h for A-1 and 28°C/192 h for A-5 and HN-3. The numbers of amoebae were determined by a haemocytometer count. The percentage of amoebae killed during the amoebicidal reaction was calculated as follows:

$$\% \text{ killed} = \frac{\text{No. of amoebae in control} - \text{No. of amoebae in test}}{\text{No. of amoebae in control}} \times 100$$

Control = medium only; Test = serum.

Measurement of CH_{50}. The CH_{50} units were measured essentially by the method of Gewurz & Suyehira (1976). Briefly, sensitized sheep red blood cells (SRBC) were incubated with the various dilutions of serum at 37°C for 30 min. Lysis was estimated by measuring spectrophotometrically the haemoglobin released.

In the measurement of haemolytic complement in experiments involving complement consumption by amoebae in the presence of EDTA or EGTA, additional Ca^{++} (0·9 mM) and Mg^{++} (3 mM) were added to the serum after incubation.

Conversion of C3. To a total of 1×10^7 amoebae (pelleted by centrifugation) was added 1 ml of either NHS, NHS + 5 mM EDTA or NHS + 5 mM EGTA/0·35 mM Mg^{++}. After incubation at 37°C for 2 h the amoebae were removed by centrifugation and the serum millipored (0·22 μm filter). Conversion of C3 to C3i was measured by crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed using essentially the method of Clarke & Freeman (1968). Briefly, samples were electrophoresed into 1% agarose at 5 V/cm for 4 h and then into agarose, containing goat anti-human C3 (Cappel Laboratories, USA), at 2 V/cm for 18 h.

Transmission electron microscopy. Amoebae were collected by centrifugation and fixed in 3%

(vol./vol.) glutaraldehyde in 0·1 M sodium cacodylate buffer with 0·002 mM $CaCl_2$, pH 7·2 for 2 h, followed by 1% OsO_4 in 0·1 M sodium cacodylate buffer and dehydrated by 10 min changes in a graded ethanol series. After several changes in 100% ethanol, the material was infiltrated with low viscosity epoxy resin (Spurr, 1969), and then polymerized overnight at 65°C. Ultra-thin sections were cut with a diamond knife on a Reichert Ultracut ultramicrotome. Sections were collected on copper grids, stained with uranyl acetate and lead citrate, and examined in a JEOL JEM 100 C transmission electron microscope.

RESULTS

Amoebicidal activity of normal human serum

A. culbertsoni strain A-1 was killed when incubated in NHS. Within 10 min greater than 80% of the amoebae were killed (Fig. 1). Serum dilutions of 1/32 were effective in causing greater than 80% killing (Fig. 2). Similar results were obtained with strains A-5 and HN-3 (Figs 1 & 2).

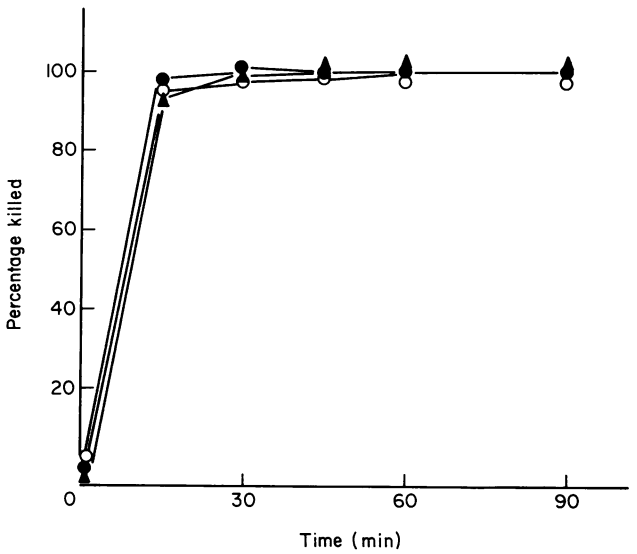

Fig. 1. Killing of *A. culbertsoni* by NHS in relation to incubation time. A-1 (○); A-5 (●); HN-3 (▲). Results are expressed as mean of five experiments each conducted in triplicate.

The events leading to amoeba death were observed by both light and electron microscopy. Within minutes of incubation in NHS, the trophozoites of A-1 showed cytopathogenic changes and disruption of the plasma membrane (Fig. 3). Further incubation resulted in complete extrusion of cellular components (Fig. 4). These changes were absent in amoebae reacted with NHS which had been heated at 56°C/30 min (Fig. 5).

Complement consumption by amoebae

The killing of amoebae by NHS was most likely caused by complement fixation by the amoebae. Thus, fixation of complement by A-1, A-5 and HN-3 was examined. The amoebae, in varying concentrations, were added to NHS and incubated at 37°C for 2 h. Following incubation, the amoebae were removed by centrifuging at 400*g* for 5 min and the supernatant assayed for haemolytic complement activity. Approximately $1–4 \times 10^6$ amoebae were capable of fixing 50% of available CH_{50} (Fig. 6). Under these conditions addition of *A. culbertsoni* A-1 to normal human serum produced conversion of C3 to C3i (Fig. 7).

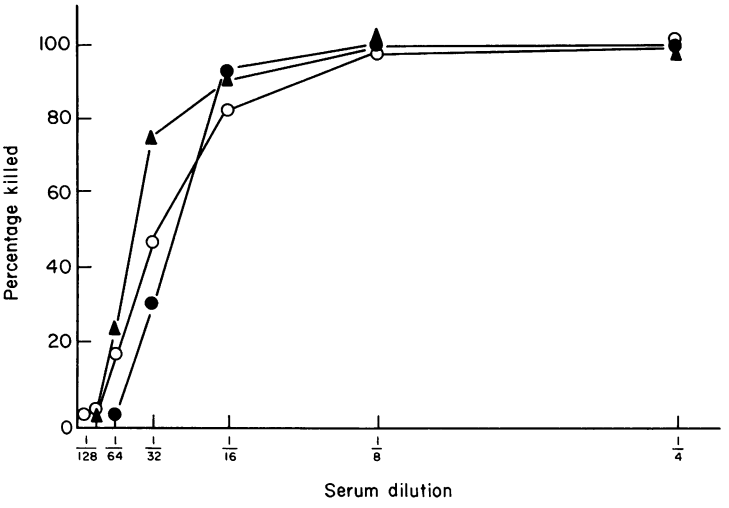

Fig. 2. The degree of amoebicidal activity in NHS. Incubation time was 45 min at 37°C. A-1 (○); A-5 (●); HN-3 (▲). Results are expressed as mean of five experiments each conducted in triplicate.

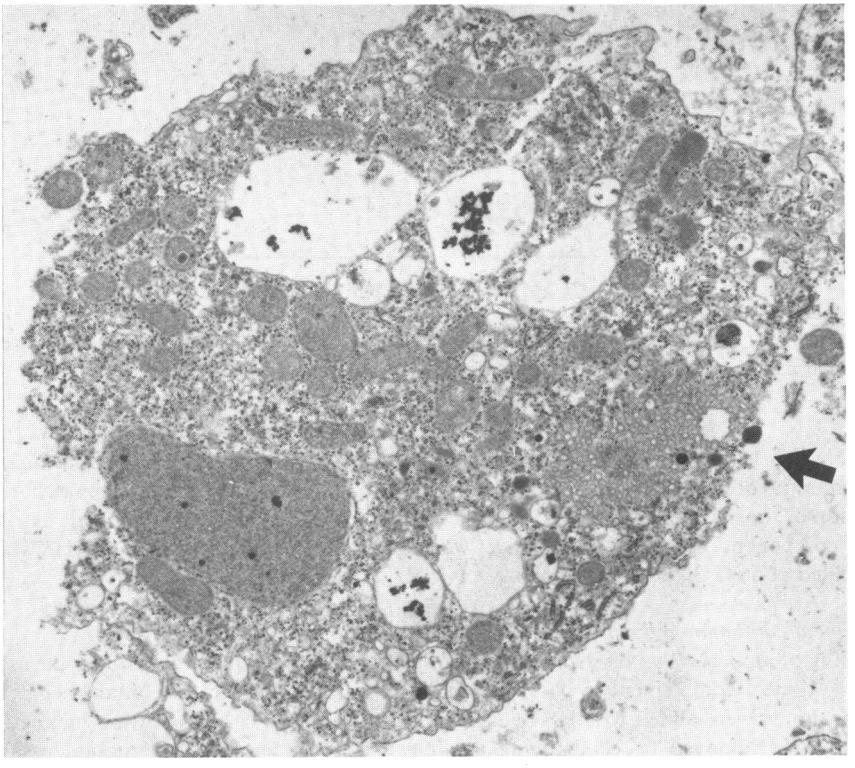

Fig. 3. *A. culbertsoni* A-1 incubated in NHS for 5 min. Shows cytopathogenic changes and damage of the plasma membrane (←); compare this with Fig. 5. Magnification ×6,900.

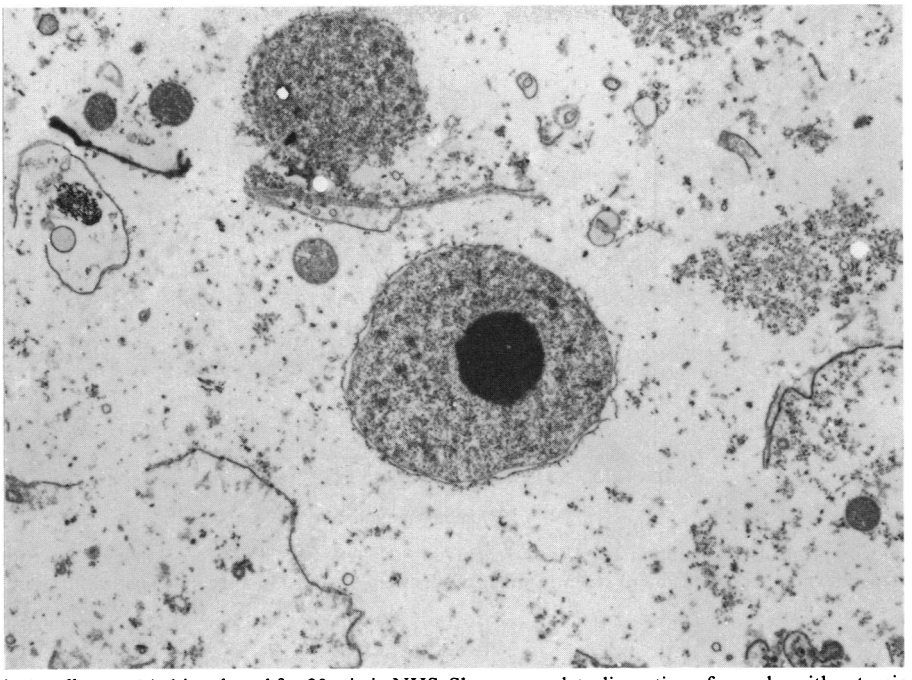

Fig. 4. *A. culbertsoni* A-1 incubated for 20 min in NHS. Shows complete disruption of amoeba with extrusion of nuclei and other cellular components. Magnification × 6,700.

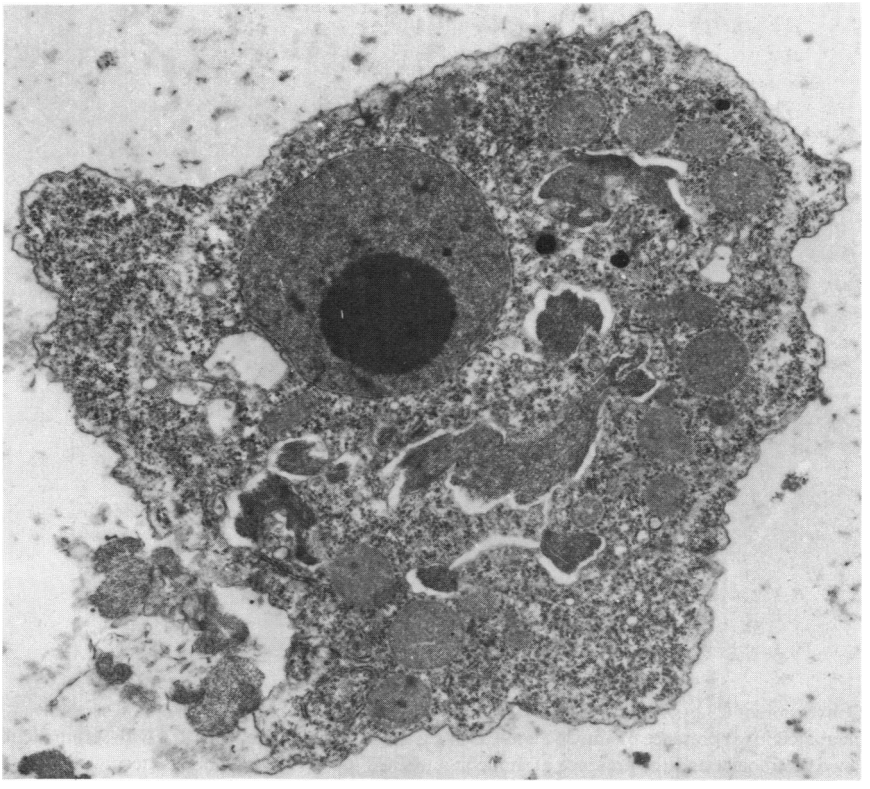

Fig. 5. *A. culbertsoni* A-1 incubated for 20 min in heated (56°C/30 min) NHS. Shows an intact amoeba. Magnification × 10,000.

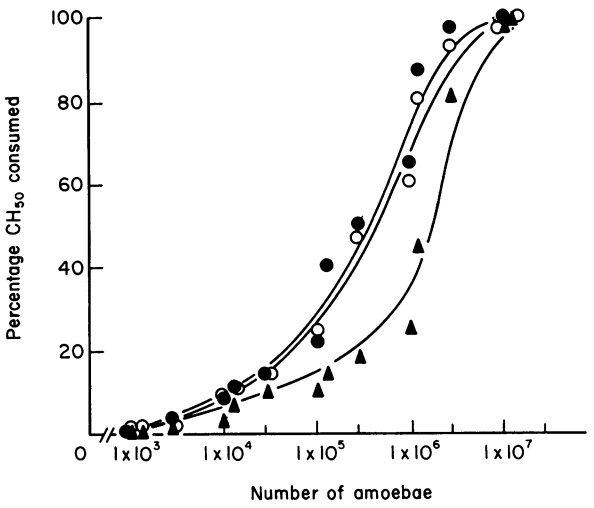

Fig. 6. Complement consumption by *A. culbertsoni* A-1 (○); A-5 (●); HN-3 (▲). Amoeba concentration represents the number of cells/ml of serum. Similar results were obtained in a second experimental run.

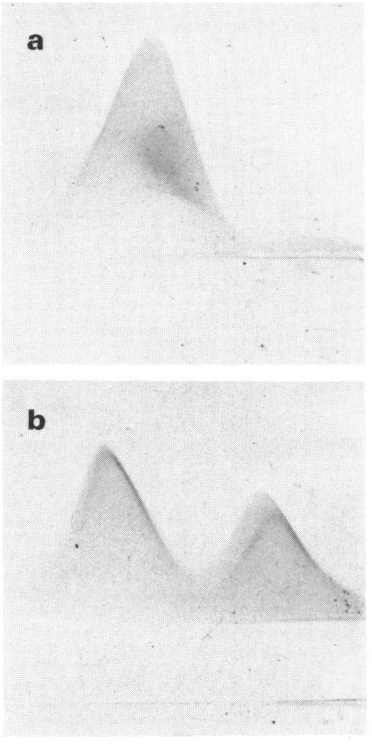

Fig. 7. Conversion of C3 by *A. culbertsoni* A-1 as measured by crossed immunoelectrophoresis. Normal human serum incubated in (a) presence of amoeba and EDTA; a similar pattern was obtained with serum incubated in the absence of amoeba and (b) presence of amoeba and EGTA/Mg^{++}; a similar pattern was obtained with serum incubated with amoeba only.

Table 1. The role of the alternative complement pathway on amoebicidal activity of NHS

Treatment of normal human serum	Percentage of amoebae killed*		
	A-1	A-5	NH-3
None	99·7	98·6	98·6
56°C/30 min	0	0	0
Zymosan	1·2	6·5	7·2
Amoeba adsorbed	99·7	98·1	96·8
EDTA, 5 mM	4·0	7·9	3·5
EGTA, 5 mM	98·8	96·6	94·2
EGTA, 5 mM + $MgCl_2$, 0·35 mM	99·4	98·5	95·4
C2 deficient human serum	99·1	98·6	98·6
Normal rabbit serum	48·9	54·6	50·0
C6 deficient rabbit serum	0	0	0

* Final serum concentration used was 25% for human and 50% for rabbit. Results are expressed as mean of three experiments each conducted in triplicates.

Alternative pathway activation of complement

Heat-inactivating NHS at 56°C for 30 min or pre-treatment with zymosan completely abolished the amoebicidal activity (Table 1). The late complement components were essential for amoebicidal activity; rabbit serum genetically deficient in the C6 complement component in contrast to normal rabbit serum was devoid of amoebicidal activity. Chelation of both Ca^{++} and Mg^{++} ions by addition of EDTA prevented the amoebicidal reaction of NHS. However, preferential chelation of Ca^{++} by addition of EGTA or EGTA/Mg^{++} had no effect on the amoebicidal activity. Human serum completely lacking the C2 component of complement, was also effective in killing the amoebae. In addition, adsorption of NHS with the respective amoebae strains did not remove the amoebicidal activity.

Table 2. The effect of EDTA or EGTA on the ability of acanthamoeba to reduce haemolytic complement

Treatment of normal human serum	Number of CH_{50} units consumed*		
	A-1	A-5	HN-3
Number of CH_{50} available	80·1	77·4	77·8
None	0	0	0
Amoeba	>78·1	70·1	59·8
Amoeba + 5 mM EDTA	8·1	7·1	10·1
Amoeba + 5 mM EGTA	>78·1	70·5	68·9
Amoeba + 5 mM EGTA + 0·35 mM $MgCl_2$	>78·1	70·5	69·1

* The results are expressed as the mean of two experiments each conducted in duplicate.

Haemolytic complement consumption by the amoebae was prevented by addition of EDTA but not EGTA or EGTA/Mg^{++} (Table 2). In addition the results showed that the conversion of C3 to C3i by *A. culbertsoni* A-1 occurred in the presence of EGTA/Mg^{++}, but not EDTA (Fig. 7).

DISCUSSION

A. culbertsoni strain A-1 was killed when incubated in NHS, with plasma membrane disruption and extrusion of intracellular components being apparent within a few minutes of incubation. The late complement components were required; killing of amoebae occurred in normal rabbit serum, but not in C6 deficient rabbit serum. During the event of amoebae killing, haemolytic complement was consumed. The amoebicidal activity of NHS was prevented by heating the serum at 56°C/30 min or treatment with zymosan. Complement activation by the classical pathway requires both Ca^{++} and Mg^{++} and hence may be inhibited by EGTA which preferentially chelates Ca^{++} while the alternative pathway may proceed as the requirement is for Mg^{++}, but not Ca^{++} (Fine *et al.*, 1972). However, EDTA which chelates both Ca^{++} and Mg^{++} blocks also alternative pathway activation. Results from the present study showed that EDTA, but not EGTA inhibited the amoebicidal activity of NHS. In addition, the ability to consume haemolytic complement of NHS was markedly reduced by EDTA, but was not affected by EGTA. C3 conversion in serum incubated with the amoeba, A-1, was prevented by EDTA, but not EGTA. Human serum which was completely deficient in the second component of complement (C2) was also effective in killing *A. culbertsoni.* Repeated adsorption of NHS with amoebae at 4°C did not remove the amoebicidal activity indicating that specific antibody is not required. The results outlined demonstrated that *A. culbertsoni* A-1 was able to activate the alternative complement pathway. Similar results were obtained with respect to a non-virulent strain of *A. culbertsoni*, A-5 (Culbertson, 1971) and HN-3 classified as *A. rhysodes* (Culbertson, 1971; Daggett *et al.*, 1980), showing that the property of alternative pathway activation is probably widely distributed amongst the *Acanthamoeba* species and indeed is not related to non-pathogenic properties.

In vivo, acanthamoeba may be killed either by membrane damage as a result of the concerted action of C5, C6, C7, C8 and C9, or by phagocytic cells following opsonization by C3b. Because of the larger size of the amoeba the latter event appears to involve a cytotoxic reaction (Ferrante, in preparation), as previously described for *N. fowleri* (Ferrante & Thong, 1980). A survey of acanthamoeba infections showed that some were associated with a background of systemic disorders; lymphoma, diabetes mellitus, skin ulcers, chronic pulmonary diseases and chronic alcoholism (Martinez, 1980). Thus infections and subsequent haematogeneous spread of acanthamoeba, which can lead to a non-treatable meningoencephalitis, may occur as a consequence of abrogation of the complement system and/or phagocytic cells (Sneiderman & Wilson, 1975).

N. fowleri, which causes an acute meningoencephalitis also activates the alternative complement pathway (Rowan-Kelly, Ferrante & Thong, 1980; Holbrook *et al.*, 1980). Infection results from instillation of contaminated water into the nasopharynx and amoeba migration to the brain by penetration of the nasal mucosa (Carter, 1972; Martinez *et al.*, 1973). Haematogenous spread of the amoeba has not been observed in human cases, and is believed to be due to amoebicidal factors in serum generated by complement activation by the alternative pathway (Carter, 1970; Ferrante, 1981).

In this manner, both *N. fowleri* and species of *Acanthamoeba* fail to spread haematogeneously, and infection in healthy individuals by naegleria appears to be only a consequence of a selective invasion route, while that by acanthamoeba is due a compromized immune system.

Phospholipids of appropriate composition in the form of liposomes can activate the alternative pathway of complement (Richards *et al.*, 1979) and thus it is not surprising that alternative pathway activation may be a widespread property of plasma membranes. Sialic acid on the surface of plasma membranes acts to prevent alternative pathway activation by increasing the affinity for C3b of β1H relative to B, so that formation of the alternative pathway convertase is blocked and in this way particles may be spared from this natural host defence mechanism. In addition, other mechanisms may operate to prevent alternative pathway activation. For example, the glycoprotein coat on the

plasma membrane of African trypanosomes prevents alternative pathway activation by masking components of the underlying plasma membrane (Ferrante & Allison, 1983). The lack of sialic acid in acanthamoeba membranes (Korn & Olivecrona, 1971; Stevens, 1977) and the absence of material (coat or capsule) external to the plasma membrane (Bowers & Korn, 1968) is consistent with the observed property of alternative pathway activation by the amoeba.

Whilst under certain conditions complement activation contributes to host protection, under others it is responsible for pathogenesis of parasitic diseases as a result of generation of mediators of inflammation. The latter may in part explain the oedema and damage to blood vessel walls which are characteristic features of acanthamoeba and naegleria infections.

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