spectrum in the range 235 to 235·9 MHz which may be due to the overlap of the other two lines. The measurements have all been made at 77° K, using lock-in detection. Further investigations to resolve the details of this spectrum are under way.

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ULTRASTRUCTURAL STUDY ON THE FATE OF SOME SPECIAL CYTOPLASMIC INCLUSION IN PATHOGENIC *NAEGLERIA AEROBIA* (SINGH & DAS, 1970)

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ABSTRACT

Naegleria aerobia Singh and Das[1] cause primary amoebic meningo-encephalitis in man and animals. The morphological changes at the ultrastructural level associated with *N. aerobia* growing in mouse brain and on its subsequent *in vitro* culture have been reported. The major differences include the staining characteristics of mitochondria under these conditions and the appearance and disappearance of some special inclusions (black bodies) observed in the cytoplasm of these amoebae during growth *in vitro*. That the rough endoplasmic reticulum plays an important role in the gradual digestion of the black bodies has been noticed and demonstrated in the figures. The nature of these bodies and their role are discussed.

INTRODUCTION

NUMEROUS membrane bound special inclusions, observed as black bodies, in the cytoplasm of *N. aerobia* from freshly isolated infected mouse brain tissue or from infected human cases have been reported[2–6]. These bodies have not been reported in amoebae maintained in stock and used for infecting mice or in amoebae cultured from infected mouse brain and passaged through repeated subcultures *in vitro*. This communication deals with the fate of these black bodies during prolonged cultivation of the amoebae *in vitro* from brain tissues.

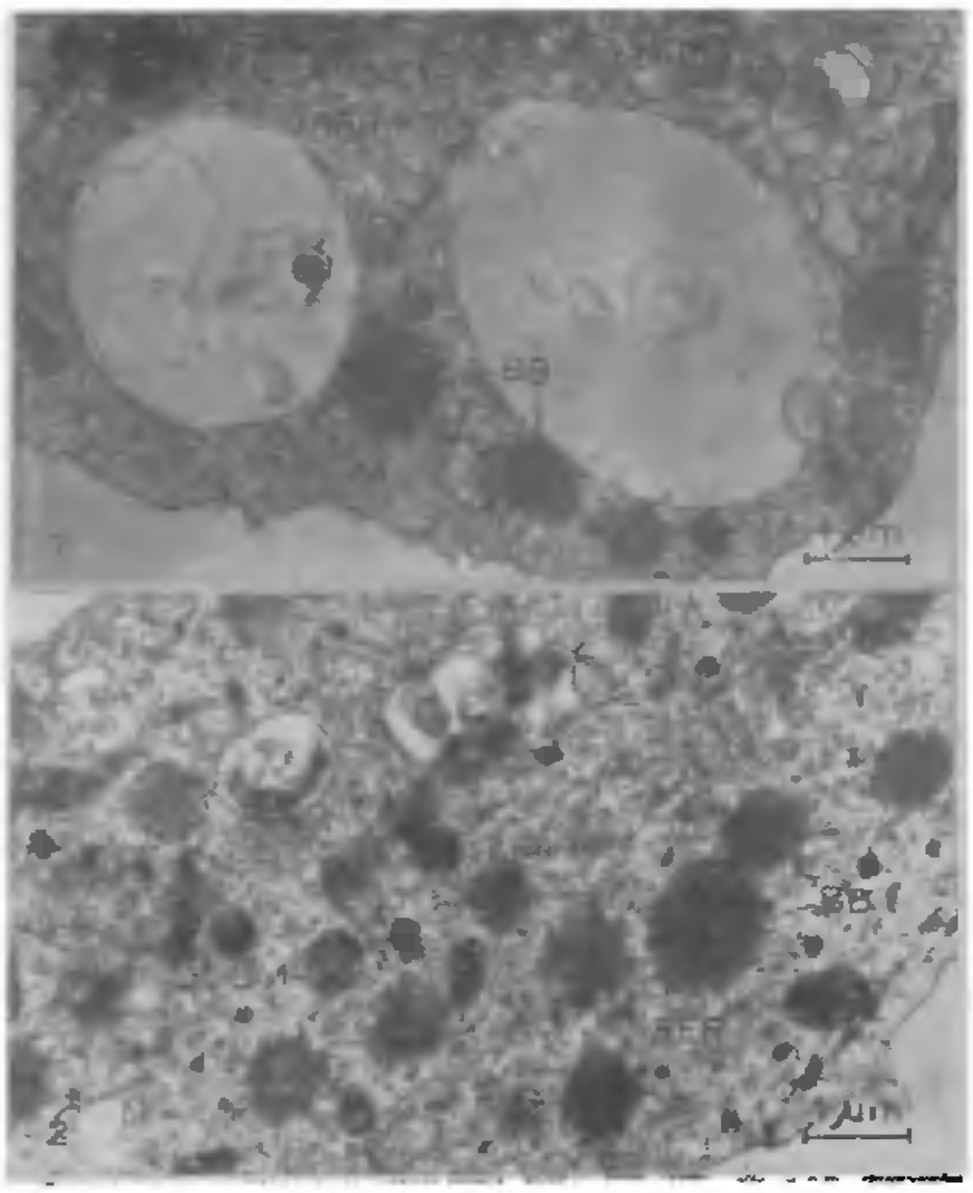
MATERIALS AND METHODS

The methods for culturing *N. aerobia* and infecting mice were carried out in a manner similar to those described by Das, 1977; Maitra, *et al.*[1,2]). A small portion of brain from sick mice was teased in a small quantity of distilled water (37° C) and a hanging drop preparation was made to demonstrate the amoebae. Large number of petri dishes were then smeared with *Escherichia coli* and seeded with the infected brain material. Subculture of these plates was made at every 24 h intervals. The amoebae were fixed for electron microscopy in a manner described below. The areas on a plate showing heavy bacterial growth and the pieces of brain tissue was scraped off and the plates were flooded with fixative containing 5% glutaraldehyde in 0·1M cacodylate buffer with 1 mM calcium chloride (pH 6·8). The amoebae which were on the margins of the streak were scraped and collected in centrifuge tubes, washed once by centrifugation (500 rpm) for 5 min and the sediment resuspended in fresh fixative for 5 h and the samples were centrifuged at 5000 rpm for 10 min to get a pallet. They were then washed 6–8 times during a period of 8–12 h in 0·1 M cacodylate buffer with 4·5% sucrose (pH 6·8) and fixed in Caulfield's[8] 1% osmium tetroxide at 4° C for 2 h. The amoebae were washed once in distilled water and kept in fresh 1% aqueous uranyl acetate overnight. The tissue was then dehydrated in graded alcohol or acetone and embedded in epon-araldite mixture (Mollenhouer[9]). Brains of mice showing symptoms after *N. aerobia* infection were fixed by intracerebral inoculation of fixative, the brain removed and processed in the manner described above. Silver sections were cut on LKB Ultrotome III using a glass knife. Sections were mounted on copper grids

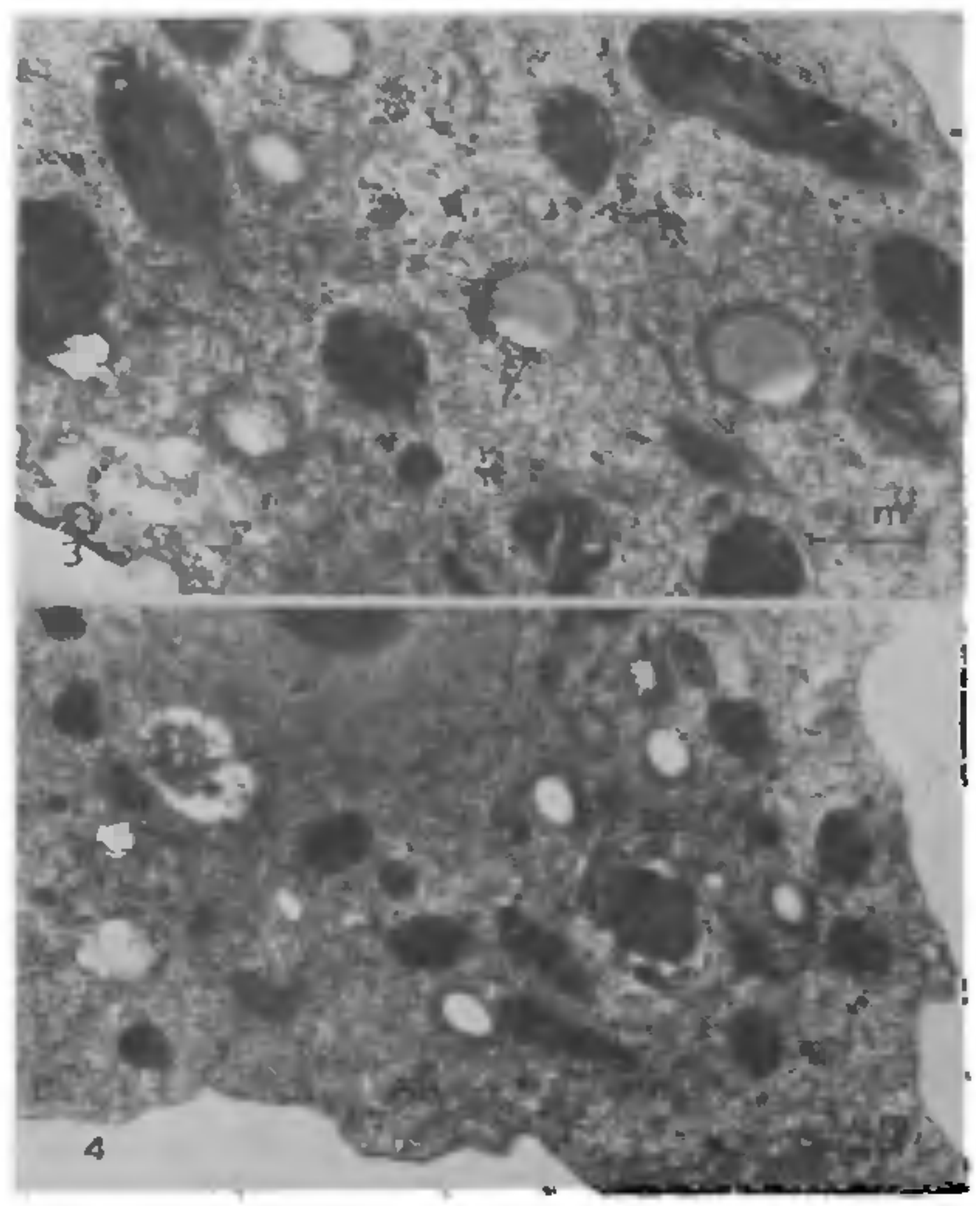
and stained in 1% aqueous uranyl acetate at 60° C for 20 min and lead citrate (Reynolds[30]) at 4° C for 5 min. Observations were made with a Hitachi HU-11E electron microscope with an accelerating voltage of 75 kV and photographed on Fuji electron microscopic orthochromatic sheet film.

Results

The ultrastructural characteristic of *N. aerobia* in infected mouse brain was similar to those described earlier[2,11]. The amoebae were more or less rounded in appearance with less pronounced pseudopodia than the amoebae maintained in cultures *in vitro*. The cytoplasm was less compact in the former than the latter trophozoites with most of the free ribosomes aggregating to form polyribosomes. A majority of mitochondria were lightly stained with the outer membrane and cristae being clearly visible. Both pinocytic vacuoles and "myelinated" food vacuoles could be seen indicating the mode of ingestion of food by both pinocytic and phagocytic processes. Smooth and rough endoplasmic reticulum was clearly seen in the amoeba cytoplasm. A conspicuous inclusions (black bodies) were seen inside the cytoplasm of amoebae taken from the infected brain tissue, but no such inclusions could be observed in amoebae used for the infection of animals. These inclusions were numerous membrane bound electron dense spherical bodies (Fig. 1). Subcultures of amoebae from infected mouse brain at 24 h intervals over non-nutrient agar with *E. coli* as food, displayed two distinct changes at ultrastructural levels. (1) The mitochondria show densely staining property and this characteristic persisted in amoebae observed at different periods during the study (Figs. 2-6). (2) The black bodies in amoebae at 24 h after culture in association with *E. coli* over non-nutrient agar plate appeared to be surrounded by rough endoplasmic reticulum (Fig. 2). These bodies were completely surrounded by the rough endoplasmic reticulum when examined in amoebae fixed at 48 h. Simultaneously, a new phenomenon of lysis of the black bodies becomes evident (Figs. 3, 4) This process of digestion mainly occurred from a point in the circumference of the black body and the area of clearing progressed inwards till the whole of the black body disappeared (Figs. 5, 6). This process can be compared to the stages in the moon during its

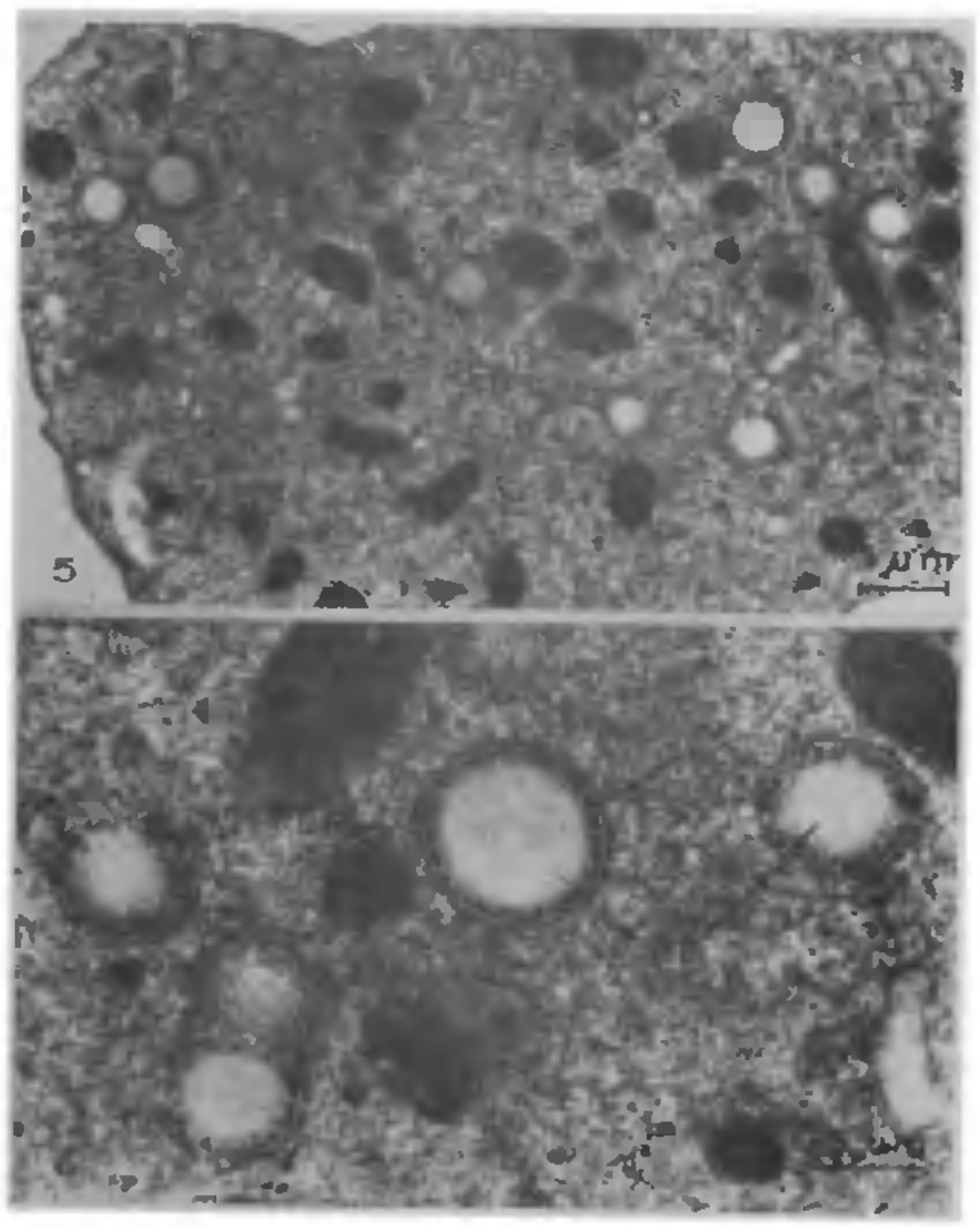


FIGS. 1–2. Fig. 1. Section of infected mouse brain showing part of an amoeba trophozoite (*Naegleria aerobia*) with mitochondria (M), rough endoplasmic reticulum (RER), pinocytic vacuole (PV). Note the spherical black bodies (BB) free from rough endoplasmic reticulum lying all over the cytoplasm. Fig. 2. Portion of an amoeba (*N. aerobia*) from *in vitro* culture 24 h after mouse brain passage showing densely staining mitochondria (DM). Note the close association of rough endoplasmic reticulum (RER) around the black bodies (BB).



FIGS. 3–4. Fig. 3. Portion of an amoeba (*N. aerobia*) from *in vitro* culture 48 h after mouse brain passage showing the complete encircling of rough endoplasmic reticulum (RER) around the black bodies (BB). Some clearing of the black bodies are also evident at this stage (arrow). Fig. 4. Part of an amoeba (*N. aerobia*) from *in vitro* culture 48 h after mouse brain passage showing advanced stage of lysis of the black bodies (BB).

waning period The leaching process at times has also been observed to begin at all areas on the surface of the black body and the process continued inwards till the black body disappeared. At 72 h, a greater clearing of black bodies was evident and a membrane surrounding the black body was clearly visible (Fig. 6) at this stage. Most often these appeared as vacuoles and showed no staining reactions with osmium tetroxide, lead citrate and uranyl acetate (Figs. 5, 6). The amoebae after subsequent subcultures were comparable in their ultrastructural morphology with those of the parent strain used for infecting the mice.



FIGS. 5–6. Fig. 5. Part of an amoeba (*N. aerobia*) from *in vitro* culture 72 h after mouse brain passage showing electron transparent black bodies (BB) after complete digestion. Note the ring of rough endoplasmic reticulum (RER) still exist around BB. Fig. 6. Part of an amoeba (*N. aerobia*) from *in vitro* culture 72 h after mouse brain passage. Note the presence of the membrane (arrow) surrounding the digested black bodies (BB) inside the ring of rough endoplasmic reticulum (RER).

DISCUSSION

There is reason to believe that the black bodies in the cytoplasm of *N. aerobia* freshly isolated from brain tissue are in the nature of stored energy material. This is evidenced by the rapid digestion of these bodies mediated through the rough endoplasmic reticulum when these amoebae are cultured *in vitro* over non-nutrient agar plates supplied with *E. coli* as food for the amoebae. In no case have these bodies been found to be excreted as such from the cytoplasm of these amoebae either in brain tissue or in culture.

It is interesting to note that while cysts of *N. aerobia* have not been reported in tissue of infected animals, yet the presence of reserve energy material in the cytoplasm of the amoebae is baffling. This may be explained on the basis of the fact that *N. aerobia* growing in an abnormal environment in the brain tissues of infected animals are exposed to complex organic materials resulting in the formation of substances that are stored in vacuoles inside the amoeba cytoplasm. Lees and Korn[12] have shown that *Acanthamoeba castellanii* can incorporate high levels of fatty acids. No harm to amoebae have been observed in such situations. Similar findings have been reported in the case of anaerobic *Entamoeba invadens*, a parasitic amoeba from reptiles[13]. In these studies it has been reported that the fatty acid composition of phospholipids is a reflection of the quantitative and qualitative occurrence of the fatty acids in the medium. If the example cited above in the case of aerobic free-living *A. castellanii* and the anaerobic parasitic *E. invadens* have a generalization for the order Amoebida, it can safely be assumed that the black bodies in the cytoplasm of *N. aerobia* freshly isolated from infected brain tissues are a reflection of metabolism of unnatural substrates by these amoebae. The return to normal culture conditions is witnessed by the rapid loss of these bodies from the cytoplasm of these amoebae. It is possible to believe that the black bodies in *N. aerobia* are not toxic materials and have no role to play in the disease processes caused by these amoebae.

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