PHAGOCYTOSIS OF *TRYPANOSOMA BRUCEI RHODESIENSE* BY PERITONEAL MACROPHAGES: A STUDY BY SCANNING ELECTRONMICROSCOPY

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SUMMARY. Phagocytosis of *Trypanosoma brucei rhodesiense* by peritoneal macrophages takes place by seizure of the trypomastigote by either end but usually by the anterior. A lamellar sheath similar to that seen in phagocytosis of the promastigote of *Leishmania* is observed, but it is smaller and does not proceed to envelop the living parasite. The attached trypomastigote becomes pitted and appears to have been killed and partially destroyed before it is completely engulfed.

INTRODUCTION

Studies by light microscopy on the uptake of *Trypanosoma brucei* by macrophages (Levaditi and Mutermilch, 1910; Lumsden and Herbert, 1967) have been concerned primarily with the dynamics of phagocytosis and the conditions under which it takes place. Stevens and Moulton (1978), using transmission electronmicroscopy (TEM), reported complete engulfment of the trypomastigote. Scanning electronmicroscopy (SEM) does not appear to have been used previously to study the phagocytosis of *T. brucei* although the related parasite *Leishmania tropica* has been the subject of SEM studies by Zenian, Rowles and Gingell (1979) and Al-Taqi and Mohammed (1981). There were conflicting reports of the speed of phagocytosis in earlier studies. In the present study by SEM of the phagocytosis of *T. brucei rhodesiense*, our objectives were: (1) to assess the completeness, or otherwise, of engulfment; (2) to observe the surface appearance of trypomastigotes reacted with macrophages in the peritoneal fluid of infected rats and from in-vitro culture, to assess changes during phagocytosis; and (3) to compare the mechanism of phagocytosis of *T. brucei* with that described for other species such as *T. cruzi* and *Leishmania*.

MATERIALS AND METHODS

Preparation of trypomastigotes. *T. brucei rhodesiense*, strain UC/D3/15/180F that had been isolated from man in Botswana (Apted *et al.*, 1963) was used in these experiments. The strain had been preserved by freezing below -70°C and had been passaged seven times since isolation. Trypomastigotes from a donor rat were freed from blood on a column of DE 52 and eluted in

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PSG (phosphate buffer, pH 7.2, with glucose 1% w/v) as used by Lanham (1968), and their normal motility and appearance verified by phase-contrast microscopy immediately before each experiment.

Observations on macrophages and trypomastigotes from infected rats. Wistar rats were infected with 10^6 parasites of *T. b. rhodesiense*. Peritoneal fluid from infected rats was examined by phase-contrast microscopy and filtered through 1.0- μ m polycarbonate "Unipore" membranes held in 13-mm aerosol holders (Bio-Rad Labs, Hollywell Industrial Estate, Watford). Glutaraldehyde 2.5% (v/v) was forced through the membrane with a syringe immediately after the trypomastigotes had been deposited and before they had dried. The membranes were placed in a fresh solution of glutaraldehyde and left overnight.

Experiments with trypomastigotes and cultured macrophages. Normal unstimulated peritoneal macrophages were obtained from outbred TO mice by the method of Stuart, Habeshaw and Davidson (1978) and cultivated as monolayers on coverslips in a tissue culture medium (TCM) consisting of Eagle's minimum essential medium with NaHCO_3 0.11% and fetal-calf serum 10%; the preparation was supplemented with antibiotics (benzyl penicillin 500 IU/ml and streptomycin sulphate 2.5 μ g/ml).

On to each monolayer of macrophages, 10^6 trypomastigotes in PSG were added. Coverslips were removed after 5 min, 15 min, 30 min, 1 h and 2 h, washed twice in TCM to remove unattached cells and deposits, fixed in glutaraldehyde 2.5% and left overnight in the fixative. Control macrophages were fixed in the same way. Control experiments with killed trypomastigotes were also performed. The trypomastigotes were killed by fixing them in glutaraldehyde 2.5% overnight; they were then washed thoroughly in PSG. Fixed trypomastigotes, 10^6 in 2 ml of PSG were added to each monolayer which was then treated as before.

Preparation of membranes and coverslips for SEM. Each preparation was washed in three baths of 0.1M sodium cacodylate buffer and dehydrated by passing through a graded series of alcohols from 50% up to two baths of absolute ethanol in 10% steps with 5 min at each grade. Specimens were impregnated with 1:1 (v/v) ethanol/Arcton (ICI) for 20 min and immersed in a "Polaron" E300 Critical Point Drying Apparatus (Polaron Equipment Ltd, Hollywell Industrial Estate, Watford) with liquid CO_2 . Each membrane or coverslip was mounted on an aluminium stub and shadowed with gold in an argon atmosphere in a Polaron Diode Sputter Coater (E5000). A Jeol JSM Scanning Electronmicroscope (Jeol UK Ltd, Jeol House, Grove Park, Colindale, NW9) was used, operating at 18–20 Kv at 20° angle of tilt.

RESULTS

Phagocytosis by peritoneal macrophages

Fig. 1 shows a trypomastigote from the peritoneal cavity that has not undergone phagocytosis. Observations on macrophages and trypomastigotes that had reacted together already in the peritoneal cavity of the rat showed that some trypomastigotes had been seized by the anterior (flagellar) end (fig. 2) and others by the posterior end (figs. 3 and 4). It was also noted that the cell membranes of the parasites that were in the process of phagocytosis had a pitted appearance; it seemed probable that they were already dead (fig. 2). The results of these observations were the same whatever the phase of infection in the rat.

Phagocytosis by cultured macrophages

Living parasites. No phagocytosis was observed within the first 5 min of interaction. Specimens fixed after 15 min showed the anterior end grasped by the macrophage (fig. 5). Specimens fixed after 30 min of interaction showed more advanced stages of phagocytosis with parts of the body of the parasite already inside the macrophage (fig. 6). After 1 h, more of the trypomastigote had been taken into the

FIGS. 1-4.—Phagocytosis in peritoneal fluid demonstrated by SEM (all $\times 30\,000$). Fig. 1. Normal trypomastigote close to macrophage but not affected by it. Fig. 2. Engulfment of trypomastigote from anterior end—note lines of pitting that follow the general course of microtubules. Fig. 3. Engulfment from posterior end—despite pitting the natural curves of the flagellum and parasite body indicate that the trypomastigote may still be alive. Fig. 4. Late stage of engulfment, only flagellum and anterior extremity protrude from a depression in the surface of the macrophage; note normal trypomastigote unattached to macrophage.

FIGS. 5-9.—Phagocytosis of live parasites by cultured macrophages demonstrated by SEM. Fig. 5. Trypomastigote trapped by anterior end, showing the cap-like protrusion, "lamellar sheath", from the macrophage grasping the parasite which appears still to be motile and undamaged; 15 min interaction ($\times 11\,200$). Fig. 6. Further engulfment of trypomastigote after 30 min; note the pitting that appears to have damaged the parasite severely ($\times 30\,000$). Fig. 7. Engulfment of parasite by the posterior end after interaction for 1 h ($\times 15\,000$). Fig. 8. Engulfment of parasite by posterior end showing lamellar sheath, after interaction for 1 h ($\times 15\,000$). Fig. 9. Higher magnification of Fig. 8 showing detail of parasite grasped by lamellar sheath ($\times 30\,000$).

FIG. 10.—Phagocytosis of live parasites by cultured macrophages demonstrated by SEM. Trypomastigote grasped by macrophage but not drawn into it, showing advanced destruction of parasite ($\times 22\ 000$).

FIGS. 11–14.—Phagocytosis of dead parasites, fixed with glutaraldehyde and washed before use, demonstrated by SEM. Fig. 11. Posterior end of trypomastigote held by macrophage; note ruffling of macrophage membrane indicating a normal process of phagocytosis and the absence of pitting on the surface of the fixed parasites ($\times 15\ 000$). Fig. 12. Anterior end held by macrophage ($\times 45\ 000$). Fig. 13. Anterior end of parasite held by modified lamellar sheath ($\times 16\ 500$). Fig. 14. Engulfment of dead parasite almost complete within 15 min ($\times 15\ 000$).

macrophage although phagocytosis still remained incomplete (figs. 7–9). Little additional information was obtained from observation of material fixed after 2 h (fig. 10).

The following general observations were made: (1) all parasites attached to macrophages were disfigured or damaged while most of the parasite was outside the macrophage (figs. 7–10) in a similar manner to those obtained from the peritoneal cavity of the rat (figs. 2–4); (2) interaction between parasite and macrophage appeared to be controlled by the latter, which developed an extension or “lamellar sheath” as described by Zenian *et al.* (1979) (figs 5, 8 and 9); and (3) the posterior end of the parasite could be the point of attachment (figs 7 and 8) but the anterior end was more commonly favoured (figs 5 and 6), and there were occasional instances (not illustrated) in which the process of attachment appeared to start in the middle of the body of the parasite rather than at one of its ends.

Dead (fixed) parasites. Stages in the phagocytosis of dead parasites were similar to those observed with the living, but the rate of uptake was faster and the process more complete; although no phagocytosis was observed during the first 5 min, the process of engulfment was virtually complete within 15 min (fig. 14). Either end of the parasite became attached, and ruffling of the macrophage membrane occurred, as with the living parasite interacting with either peritoneal or cultured macrophages, but no part of the fixed parasite showed signs of damage during phagocytosis in contrast to observations with the living parasite.

DISCUSSION

Our observation that phagocytosis is incomplete within 1 h differs from that of Lumsden and Herbert (1967) who noted that “the speed of phagocytosis was impressive; trypanosomes were found attached to, and ingested by macrophages in large numbers in the first few minutes after their addition to cultures”. In our work no phagocytosis occurred within 5 min, either of living or dead parasites. On the other hand, our results agree generally with those of Stevens and Moulton (1978) on *T. brucei* and of Zenian *et al.* (1979) on *Leishmania*. We do not regard divergence from the results of Lumsden and Herbert (1967) as significant because of the differences in the techniques used and in the criteria for assessing the progress of phagocytosis. Moreover, Stevens and Moulton (1978) pointed out that the degree of phagocytic activity varies with the stage of infection and it is possible that Lumsden and Herbert (1967) used trypomastigotes from a stage of infection when phagocytosis was maximal whereas there was less activity at the stages at which our experiments and those of Stevens and Moulton (1978) were performed; no suggestion has been made, however, that the mechanism of phagocytosis varies with the stage of infection.

TEM involves the examination of thin sections and, unless serial sections through a complete macrophage are studied, it is unlikely to demonstrate with certainty that the phagocytosis of a trypomastigote was complete and that parts of the parasite did not protrude. Stevens and Moulton (1978) describe the degradation of a trypomastigote within the macrophage as taking place in a phagosomal vacuole with which lysosomes had fused; they assumed that the whole process occurred within the body of the macrophage by a mechanism similar to that proposed by Hart and Young (1975) for the destruction of yeast cells by macrophages. Nevertheless the mechanism of

destruction that they put forward is not incompatible with our view that partial destruction of the trypomastigote has occurred before engulfment of the parasite is complete. Our observation of pitting of the live trypomastigote, once it has been grasped by the macrophage, suggests that the actions of the macrophage in dissolving the trypomastigote are not confined to its cytoplasm but that secretion of substances that are actively trypanolytic may also occur from the surface. Because the pitting occurred when living trypomastigotes were grasped by macrophages both in culture and in the rat peritoneum but not when dead trypomastigotes were used, it is concluded that the pitted trypomastigotes were healthy before being grasped by the macrophage and were not moribund before the process began.

Although it may be argued that the mechanism of phagocytosis may be different depending upon whether the macrophage has been sensitised or the trypomastigote subjected to antibodies, the similarity of our results with both peritoneal and unsensitised cultured macrophages, suggests that mechanisms of attachment and dissolution of the trypomastigote do not depend upon sensitisation. Our results, however, give no information on the mechanism of final dissolution of the parasite, which is likely to follow that proposed by Stevens and Moulton (1978).

While the flagellar end of a flagellate is accepted, by convention, as the "anterior end", trypomastigotes on the one hand and promastigotes and epimastigotes on the other, differ in that the kinetoplast is posterior in the former and anterior in the latter. There is little consensus as to how promastigotes of *Leishmania* enter the macrophage. The observations of Pulvertaft and Hoyle (1960) suggested that the posterior end made first contact although subsequent penetration was by the anterior end; this appeared to be confirmed by Akiyama and Haight (1971). Miller and Twohy (1967), however, observed penetration by the anterior end, and Zenian *et al.* (1979) and Al-Taqi and Mohammed (1981) both stated that although attachment and penetration is predominantly by the anterior end, posterior penetration may also occur. Similarly reports on penetration by *Trypanosoma* tend to differ. Dvorak and Schmunis (1972) and Nogueira and Cohn (1976) both reported that trypomastigotes and epimastigotes of *T. cruzi* are attached, in culture, by the posterior end. Liston (1975) and Liston and Baker (1978) stated that epimastigotes of *T. dionisi* enter by the posterior end but that the trypomastigotes enter by either end. As with the trypomastigotes of *T. dionisi*, the blood trypomastigotes of *T. brucei rhodesiense* from Botswana, whether alive or fixed and whether reacting with peritoneal or cultured macrophages, are shown in the present work to be attached by either end although attachment by the anterior end is the more usual; subsequent engulfment of the parasite appears to follow from the site of initial attachment.

Zenian *et al.* (1979) and Al-Taqi and Mohammed (1981) both noted a characteristic "lamellar sheath" that progressed up the flagellum of the promastigote, no doubt controlling its struggles, and gradually engulfing the whole parasite after about 1 h. We also noted a similar sheath (described by Stevens & Moulton (1978) as a pseudopodium) passing up the flagellum (fig. 5), but the phenomenon was not as well defined as in *Leishmania*, presumably because in this parasite the promastigote remains alive and active so that it has to be grasped more firmly by the macrophage for engulfment to be effected. In *T. brucei rhodesiense*, on the other hand, the trypomastigote shows signs of damage even at an early stage of phagocytosis and its death seems to have taken place before the engulfment of the parasite is complete. It is

important, however, to draw the distinction between the phagocytosis of *T. brucei rhodesiense* by macrophages, and the penetration of other types of cell in which the parasite remains alive to emerge later.

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