A MORPHOLOGICAL STUDY OF THE INTERACTION BETWEEN TRYPANOSOMA CRUZI AND RAT EOSINOPHILS, NEUTROPHILS AND MACROPHAGES IN VITRO

COLIN J. SANDERSON
Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, England

AND WANDERLEY DE SOUZA
Instituto de Biofisica, Centro de Ciências da Saúde, Universidade Federal de Rio de Janeiro, Rio de Janeiro, Brasil

SUMMARY

The interaction between trypomastigotes of T. cruzi and purified rat eosinophils, neutrophils and macrophages has been studied. Macrophages induced by dextran, became infected and the parasites multiplied as amastigotes, eventually being released as trypomastigotes into the medium. Eosinophils show an antibody-dependent phagocytosis, while neutrophil phagocytosis is enhanced by antibody. Electron microscopy showed that once inside the granulocyte phagosome, the trypomastigotes transform to amastigotes as a prelude to degeneration. This occurs as material apparently of granule origin accumulates in the phagosome. Eosinophils have the capacity to eject incompletely degenerated amastigotes into the medium. This phenomenon is discussed in relation to previous time-lapse cinematography studies of the lysis of chick erythrocytes by eosinophils, and reports that eosinophils release granule contents by means of a secretory vesicle.

INTRODUCTION

The factors influencing the outcome of infection of a mammalian host by T. cruzi remain largely unknown. Insufficient is known about the interaction between the parasite and the cells of the defence systems to provide information about the mechanisms likely to be active in curtailing infection, or the means by which the parasite can avoid the defence systems to establish a chronic infection. Evidence is accumulating that macrophages can provide a site for the multiplication of the parasite (Nogueira & Cohn, 1976; Milder, Kloetzel & Deane, 1977). Lymphoid K cells which have cytolytic activity in vitro against antibody-coated tumour cells, appear to have no activity against T. cruzi (Sanderson, Lopez & Bunn Moreno, 1977).

The life cycle of T. cruzi consists of 3 main morphological forms. Epimastigotes are the dividing form in the gut of the insect vector. They grow readily in cell-free media, containing haemoglobin, and for this reason have been widely used for in vitro studies. In the mammalian host there is a cycle between the non-dividing trypomastigote in the blood, and the dividing intracellular amastigote form. This cycle can be reproduced in tissue culture; blood trypomastigotes penetrate tissue culture cells and transform to the dividing amastigote form. After a number of generations the amastigotes transform to trypomastigotes and break out of the cell to initiate a new cycle.
Eosinophils and neutrophils have activity in vitro against epimastigote forms of the parasite as measured by the release of \(^{3}H\)-labelled RNA (Sanderson et al. 1977; Lopez, Bunn Moreno & Sanderson, 1978). The present study was undertaken to obtain morphological confirmation of the isotope release cytotoxic data, and to study the sequence of events when trypomastigotes are encountered by granulocytes. It is shown that the trypomastigotes are phagocytosed, and then undergo transformation to amastigotes as a prelude to degeneration. Eosinophils exhibit a curious phenomenon, in which incompletely degenerated parasites are released from the phagosome to the exterior of the cell.

**Materials and Methods**

**Parasites**

Experiments with epimastigotes were carried out with the Y strain of *Trypanosoma cruzi* grown in modified Warren's medium as described previously (Sanderson et al. 1977). The strains Emereledo and Peru were obtained from the London School of Hygiene and Tropical Medicine coded as LUMP 634 and LUMP 1287, respectively. Monolayers of MRC-5 human diploid cells were infected with blood forms from an infected mouse. After 6 - 9 days trypanomastigotes released from infected cells were harvested by centrifugation. The preparations used in these studies were at least 98% trypomastigotes, the remaining parasites were mainly amastigotes, with occasional intermediate forms. The Y strain was obtained from Dr G. A. Schmufiis (Instituto de Microbiologia, Universidade Federal de Rio de Janeiro). Trypomastigotes were separated from infected, defibrinated mouse blood by differential centrifugation.

**Effector cells**

Eosinophils were obtained from either AM-2 rats (kindly supplied by Prof. S. T. Torres, Universidade Federal Fluminense, Niteroi) or from August strain rats (obtained from the National Institute of Medical Research, Mill Hill, England). Both these strains have a high proportion of eosinophils in the peritoneal cavity. Neutrophils and macrophages were obtained from Agus rats (obtained from Bantin and Kingman Ltd, Yorkshire, England) 15 h after an intraperitoneal injection of 5 ml of 5%, dextran (mol. wt. 5 to 40 x 10⁶). Cells were purified by centrifugation on 2-step gradients of Metrizamide (Nyegaard and Co., A/S, Norway) (Sanderson & Thomas, 1977). Metrizamide was first made up as a 35.3% solution in water (300 mosmol) and different densities were prepared by dilution in isotonic phosphate-buffered saline (pH 7.4). Two per cent foetal calf serum and 1.5 μg/ml deoxyribonuclease (type DN-C, Sigma) were added. Centrifugation was carried out at 1000 r.c.f. for 15 min. Eosinophils were purified using a layer of 17.5% metrizamide overlayed with 16.5% metrizamide and the cells in medium layered on top. After centrifugation the top interface containing mainly macrophages and lymphocytes was discarded and eosinophils recovered from the lower interface. The pellet contained mainly mast cells, erythrocytes and dead cells. The process was then repeated to obtain eosinophils of higher purity. Neutrophils and macrophages were separated on Ficoll-Hypaque. Macrophages were recovered from the interface, and the pellet cells containing mainly neutrophils were further purified using 20 and 17.5% metrizamide in a stepwise gradient. The top interface contained mainly macrophages which were discarded, the lower interface contained neutrophils.

**Cytotoxicity assay**

Effector cells (2 x 10⁶), trypanosomes (4 x 10⁶) and antiserum (final dilution 1:20) were mixed in small plastic tubes (Walter Sarstedt Ltd, Catalogue No. 669) in a total volume of 100 μl of medium (RPMI 1640 containing 10 mM HEPES and 10% inactivated foetal calf serum). After incubation at 37 °C for 1 - 4 h the cells were either transferred to a glass slide with the aid of
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a cyt centrifuge (Shandon-Elliot Ltd, London) or the cells were pelleted by centrifugation and fixed for electron microscopy.

Antiserum was obtained 26 days after an injection of approximately $10^6$ blood forms (obtained from mouse blood) of the Esmereld0 strain, into rats. The serum was pooled and was inactivated at $56 ^\circ C$ for 30 min.

**Fixation and staining**

Glass slides for examination by light microscopy were fixed in methanol and stained with Leishman's stain. The pellets were fixed in 2.5% glutaraldehyde in 0.09 M cacodylate buffer, pH 7.2 containing 2mM CaCl$_2$. After 1 h at 22 °C the fixative was removed and replaced by cacodylate buffer. The pellets were stored at this stage, and those prepared in England were transported to Brasil. Subsequently the pellets were fixed in 1% uranyl acetate, dehydrated in ethanol and the pellets embedded in Epon. Sections were examined in an AEI EM6-B electron microscope at the Instituto de Biofísica, Universidade Federal de Rio de Janeiro.

**RESULTS**

**Light microscopy**

The phagocytic activities of granulocytes and macrophages against Esmereld0 strain are shown in Table 1. Similar results were obtained with tissue culture trypomastigotes of Peru strain, and blood trypomastigotes of Y strain. Higher values may be obtained with a greater excess of parasites, but these lower ratios minimize the possible effect of active penetration by the parasites which might distort the phagocytic index. Eosinophils show phagocytosis in the presence of antibody, but very little in its absence. Neutrophils show a significant amount of phagocytosis in the absence of antibody, but the index is increased considerably by the presence of antibody.

**Table 1. Phagocytosis of trypomastigotes**

<table>
<thead>
<tr>
<th>Effector cell</th>
<th>Antibody</th>
<th>1 h</th>
<th>4 h</th>
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<tr>
<td>Eosinophils</td>
<td>+</td>
<td>11-34</td>
<td>7-23</td>
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<tr>
<td></td>
<td>-</td>
<td>0</td>
<td>0-2</td>
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<tr>
<td>Neutrophils</td>
<td>+</td>
<td>14-19</td>
<td>17-25</td>
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<tr>
<td></td>
<td>-</td>
<td>2-9</td>
<td>6-7</td>
</tr>
<tr>
<td>Macrophages</td>
<td>+</td>
<td>32-50</td>
<td>38-44</td>
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<tr>
<td></td>
<td>-</td>
<td>3-4</td>
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* Range of values in 3 experiments.

Trypomastigotes incubated in medium over 24 h showed no tendency to transform to amastigotes or epimastigotes. In all the cell types the parasites inside the cell appeared to be mostly amastigote forms, suggesting rapid transformation from the trypomastigote after uptake by the phagocytes. Many of the parasites inside eosinophils and neutrophils showed only slight staining, and in some only the kinetoplast was visible inside the cells, suggesting degeneration of the parasites. In cultures containing eosinophils plus antibody, a few amastigotes were observed outside the cells. The
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frequency was too high to be accounted for by the amastigotes in the original preparation of trypomastigotes, and too low to alter significantly the different counts, they were swollen and pale, suggesting partial degeneration. It is thought that these amastigotes were expelled from the eosinophils (see below).

Macrophages were allowed to phagocytose Esmereldo strain and then kept in culture. Amastigotes could be seen in the cytoplasm, after 3 days, and after 6 days virtually all of the cells contained large numbers of amastigotes, and trypomastigotes were released into the medium.

Electron microscopy

Eosinophil control preparations without antibody contained only extracellular parasites, whereas neutrophils did show some phagocytosis without antibody, reflecting the results obtained by light microscopy. All the figures are taken from cultures containing antibody.

T. cruzi is easily recognizable by the characteristic kinetoplast, subpellicular microtubules and flagellum with axonemal complex with a 9 + 2 pattern. Trypomastigotes can be recognized by the position of the flagellum which lies outside the body, and by the kinetoplast which consists of diffuse strands (Fig. 2). Amastigotes can be recognized by the almost spherical shape of the body, and the dense disk-shaped kinetoplast. The short flagellum is within the body. Epimastigotes have a kinetoplast similar to that of the amastigotes, and in cross-sections of the parasite, the flagellum is within the body (Meyer & Queiroga, 1960; Rodriguez & Marinkelle, 1970; Sanabria, 1963, 1964; De Souza & Chiari, 1977).

Interaction with eosinophils

Figs. 1-4 show different stages of the interaction between trypomastigotes and eosinophils. Fig. 1 shows trypomastigotes in contact with an eosinophil and Fig. 2 shows 2 trypomastigotes inside an eosinophil. The space between the parasite (right-hand side) and the phagosome membrane is empty. The space between the parasite on the left-hand side and the phagosome wall contains a small amount of electron-dense material (arrow). This electron-dense material is more clearly seen in Figs.

Fig. 1. Trypomastigotes (t) and an eosinophil. Two of the trypomastigotes are in contact with the eosinophil. Incubation, 1 h. ×15,000, bar = 1 μm.

Fig. 2. A trypomastigote cut in cross-section through the region of the nucleus (n), and another with the kinetoplast cut in longitudinal section (k). The arrow indicates the layer of electron-dense material between the parasite and the phagosome membrane. Incubation, 1 h. ×22,000, bar = 1 μm.

Fig. 3. Two trypanosomes inside an eosinophil, the lower one has amastigote morphology with dense flattened kinetoplast (k). Arrows show electron-dense material between parasite and phagosome membranes. Incubation, 1 h. ×22,500, bar = 1 μm.

Fig. 4. Degenerated trypanosome (t) inside an eosinophil. The arrows show the wide band of electron-dense material, between the parasite and the phagosome membrane. The curved arrow shows part of a trypomastigote undergoing phagocytosis. Incubation, 4 h. ×22,500, bar = 1 μm.
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3 and 4, and one of these parasites is clearly of amastigote morphology. Fig. 4 shows a wide band of electron-dense material between a degenerating parasite, and the membrane of the phagosome (arrows).

Figs. 5 and 6 show interactions between epimastigotes and eosinophils. Fig. 5 shows several stages, from contact (top right) to various stages of degeneration within phagosomes. Two structures resembling granules (g) are visible apparently fusing with the phagosome membrane. These granules do not have the electron-dense band of basic protein but consist of granular material similar to that in the matrix of intact granules, which is also similar to the electron-dense material seen in the space between the parasite and the phagosome membrane. In Fig. 6 a phagosome containing an epimastigote appears to have opened to the outside of the cell (curved arrow) and dark granular material seems to be spilling out of the phagosome into the surrounding medium.

Figs. 7–9 show interactions between eosinophils and trypomastigotes at higher magnification. The 2 membranes (parasite and phagosome) are clearly seen in Fig. 7, which appears to be soon after phagocytosis, as there is no granular material in the space between the membranes. A later stage is shown in Fig. 8, with a wide zone of dense granular material (arrows). Two structures resembling granules can be seen fused to the phagosome. As in Fig. 5 the basic protein core is not visible, leaving only the granular matrix material. Fig. 9 shows a group of amastigotes which, at least in the plane of sectioning, show no interaction with eosinophils. All of these amastigotes are covered by a thin layer of granular material similar to that seen in phagosomes, suggesting that they have been taken up into phagosomes and then expelled. It should be noted that the starting preparation of parasites in this experiment contained 99% trypomastigotes, and so it is unlikely that these amastigotes represent part of the population of amastigotes contaminating the original suspension of trypomastigotes.

Interaction with neutrophils

Neutrophils show similar types of reaction as eosinophils to trypomastigotes and epimastigotes. Fig. 10 shows a trypomastigote within a neutrophil in which the membranes of both parasite and phagosome are visible (arrows). Neutrophil granules (g) are smaller and homogenous in comparison with the eosinophil granules. Figs. 11 and 12 show parasites within neutrophils. There is a wide band of granular material between the 2 membranes (arrows), the parasites are of amastigote morphology with...
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vacuoles suggesting degeneration. In Fig. 12 a number of granules can be seen in close proximity to the phagosome.

DISCUSSION

Three strains of T. cruzi have been used in this study, Peru and Y strains cause acute fatal infections in mice, Esmerelo strain causes a subclinical infection in which the animals have a parasitaemia between 7 and 21 days detectable by direct blood examination. The parasitaemia then disappears and the animals remain chronically infected over several months and probably for life (unpublished observations). This pattern is similar to the disease pattern in man, where acute symptoms are relatively uncommon, thus this strain in rodents appears to be a better model for the disease in man than the strains causing acute fatal infections. For this reason, most of the work has been carried out with the Esmerelo strain.

As a comparison with the phagocytosis by granulocytes, macrophages induced by dextran and obtained as a byproduct of the neutrophil purification procedure, have been included. The interaction between T. cruzi and macrophages has been studied by several different groups, and there appear to be differences in the outcome of infection of macrophages by different strains of T. cruzi. For example, Ernestina strain was killed by unstimulated mouse macrophages (Dvorak & Schmunis, 1972) whereas Y, PF and Tulahuen strains survived and multiplied in both normal and thioglycollate-induced macrophages (Nogueira & Cohn, 1976). Milder et al. (1977) found that while the F strain was killed by normal hamster macrophages the Y strain survived and multiplied. It was therefore important to establish the fate of Esmerelo trypomastigotes in macrophages and it was found that they survived and multiplied as amastigotes, eventually being released as trypomastigotes in the medium. With a high number of parasites in the inoculum, virtually 100% of the macrophages became infected.

In eosinophils and neutrophils degeneration occurred with all the strains tested, so that under the light microscope only the kinetoplast remained visible. These studies gave essentially similar results to those obtained by the release of 3H-labelled...
Fig. 10. A trypomastigote inside a neutrophil. The membrane of the parasite with the kinetoplast (k) and the phagosome can be seen (arrows). The electron-dense granules (2 labelled g) can be seen in the vicinity. Incubation, 1 h. × 90000, bar = 0.1 μm.

Fig. 11. A partially degenerated trypanosome (t) of amastigote morphology inside a neutrophil phagosome. A wide band of material can be seen between the parasite and the phagosome membrane (arrows). Incubation, 4 h. × 45000, bar = 0.1 μm.

Fig. 12. A partially degenerated trypanosome (t) of amastigote morphology inside a neutrophil. The material between the parasite and the phagosome membrane is visible (arrows) and 2 granules (g) can be seen apparently entering the phagosome. Incubation, 4 h. × 30000, bar = 1 μm.
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RNA (Lopez *et al.* 1979), in that eosinophil phagocytosis was largely dependent on the presence of antibody. Neutrophil phagocytosis occurs in the absence of antibody, although the reaction is enhanced by antibody.

Eosinophils (reviewed by Butterworth, 1976) contain granules consisting of a granular peroxidase-positive matrix (Cotran & Litt, 1969) and a crystalloid core containing the major basic protein (Lewis, Lewis, Loegering & Gleick, 1978; Brown & Wood, 1978). The electron micrographs suggest the following sequence of events in the lysis of *T. cruzi* by eosinophils. Contact is antibody dependent, and trypomastigotes are phagocytosed. Granule fusion to the phagosome occurs and at this stage the crystalloid core of the granule is no longer visible. There is an accumulation of electron-dense material which appears to have come from the eosinophil granules, in the space between the phagosome membrane and the parasite membrane. Parasites in various stages of degeneration were observed. Although neutrophil granules are less distinctive and without a crystalloid core, the sequence of events appeared to be similar.

An unexpected stage in the degeneration of the trypomastigotes in both eosinophils and neutrophils is the transformation to the amastigote form in the phagosome, which occurs within 1 h of phagocytosis. This observation raises the possibility that this transformation is triggered by the damaging effect of lysozomal enzymes, as there would appear to be little else in the relatively hostile environment of a phagosome.

The mechanism of eosinophil degranulation observed in this study resembles that described by Zucker-Franklin & Hirsch (1964) and Cotran & Litt (1969) in which the granule fuses with the membrane of the phagosome and the granule contents empty into the phagosome. We could find no evidence for granule fusion with other granules or a secretory vesicle, as discussed by McLaren, MacKenzie & Ramalho-Pinto (1977). The rapid rate of eosinophil degranulation (Archer & Hirsch, 1963) makes it unlikely that granule fusion with the phagosome or cell membrane will be observed, unless a lot of material is examined. On the other hand the channels seen by Skinnider & Ghadially (1974) and by McLaren *et al.* (1977) in which electron-dense material of granular origin, was apparently emptying to the outside of the cell, may be due to regurgitation of material from phagosomes. The rapid membrane movement and deformation of eosinophils, seen as repeated phagocytosis and regurgitation of erythrocytes by time-lapse might give the impression of vesicles and channels in fixed preparations. Clearly therefore the difference between secretion via a vesicle and classical granule fusion with a phagosome, followed by regurgitation, is slight and requires the demonstration that the proposed secretory vesicles are different from phagosomes.

Although no evidence is yet available on the biological role of eosinophils or neutrophils in *T. cruzi* infection, and despite the fact that macrophages appear in many cases to support multiplication of the parasite and that lymphoid K cells appear to have little or no activity against antibody-coated parasites, it is encouraging to find that granulocytes provide one system capable of destroying the parasite and are therefore potentially important in the pathogenesis of the infection.
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