STUDIES BY ELECTRON MICROSCOPY OF THE GIANT FORMS OF SOME AFRICAN AND SOUTH AMERICAN TRYPANOSONES FOUND OTHER THAN WITHIN THEIR MAMMALIAN HOST

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Abstract. Giant multinucleate cyst forms of T. brucei group trypanosomes and 2 South American species were found in culture systems and within the insect host cells by electron microscopy. The African stocks within the testes mid-gut cells, an unidentified Brazilian trypanosome within the bug's gut cells and Trypanosoma rangeli within the muscle layers surrounding the bug's salivary gland. The various forms found were similar in that they contained varying numbers of large vacuoles, usually lined by subpleural tubules into which appeared to bud the various organelles seen in normal trypanosomes and which were produced in considerable numbers within the body of these giant forms. These large vacuoles were seen opening to the exterior and liberating what could be new small forms. Sometimes direct budding of new small forms was observed directly from the periphery of the giant form. The possibility that these giant forms may arise from some type of fusing of individual trypanosomes, that they may perhaps give rise to new individuals and that such a process might provide a mechanism for genetic exchange is discussed.

Early this century, when the life cycles of various species of trypanosomes were being considered, interest in multinucleate (giant or "cyst") forms was widespread. Ormerod (1979) has reviewed this early work on these forms found in African trypanosomes in the mammalian host. One of the earliest reports of giant forms within the testes fly was by Noury (1906), who speculated on the question of their being related to possible "male" and "female" forms. Minchin and Thomson (1911) described "echogony-like" forms of Trypanosoma lewisi within insect cells. Robertson (1912) noted multinucleates in the gut of testes flies some two weeks after infected feeds. However, with the realisation that the "male" and "female" forms represented changing forms in the parasites' life cycle, there followed a decline in interest, too, in these "giant or cyst" forms of trypanosomes. Muniz (1977) reported giant forms in T. crui cultures, but little else appeared until Deane and Mildred described, first at the light level (1969) and later (1972) using electron-microscopy, the presence of "cyst" forms in cultures of T. congoense. Jadin and Le Ray (1969) reported similar forms in cultures of T. evansi brucei and T. b. gambiensis at light level. Finally, Otieno et al. (1976) illustrated many giant forms found in the haemolymph of testes whose homoeosomic cavity had been injected with blood forms. These later authors all described similar forms: giant rounded cells containing many nuclei and flagella; and, at EM level, large numbers of further organelles, such as kinetoplasts, mitochondria, granules, etc., were recognised.

In this work, we examine the appearance of multinucleate forms found in three stocks of the T. brucei group during development in the testes fly, in culture and in testes organ cultures, and we compare these with similar forms in the stercorarian trypanosome T. rangeli in Rhodius prolixus and an unidentified trypanosome in the gut of wild-caught R. pictipes. The possible significance of these various forms is discussed.

MATERIALS AND METHODS

Testes flies. Glossina morsitans morsitans pupae were supplied twice monthly by the Testes Research Laboratories of Bristol University and kept at 25 °C in the dark at a relative humidity of approximately 80%. The flies were maintained under similar conditions during their life time and membrane fed (Mow et al. 1977) on synthetic blood meals (Evans 1979) four times a week.

Trypanosomes. The three stocks of African trypanosomes used were:

1. T. brucei (KU 8857)
2. T. congoense (KU 8858)
3. T. gambiense (KU 8859)
RESULTS

In trypanosomes. All three stocks of the T. brucei group produced giant forms within the mid-gut epithelial cells of G. m. moorae (Plate I, Figs. 1, 2). These forms were found whether the flies were fed directly on parasitic mammals (rat or mouse), or fed via synthetic blood meals infected with blood forms or cultured procyclic trypanosomes. The multinucleate trypanosomes were usually located between the nucleus and the cell wall. Pairs of the cells of the anterior mid-gut region. They were not observed in the fly in other tissues, at or on the surface of the alimentary tract. They appeared from 30th day after the first infected feed, using 180 STI procyclic forms in the artificial blood meal; after 24 days following infection with the blood forms of this stock and at this time using procyclic forms of LYMPS 1026; and often after only 15 days using STIB 348T procyclic forms in a synthetic blood meal. In section, these forms were coccobacillae, which could be seen by a unit membrane within which there appeared to be several individual trypanosomes (e.g. Plate I, Figs. 1, 2), the pellicles of each of which were lined with sub-pellicular tubules and contained all the organelles of a normal trypanosome.

In cultures of the trypanosomes. Two organ cultures were studied, whole guts, and head/salivary-gland preparations. These were seeded with procyclic forms grown in EBL medium. Multinucleate forms appeared intracellularly in whole gut cultures after 7 days using both 180TSTI and STIB 348T and also in the surrounding medium (Plate II, Figs. 5, 6). Giant cyst forms (Plate I, Figs. 3, 4; Plate III, Fig. 11; Plate IV, Fig. 12) were found after 2 weeks in whole body cultures salivary gland cultures in the surrounding medium, but not in the trypanosomes. LYMPS 1026 was not used for these organ culture experiments.

In culture. After 4 to 5 days growth in the culture medium all three stocks produced large numbers of giant forms reaching up to 10% of the total population. Their overall size often exceeded 100 μm (Plate III, Fig. 11; Plate IV, Fig. 13) and contained large numbers of the various organelles found in normal trypanosomes, i.e. nuclei (Plate II, Fig. 7; Plate III, Figs. 9, 10) flagella (e.g. as in Plate I, Fig. 8) mitochondria (Plate II, Fig. 8) and kinetoplasts (Plate I, Fig. 4).

At the time that these “cyst forms” appeared other trypanosomes were seen, apparently attached to each other in an unusual way (Plate IV, Figs. 13–15; Plate VI, Fig. 20). This phenomenon is discussed below.

Many giant forms contained vacuoles (Plate II, Fig. 6; Plate V, Fig. 18) which were usually lined with sub-pellicular tubules. From the walls of these vacuoles (or cysta small trypanosome — like organisms appeared to bud (Plate II, Fig. 8; Plate IV, Fig. 13; Plate V, Fig. 17) and these contained all the organelles that are normally present in a trypanosome. Budding also occurred directly from the pellicle of giant forms (Plate III, Figs. 9–11; Plate IV, Figs. 12, 16).

Several giant trypanosomes (Plate I, Fig. 4; Plate V, Fig. 19), whose vacuoles opened to the outside, were noted apparently liberating a number of small trypanosome-like organisms into the surrounding medium.

T. rangeli in R. prolixus. In contrast to the T. brucei giant forms, those of T. rangeli were found in the anterior mid-gut cells, and not within the gut cells. As early as 7 days after an infected feed, giant forms were found among the trypanosomes penetrating into the salivary glands from the hemocoelomic cavity (Plate V, Figs. 21, 22). These forms were present in large numbers, often giving the gland surface the appearance of a miniature "bunch of grapes." They appeared to give rise to large numbers of new individuals which continued their penetration into the parenchyma of the gland. The giant forms were never found within the main body of the gland.

Culture forms of T. rangeli. These were smaller than those found in similar preparations of the T. brucei group, and were mainly cystic. Multiple flagella appeared as with the African form.

Finally, among wild-caught Rhodius picipes, there were found other giant forms (Plate VI, Fig. 23) within the bug's mid-gut cells, these forms contained multiple organelles within vacuoles. These trypanosomes were not positively identified but were probably a species of Megatryptonym.

DISCUSSION

The term giant forms, multinucleate (Robertson 1912), "cyst-like bodies" (Deane and Milder 1966, 1972, Ellis and Evans 1977) or "forms kystiques" (Jadin and Le Ray 1969) have all been used, interchangeably, to describe these large usually vacuolated trypanosomes, sometimes referred to as (e.g. flagella), or whole moving organelles either within, or attached to the original individual.

The first modern explanation offered for these forms were put forward by Deane and Milder (1966) based on their work at light level using cultures of T. congolense. They suggested three possibilities:

i) That feeding epimastigote rosettes, which normally ingest debris, could perhaps take up whole trypanosomes — a form of cannibalism.
ii) That several trypanosomes could penetrate into large dying trypanosomes.
iii) That a complex, probably sexual process was occurring.

By 1972, according to their electron microscopic investigations, they had accepted the last possibility and suggested the following sequence: the epimastigotes fused around their flagellar pocket; that the DNA-containing organelles (nuclei and kinetoplasts with their conjoined mitochondria) underwent repeated division and were perhaps extruded into the large vacuole made up of the “parents” fused flagellar pockets; that the walls of this vacuole became thin and eventually the complete new epimastigotes were liberated to live independently outside the bodies of the “parents” which now disintegrated. These authors did not regard this form of reproduction, presumably involving genetic interchange, as necessarily a common form of reproduction in T.
cor relief, stressing the sexual activity is often only incurred under certain specific conditions, such as changes in the food supply, changes of environment (including hosts) or under the influence of hormonal changes.

Figs. 15, also 13 and 20 illustrate an unusual association between trypanosomes which were seen regularly in cultures of all three African trypanosome stocks used in this work and also in the organ culture preparations. The highly osmophilic material shown in Fig. 15, possibly lipoprotein, is characteristic of these areas of association.

When trypanosomes divide by binary fission, the subpellicular tubes at areas newly separated, are roughly parallel to each other, regardless of the orientation of the microtome section, as in Fig. 14. In Fig. 15 however, the subpellicular tubes in the two trypomastigotes are arranged at right angles to each other, those of T1 parallel to the page surface, those of T2 perpendicular to it. Thus while Fig. 14 could well represent part of the division process of a single trypanosome, such an explanation seems inadequate for Fig. 15, which could be showing part of a fusion process between two mature organisms. The unusual osmophilic material between the two opposed surfaces is closely seen in Fig. 15.

In their paper, Deane and Milder (1972) concentrated on areas looking very similar to those of the sleeping sickness trypanosomes arrowed in Fig. 13 and 20 as being the points of fusion between two trypanosomes. They suggested that this fusion occurred only at the edges of apposed flagellar pockets, and believed that these were the old edges of such pockets. According to their hypotheses, these fused pockets enlarge to produce the large vacuoles in cyst forms.

Figs. 3, 6 and 13 illustrate these vacuoles, the larger ones usually being lined with subpellicular tubules, which are arranged at the same sort of spacing intervals as are found beneath the pellicle of normal trypanosomes. In vertebrate, culture and vector forms of T. brucei group trypanosomes, the normal flagellar pocket membrane is associated with subpellicular tubules and these are grouped together, leaving the rest of the pocket membrane unlined (Ellis and Ormerod, unpublished). These four tubules are continuations of the four found in association with the special area of endoplasmic reticulum (Taylor and Godfrey 1969) lying directly beneath the flagellum. They can be traced from the base of the pocket near the kinocystoplast to the anterior extremity of the trypanosome body. A large vacuole created out of the fusion of two apposed flagellar pockets could therefore only have, initially, these four tubules as part of each trypanosome flagellar pocket to line it. Fusion at other areas, which have regular subpellicular tubules (which are known to proliferate extensively during binary fission) might seem better candidates to produce the large number of tubules necessary to line these very large vacuoles.

While many of the cyst forms seem have several vacuoles, each lined with tubules (Fig. 13), others (Fig. 11) do look as if their single cyst is derived from an enlarged flagellar pocket (in this case fully lined with tubules) into which only flagella seem to have been protruded. In Fig. 13 the largest vacuole also contains only flagella, and is unlined with tubules, while many smaller vacuoles are clearly seen to be fully lined with tubules. Moreover, both those lined with subpellicular tubules, and those without tubules, were followed in serial sections and found to be true vacuoles within the organism and not invaginations of the pellicle.

In general this complex process does not seem to be related to any of the recognised multiple division processes (e.g. rosette formation etc.) known to occur in some, but not brucei group, trypanosomes.

Figs. 1 and 5 show various giant or cyst forms found either in testes fly organ cultures, or within normal midgut cells of infected tsetse. The majority of the "individuals" (if so they be) are all still within an enclosing trypanosome membrane which is quite separate from any membrane originating from the host cell. This arrangement is most clearly shown in Fig. 5 where the cyst forms' enclosing membrane is well away from and not part of any host cell material.

If the cyst forms do produce new individuals they would appear to do so by a process of "budding off" into the vacuole described. All the normal trypanosome's organelles can be found in these budding forms, though often, as seen in Fig. 6 for these, only certain ones may be seen. This "budding" may also occur directly from the trypanosome body to the outside (Figs. 9, 12 and 16), possibly without the creation of the special vacuole. Figs. 4 and 19 appear to show the final liberations of the new individuals from the "parent". Fig. 18 shows a group of these forms outside but still apposed to the body surface, the others just lining the inner surface of the vacuole.

Within the testes fly, cyst forms were not found with the lumen of the gut, not lying beneath the basement membrane of the gut epithelium; they were only seen during the passage of trypanosomes through these gut cells (Fig. 2). These observations suggest that these forms arise only within the gut cells and then could either die off, or give rise to new individuals which then continue their journey as normal mononuclear trypanosomes. Because no dead cyst forms were ever found in the gut cells, it is possible that this last explanation could perhaps be the correct one.

To sum up, there is some evidence that an unusual association between trypanosomes can be seen, most commonly in culture and organ culture preparations. If fusion between trypanosomes does occur it is probably not limited to areas surrounding the flagellar pocket. If the giant forms do result from such a fusion, the large tubulo-lined vacuoles that are seen are not formed from enlarged flagellar pockets alone, since many of the cyst forms contain several such vacuoles. Our work suggests that the interpretation of Deane and Milder (1972) of such vacuole formation does not seem to apply to African trypanosomes. The creation of new individuals, if it occurs, does so by budding into the vacuole. Giant cyst forms may be liberated to the outside by the rupture of the vacuole. Cyst forms, apparently behaving in a similar fashion to those found in the various culture systems were observed in the gut cells of the tsetse during the passage of the trypanosomes across them following penetration from the entoprophitric space.

In culture of T. rangeli, although some giant and multi-organellate forms were found they were unusually far less frequent than those seen in the vacuoles of the different African trypanosomes. It may be that if these forms generally are a response to unfavourable conditions, our cultures of T. rangeli may not have been followed for a sufficient time.

The giant forms found in the bug, exclusively in the outer tissues of the salivary gland, were usually without the large cysts seen so regularly in the salivarian trypanosome. They appear to represent multiple division within an enclosing membrane, rather than a process of budding into vacuoles which was the principal pattern seen in the preparations of the three African trypanosomes. However the cyst forms seen of the unidentified trypanosome in the bug's gut cells (Fig. 23) with its large flagellated vacuoles, looked remarkably like the giant forms of the salivarian trypanosomes examined, both within the testes and in culture.

While all these various giant forms is difficult to assess without any evidence of genetic inter change or nuclear shuffling. For if one suggests that these forms are not dead-end aberrant malformations of a disorganised binary fission process, then the possibility of them being a product of some "sexual" process must be considered. To date, no clear evidence has been produced to indicate genetic interchange between (genetically) marked individuals and populations, though there is some interesting evidence of genetic drift in Africa (Gibson 1970) as seen in widely scattered isolates collected from all over this continent. Also using
isoenzyme studies on various African stocks, Tait (1980) has recently produced further evidence of random mating and recombination among trypanosomes. As this indirect evidence of sexual activity accumulates, it is possible that some of the processes discussed here may eventually be found to be involved.

REFERENCES


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Fig. 1. A giant form in a testes mid gut cell 31 days after emergence of the fly and 22 days after a second infected blood meal. The flagella probably belonging to each of the seven separate trypomastigotes within the giant form are marked "a" with "a'"; "b" with "b'" etc. (x21 400). Fig. 2. A low power of the testes mid gut cell containing the giant form in Fig. 1 (arrowed) showing its location. H = hemocoele. M = microvilli lining the gut lumen. N = nucleus (x28 800). Fig. 3. A section of a giant, or multiple division, form in a testes organ culture preparation (head and salivary gland). A possible new individual is marked within it (thick arrow). The subpellicular tubules lining the vacuoles within this organism are marked with small arrows (x14 270). Fig. 4. A multinucleate form from the same preparation as Fig. 3 also apparently releasing new forms (T). Note the complexity of the lower dividing kinetoplast (K) (x 5 700).

Fig. 5. A giant form in a 7 day old whole testes gut organ culture. The surrounding membrane is arrowed and smaller organisms are marked (T) (x 14 400). Fig. 6. An enlargement of a flagella-filled vacuole from a giant form from a similar preparation to Fig. 5. The subpellicular tubules lining the vacuoles are arrowed. (x 38 800). Fig. 7. A multinucleate form in a 5 day old trypomastigote culture preparation. A normal organism (containing two vacuoles) lies below the giant form (x5 600). Fig. 8. A section of a giant form from a similar culture to Fig. 7 with a form (x) budding into a tubule-lined vacuole. M = mitochondrion. (x 16 800).
**Fig. 9.** A multinucleate form (cultured as above) with a budding form still attached (thick arrow). Other forms apparently detached (thin arrow) are seen nearby (× 3 600). Note the relative sizes of the giant form, the normal trypanosome to its left and the small (arrowed) forms to the right. Fig. 10. A giant form from the same culture as Fig. 9, showing many nuclei. New forms (arrowed) are seen budding out into the medium (cf. Fig. 8) (× 3 600). Fig. 11. A giant form from a tissue culture culture (head and salivary gland) showing the complexity of its structure. A small form budding out into the medium is arrowed. (× 8 650).

**Fig. 12.** An enlargement of a budding structure similar to that seen in Fig. 11, from a similar culture preparation (× 35 700). Fig. 13. A giant form from a culture preparation showing a similarity in shape to those cyst forms demonstrated by Deane and Milder (1972) in T. congoense. The points at which those authors believed the two "parent" trypanosomes were fused are arrowed. The curved arrow marks several budding forms. Flagella are seen within and without the large central vacuoles which are unhinged by tubules, unlike the surrounding smaller vacuoles which have a similar lining to that shown for example in Figs. 3, 8 or 15 (× 6 000). Fig. 14. Two trypanosomes (T1 and T2) in a culture preparation, separated by osmiophilic material, and whose subpellicular tubules (arrowed) run parallel to each other, and therefore probably are the same forms in the process of division (× 30 000). Fig. 15. Two other trypanosomes from this same culture, but with the tubules of T1 (arrowed) running parallel to those of T2 (arrowed arrow). It is unlikely that such an
Fig. 17. Three organisms (T) budding into a tubule-lined vacuole within a cultured giant form. The attachment of one of the budding forms is arrowed (×16 000). Fig. 18. Part of a tubule-lined vacuole within a cultured giant form showing flagella within and without (thick arrow). The thin arrow marks a group of tubules, often found as here shown, within the cytoplasm of a giant form and not directly associated with any membrane system (×21 000). Fig. 19. A cultured giant form apparently liberating small organisms (I) to the exterior by the rupture of the large vacuole. Flagella unassociated with the main tubule-lined vacuole are arrowed (cf. Fig. 18). Normal trypanosomes lie below this multinucleate (×8 250).

Fig. 20. A cultured multinucleate, apparently made up of two individuals (cf. Fig. 13) whose junctions are arrowed. Many nuclei are present as well as 3 small forms (T) (×8 750). Fig. 21. A giant form of T. rangeli in the outer layers of a salivary gland of Rhodius prolixus, with several separate forms (T) together with their associated flagella (cf. Fig. 1) (×30 000). Fig. 22. Two giant forms of T. rangeli lying within the hemocoele/basement membrane complex of a salivary gland of R. prolixus. The outer membrane of the giant forms (arrowed) surround several smaller forms (×9 100). Fig. 23. Giant forms of an unidentified species of trypansome (probably a megatrypanosome) containing many organelles within the vacuoles. The giant forms lie within the midgut cells of a wild caught R. prolixus (×7 200).