THE FORMATION OF FILOPODIUM-LIKE PROCESSES BY *TRYPANOSOMA* (TRYPANOZOOON) BRUCEI

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SUMMARY

Bloodstream forms of *Trypanosoma* (Trypanozoon) brucei were observed in vitro to possess long filaments arising from both the posterior end and the tip of the flagellum, and also to become attached to various substrates, including themselves. The filaments were examined by negative staining and thin-section techniques and found to have a structure resembling filopodia. However, as their functional significance is still questionable, their designation as filopodia must be considered provisional. The possible functions of these structures, such as mediation of attachment, and release of antigenic material, are discussed.

INTRODUCTION

The morphology of trypanosomes is well known from light microscopy and from recent electron-microscope studies (Vickerman, 1962, 1969; Sanabria, 1963; Judge & Anderson, 1964; Anderson & Ellis, 1965; Fuge, 1968). However, examination of living bloodstream forms of *Trypanosoma* (Trypanozoon) brucei has revealed some additional and unexpected structures and activities. This paper presents our observations on the presence of long processes which may arise from either the anterior or posterior extremities of the trypanosomes. As these processes are filamentous protrusions of the trypanosome's pellicle containing a central cytoplasmic core, they resemble filopodia (Kudo, 1966). However, as their functional significance is not completely understood we suggest that provisionally they should be considered as filopodium-like processes.

MATERIALS AND METHODS

Bloodstream forms of *Trypanosoma* (Trypanozoon) brucei Plimmer and Bradford, 1899 were derived from stabilates (Lumsden & Hardy, 1965). These were populations of single antigenic type (ETat 5) set up by the methods described by Lumsden, Herbert & McNeillage (1967) and preserved, as infected mouse blood, in glass capillary tubes at −79 °C (Cunningham, Lumsden & Webber, 1963). All were derivatives, through 4–10 passages in mice, of stabilate TREU 164, 21st-passage material of a strain originally derived from metacyclic trypanosomes obtained from *Glossina pallidipes* Austen at Lugala, Busoga District, Uganda, in 1960. Details of the passage history of that stabilate are given by McNeillage, Herbert & Lumsden (1969).

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White mice from the colony of the Connaught Medical Research Laboratories were infected by a large (about antilog 6 ID₃₀) inoculum, intraperitoneally, and studies were made of the forms composing the bloodstream trypanosome populations 3–5 days later, during the increase and at the peak of the first parasitaemic wave. Mice were bled from the retro-orbital sinus by puncture with heparinized microhaematocrit capillaries.

For study of preparations in vitro the ‘buffy’ layer after haematocrit centrifugation of the capillary samples of blood was examined either directly or after resuspension at room temperature (20–24 °C) or at 37 °C in mouse serum or in Tyrode’s solution (Paul, 1965) or buffered salts solution (Cunningham, Lumsden & Webber, 1963). Living trypanosomes were examined by phase-contrast microscopy in baseline-sealed slide preparations. In order to retard the trypanosomes’ activity, they were suspended in solutions of 2, 4, or 8% methyl cellulose (Dow Chemical’s Methocell) prepared in Tyrode’s solution, pH 7·8. Photomicrographs were taken with stroboscopic flash.

One experiment was conducted to follow the occurrence of filopodia on trypanosomes at various times during the parasitaemia in the host. Six groups of 4 mice each were infected with each group receiving a serial one-tenth dilution of inoculum. Numbers of circulating trypanosomes in each mouse were determined daily by haemocytometer counts. When trypanosomes were detectable, daily capillary samples were taken by retro-orbital sinus puncture and the trypanosomes concentrated by haematocrit centrifugation. An aliquot of trypanosomes was immediately suspended in 4% methyl cellulose-Tyrode’s and examined with phase contrast under 100 x oil immersion. Times were recorded between the end of slide preparation and the observation of the 1st, 2nd, 3rd and 4th trypanosome-bearing filopodia.

Electron-microscope observations were made on trypanosomes either direct from blood or after suspension in physiological salt solutions (as above). They were examined by both negative-staining and thin-section techniques.

Negatively stained preparations were made in 3 ways: (a) by direct suspension with 2% phosphotungstic acid (PTA), pH 7·2, on carbon-Formvar coated grids; (b) by direct suspension with isotonic ammonium molybdate (310 m-osmol); (c) by suspension with 2% PTA after fixation in 5% glutaraldehyde-cacodylate at room temperature for 20 min and washing with Tyrode’s solution. The isotonic staining technique served as a control of osmotic damage to trypanosomes, which does occur in those organisms first exposed to the hypotonic PTA solution. Pre-fixation in glutaraldehyde produced specimens more comparable with those examined by thin sectioning.

Trypanosomes examined as thin sections were treated as follows: they were concentrated by haematocrit centrifugation in capillaries and then extruded directly into 5% glutaraldehyde in 0·1 M cacodylate, pH 7·2–7·4; the plug so formed was cut into small pieces and fixed at room temperature for 20–30 min, then washed for 2 h at ~4 °C with 0·1 M cacodylate containing 0·25 M sucrose, and post-fixed at ~4 °C in 1% osmium tetroxide in 0·1 M cacodylate. Following ethanol dehydration and propylene oxide treatment, the pieces were embedded in Epon-Araldite mixture. Sections were stained with uranyl acetate followed by lead citrate.

RESULTS
In vitro observations

Phase-contrast examination of living trypanosomes in various media revealed the presence of filopodium-like processes (which will be called filopodia in this paper) associated with both the flagellum tip and the posterior end of the organisms (Figs. 1–3). They were most clearly seen on trypanosomes separated from host plasma and resuspended in physiological salt solutions either at room temperature or 37 °C. However, in these media or in plasma, the activity of the trypanosomes prevented filopodia from remaining in focus for adequate observation (Fig. 1). When their activity was retarded by suspension in methyl cellulose, they were easily studied and photographed (Figs. 2, 3). In such preparations, filopodia, sometimes extending to
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about 70 μm long were seen trailing behind trypanosomes moving either anteriorly or posteriorly. When the trypanosome reversed its direction of movement, filopodia trailing behind became appressed to its body, giving the impression of shortening, but were freed from the body if the direction of movement again reversed. Filopodia sometimes occurred simultaneously on both anterior and posterior ends. Clumps of them were seen on coverslips or slides, apparently detached from living trypanosomes (Fig. 4).

Also, trypanosomes became attached to the glass substrate, to particles, or to each other. Frequently, when trypanosomes were attached to the glass substrate by the posterior end, short filopodia spread over the glass. Organisms might attach by either end and, in a few instances, simultaneous attachment by both ends occurred. Trypanosomes adhered to each other, either directly posterior end to posterior end, or with the posterior end of one to the posterior side of another. Electron micrographs of negatively stained trypanosomes apparently attached in the latter manner show filopodia from the attaching trypanosome applied to the surface of the second (Fig. 12). Trypanosomes attached in this way appeared to be firmly adherent as they moved together for some distance through viscous methyl cellulose solutions, often colliding with, but not attaching to, other organisms. Flagella of 2 trypanosomes were also seen to come into contact, and as the trypanosomes separated, intertwined filopodia could be seen stretching between the tips of the flagella. Some also attached posterior end to flagellar tip. Freshly isolated trypanosomes examined *in vitro* often showed intense flagellar activity and attachment was not obvious; however, within a short time their activity decreased and attachment and filopodia could be observed.

Not all trypanosomes produced filopodia. Indeed, one gained the impression from *in vitro* examination that they appeared at varying periods after isolation from the host. Frequently, filopodia were seen after much shorter time intervals in preparations where the host serum was largely replaced by physiological salt solutions. Thus, trypanosomes from the same blood sample, suspended in Tyrode's solution showed many filopodia 15 min after preparation, while none were visible on those suspended in serum 30 min after preparation. In some instances the rate of filopodium formation seemed to depend on the population density of trypanosomes, those in heavy parasitaemias forming them in shorter times than those in lighter parasitaemias on the same day of infection. Some of the variability in the appearance of filopodia may have been due to the examination of very heavy infections produced by massive inocula. This is apparent from the results of the later experiment in which 6 groups of mice were infected with inocula in a serial one-tenth dilution. No mice survived the first wave of parasitaemia, but succumbed when the concentration of trypanosomes reached \(1-2 \times 10^6\) per mm\(^3\) of peripheral blood. In those groups reaching these parasitaemias in the shortest times (3-4 days) filopodia were not as readily seen as in the group in which the parasitaemia developed most slowly (7 days). Indeed, in the latter group, filopodia were seen as soon as a preparation could be examined. Yet, in some mice, there appeared to be an increase in number and length of filopodia with increasing time *in vitro*, indicating further elaboration *in vitro*.

Filopodia have also been seen on uncentrifuged trypanosomes examined directly
in whole blood suspended in methyl cellulose. If large numbers of trypanosomes were crowded into a single slide preparation, many trypanosomes died, producing 'ghosts' in various stages of rupture. When such ghosts formed rapidly, before living forms had produced visible filopodia, no filopodia were evident on the ghosts. Conversely, when ghosts formed only after living trypanosomes had produced visible filopodia, ghosts too showed filopodia. Thus filopodium formation was not related to the death of the animal.

**Fine structure of the filopodia**

Negative staining of unfixed trypanosomes produced specimens in many ways comparable to film preparations classically examined by light microscopy. External morphology was most clearly shown, although some internal structures (e.g. subpellicular microtubules and nucleus) were also visible. Two features were of immediate interest: first, the filopodia which could be seen arising from both anterior and posterior extremities of the trypanosomes; second, the characteristic truncate morphology of the posterior extremity of the trypanosome body.

Filopodia were evident in greatest abundance in preparations made from trypanosomes incubated *in vitro* in physiological salt solutions, although they were also seen in specimens prepared as rapidly as possible after removal from the host (3-4 min delay). They were seen arising from the tip of the flagellum and from the posterior end of the trypanosome (Figs. 5-13).

Filopodia usually appeared uniform in diameter, measuring approximately 50-60 nm, although a few were more variable. Globular thickenings sometimes occurred along their thread-like length (Fig. 11) or at their extremities. Filopodia extended freely from the extremities of the trypanosomes, were clumped along the trypanosome body, or lay free on the supporting film with no attachment to trypanosomes. Branching was seen occasionally in unattached filopodia. Globular extrusions occurred at the flagellar tips of some trypanosomes (Fig. 8), and 'filopodia' sometimes arose from these. In some organisms up to eight arose simultaneously from the posterior end.

Higher-magnification micrographs resolved a fringe-like layer, about 13.5 nm thick, surrounding the filopodia and extending over the surface of the trypanosome flagellum and body as well (Figs. 6, 7, 10, 11). This fringe is interpreted as the external cell coat of the pellicle. The fringe around globular thickenings of the filopodia was thinner, measuring about 7.5 nm (Fig. 11).

The truncate shape of the posterior end of the trypanosome was variable. Observations of living organisms (e.g. Fig. 2), as well as electron micrographs (e.g. Fig. 13), gave the impression that the posterior end was flattened or wedge-shaped, especially in those animals having the broadest, most truncate appearance. Attached pairs of trypanosomes suggested a sucker-like mode of attachment with filopodia extending from the margin of the attached trypanosome (Fig. 12).

The above observations, made from organisms negatively stained with hypotonic PTA solutions, were confirmed in specimens stained with isotonic ammonium molybdate. Filopodia were also seen on trypanosomes negatively stained after fixation in glutaraldehyde, indicating that they are not produced during the staining pro-
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The width of filopodia, however, was greater in pre-fixed than in non-fixed specimens. High-magnification micrographs reveal a peripheral fringe about 9-5 nm thick and an inner core of about 100 nm, the major difference in diameter being determined by the core (Fig. 9). The surface of the body of prefixed trypanosomes was much more irregular than that of non-fixed trypanosomes. This was probably due to stiffening of the pellicle and its subsequent distortion when dried on the carbon-grid substrate.

Thin sections of trypanosomes fixed after in vitro incubation in Tyrode's solution when filopodia were clearly present by phase-contrast examination showed many circular and elongated profiles between sections of trypanosomes (Figs. 14-17). The profiles were bounded by layers characteristic of the trypanosome pellicle (Fig. 17). The dimensions of these profiles agreed closely with those of filopodia in pre-fixed negatively stained preparations. Occasional sections through the posterior end of the trypanosome body showed a finger-like evagination of the pellicle (Figs. 14-16).

DISCUSSION

The filament-like structures seen on living trypanosomes, the processes arising from both anterior and posterior ends of trypanosomes as seen by negative-staining techniques, and the profiles seen in thin sections of trypanosomes are evidently the same structures. Thin sections show these to be evaginations of pellicle and cytoplasmic matrix and indicate their similarity to the form of filopodia. The diameter of these filopodia, however, varies with preparation techniques. Measurements from thin sections and pre-fixed negatively stained specimens, which are more reliable, suggest a diameter of about 100-150 nm. When trypanosomes are negatively stained without prior fixation, the cytoplasmic component of filopodia shrinks or clumps to give thin threads with globular swellings. This results in a thickening of the cell coat in areas of shrinkage and thinning of the cell coat in areas of swelling. Only the thinner dimensions for filopodia as seen in unfixed negative stains were given in an earlier report of these findings (Wright & Lumsden, 1969). The filaments seen by phase-contrast microscopy may be the images of several filopodia lying together. The filopodia of trypanosomes may resemble long filamentous pseudopodia produced by platelets during viscous metamorphosis—a part of the process of blood clotting (MacFarlane, 1961; Marcus & Zucker, 1965). They also resemble stromalytic forms seen on ghosts of mammalian erythrocytes (Baker, 1964, 1967) and membrane projections produced on leucocytes by digitonin treatment (Graham et al. 1967). Although these forms of membrane protrusions are produced under non-physiological conditions (osmotic shocking, or treatment with poisons or surface-activating agents), Baker (1967) has suggested that stromalytic forms of erythrocytes may be related to fragmentation of cells in vivo seen in some pathological conditions. Several features of the structures found on trypanosomes, however, distinguish them from these membrane 'artifacts': (1) their presence in serum and physiological solutions immediately after isolation from the host; (2) their presence in negatively stained preparations of pre-fixed trypanosomes, and in such preparations made with isotonic stain;
their excessive length; and (4) the absence of true stromalytic forms on erythrocytes seen in the same preparations. Similarity in appearance may well be due to similar forces acting on such membrane protrusions; however, the stimulus for formation is more physiological.

Observations on living trypanosomes indicate that formation of filopodia is not concurrent with the death of the organisms. Whether they also occur on trypanosomes in vivo is uncertain. If the increase in parasitaemia in the host was rapid (as produced by massive inocula), filopodia were usually not seen on trypanosomes immediately after isolation but they were formed if the trypanosomes were incubated in vitro in physiological salt solutions. Conversely, if the increase in parasitaemia was gradual, filopodia were seen immediately on isolation (within 1–2 min). The conclusion seems inescapable that bloodstream forms of *T. brucei* are capable of forming extensive filopodia. The surface area of a long filopodium was estimated from one photomicrograph (Fig. 3), as the area of a cylinder, and compared with the surface area of the trypanosome body estimated as a central cylinder plus 2 cones. The filopodium presented a surface area equivalent to 39% of the total surface area of the trypanosome—a significant quantity of pellicle to be produced, and perhaps maintained. This indicates a further feature distinguishing these trypanosome filopodia from erythrocyte stromalytic forms. The trypanosome must presumably actively synthesize this pellicle material, whereas with increasing production of stromalytic forms no new cell membrane is formed and the erythrocyte ‘body’ decreases in size (Baker, 1964).

The functional significance of filopodia is uncertain. The attachment activities of trypanosomes seen in vitro may be mediated by them. Pairs of attached trypanosomes seen in negatively stained preparations suggest this most strongly. Bardsley & Harmen (1969) suggest that fluctuation in peripheral parasitaemia in *Trypanosoma rotatorium* may be due to attachment of the trypanosomes to tissues such as liver, kidney, or heart. Such in vivo attachment could be mediated by filopodia in *T. brucei*.

Filopodia are probably not related to establishment of trypanosomes in the invertebrate vector, as the trypanosomes studied were harvested early in the parasitaemia before ‘short forms’, believed to be infective to the insect vector (Wijers & Willett, 1960), had appeared.

Since the filopodium-like processes are extensions of the trypanosome’s surface, bearing the same cell coat material as the remainder of the pellicle, they have important immunological implications. Detached filopodia, or substances derived from them, may constitute the antigens which trypanosomes are believed to release into the environment, the metabolic antigen of Thillet & Chandler (1957) and the exoantigen of Weitz (1960). In fact it is most likely that filopodia would be formed by trypanosomes during techniques used for antigenic analyses, as the methods usually entail maintenance of trypanosomes for some time at room temperature before tests are performed. Indeed, filopodia have been seen on trypanosomes of the stabilates used as inocula in this study. If filopodia are a source of antigens, their production may be expected to vary with the stage of the infection, depending, perhaps, on the degree of antibody response on the part of the host.

Filopodia may occur on other flagellate Protozoa. The ‘curious’ form of *Trypano-
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soma lewisi, showing a greatly attenuated posterior end, recorded by Strickland & Swellengrebel (1910), may have been a form trailing numerous bunched filopodia. Molloy & Ormerod (1964) observed a 'fibril' associated with the posterior end of Trypanosoma rhodesiense. Honigberg & King (1964) suspected that filopodia were formed by Trichomonas vaginalis; and structures of this sort, about 50 μm long, have been recently observed in T. vaginalis (D. D. Hosie, personal communication).

This investigation was supported partly by National Research Council of Canada Grant A-3757.

REFERENCES


(Received 23 December 1968—Revised 12 June 1969)

Figures 1-4 are phase-contrast photomicrographs of *Trypanosoma (T.) brucei*, taken with stroboscopic flash. Figures 5-17 are electron micrographs.

Fig. 1. Living trypanosome in serum showing a long filopodium arising from the tip of the flagellum (arrow). × 2400.

Fig. 2. Living trypanosome in 4% methyl cellulose-Tyrode’s solution, showing a long filopodium arising from the truncate posterior end and adhering to a particle at its free end. × 1700.

Fig. 3. Living trypanosome in 4% methyl cellulose-Tyrode’s solution; the filopodium arising from the flagellar tip is estimated to be 57 μm long, the trypanosome 23 μm long. × 2100.

Fig. 4. A clump of filopodia in serum, no longer attached to trypanosome. × 2600.

Fig. 5. The tip of a flagellum with 2 long filopodia and a small globular extrusion. × 59 000.

Fig. 6. The posterior end of a trypanosome with 4 long filopodia and 2 smaller globular extrusions. The longitudinal striations seen in the body in this figure and in Figs. 12 and 13 are impressions of the subpellicular microtubules. × 40 800.

Fig. 7. Posterior end of a trypanosome with several irregular filopodia showing the fringe-like cell coat. × 62 700.
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Fig. 8. The tip of a flagellum with a large globular extrusion from which 2 filopodia arise (arrows). × 40,200.

Fig. 9. A filopodium on the posterior end of a trypanosome first fixed in glutaraldehyde and then negatively stained. Note the difference in dimensions of the cell coat and cytoplasmic core as compared with unfixed negatively stained filopodia (Fig. 11). × 97,300.

Fig. 10. The tip of a flagellum with an irregularly shaped filopodium. The fringe-like layer of the cell coat can be seen. × 24,000.

Fig. 11. Higher magnification of 2 filopodia negatively stained without prior fixation. The cell coat is thick in narrow regions of the filopodia and thinner over globular regions. × 146,000.
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Fig. 12. The posterior end of 2 attached trypanosomes. Note filopodia (arrows) extending over the surface of the other trypanosome. × 22,000.

Fig. 13. A bluntly truncated posterior end of a trypanosome with several short, globular extrusions of pellicle (arrows). × 28,500.

Fig. 14. Section through the posterior end of a trypanosome showing the origin of one filopodium and a cross-sectioned profile of another (arrow). × 91,200.

Fig. 15. Oblique section through the posterior end of a trypanosome, showing the base of 1 filopodium and oblique sections of 2 others (arrows). × 72,200.

Fig. 16. Section through the posterior end of a trypanosome which has just grazed the cell coat extending over the left-hand filopodium. A base of a filopodium may be indicated at the right-hand corner (arrow). × 73,600.

Fig. 17. Cross-section through a filopodium, showing the cell membrane and cell coat similar to that of the pellicle of adjacent trypanosome bodies. × 211,000.