Trypanosoma brucei spp. development in the tsetse fly: characterization of the post-mesocyclic stages in the foregut and proboscis

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SUMMARY

Post-mesocyclic development of *Trypanosoma brucei* in the tsetse fly in its migration from midgut to salivary glands, was revisited by sequential microdissection, morphometry and DNA-cytofluorometry. This development started by day 6 after the infective feed, with passage of mesocyclic midgut trypomastigotes through proventriculus and upward migration along foregut and proboscis to the salivary gland ducts. Kinetics of salivary gland infection showed that colonization of the salivary glands by epimastigotes occurred only during the time-limited presence of this developmental phase in the foregut and proboscis. Post-mesocyclic trypanosomes in the foregut and proboscis were pleomorphic, with 4 morphological stages in various constant proportions and present all through from proventriculus up to the salivary gland ducts: 67% long trypomastigotes, 27% long epimastigotes, 4% long epimastigotes undergoing asymmetric cell division and 2% short epimastigotes. Measurements of DNA content demonstrated a predominant tetraploidy for 67% of these trypanosomes, the remainder consisting of the homogeneous diploid short epimastigotes and some long epimastigotes. According to the experimental data, the following sequence of trypanosome differentiation in the foregut and proboscis is proposed as the most obvious hypothesis. Incoming mesocyclic trypomastigotes (2N) from the ectoperitrophic anterior midgut start to replicate DNA to a 4N level, are arrested at this point, and differentiate into the long epimastigote (4N) which give rise, by an asymmetric cell division, to 2 unequal, diploid daughter cells: a long, probably dead-end long epimastigote and a short epimastigote. The latter is responsible for the epimastigote colonization of the salivary glands if launched at the vicinity of the gland epithelium by the asymmetric dividing epimastigote.

Key words: *Glossina*, *Trypanosoma brucei*, life-cycle, post-mesocyclic stages, differentiation, ploidy.

INTRODUCTION

African trypanosomes are haemoflagellate protozoa which are the causative agents of sleeping sickness in humans and 'nagana' in cattle. These parasites exhibit a 2-phase life-cycle alternating between the blood-feeding tsetse fly (*Glossina* spp.) and the mammalian host. As compared with the other African salivarian trypanosomes, *Trypanosoma brucei* spp. has the most complex developmental cycle throughout the alimentary tract of the tsetse fly. Morphogenetic events at the initial and terminal phase in the vector are well documented, i.e. colonization of the midgut by transformation of ingested bloodstream forms into multiplicative procyclic trypomastigotes and, metacyclogenesis in the salivary glands where epimastigotes, attached to the gland epithelium, represent a second multiplicative stage and undergo differentiation into the final metacyclic trypomastigotes, infective to the mammal (Tetley & Vickerman, 1985; Vickerman, 1985; Tetley *et al*. 1987; Vickerman *et al*. 1988). In contrast, few data are available about the intermediate phase, corresponding chiefly to the migration of *T. brucei* from midgut through proventriculus to salivary glands, apart from early descriptions by Bruce *et al*. (1911), Robertson (1913), Taylor (1932), Lewis & Langridge (1947) and, more recently, Steiger (1973). Even the pathway the trypanosomes follow to reach the salivary glands is not well documented. However, this migratory phase may be of major biological significance for the completion of the parasite developmental cycle in the tsetse vector. Indeed, it has been shown that the latter depends on the ability of the trypanosome to get through 2 successive developmental barriers: colonization of the midgut and, establishment in the salivary glands (Harmsen, 1973; Le Ray, 1989). These two barriers are independent of each other

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and in most cases trypanosome development is blocked at the procyclic (midgut) phase if established. The present work focuses on the characterization of the post-mesocyclic migratory stage in *T. brucei* development in the tsetse fly, i.e. its kinetics, its morphological stages and its biological significance, as a prerequisite to the study and understanding of molecular determinants controlling the maturation of procyclic trypomastigotes into the metacyclic trypomastigotes.

MATERIALS AND METHODS

Tsetse flies

Male flies from the *Glossina morsitans morsitans* colony maintained at ITMA (Institute of Tropical Medicine, Antwerp) were used throughout the study. This colony originated from pupae collected in Kariba (Zimbabwe) and Handeni (Tanzania). A description of its rearing history, started in 1967, can be found in Elsen, Van Hees & De Lil (1993). This colony is maintained on rabbits at 25 °C and 65 % relative humidity and its characterized by a high intrinsic vectorial capacity.

Trypanosomes

A highly transmissible *Trypanosoma brucei brucei* strain was used, derived from the stock *T. b. brucei* EATRO 1125 AnTAR 1 (Le Ray *et al*. 1977). This pleomorphic strain was cyclically transmitted by tsetse after which it was mechanically passaged by syringe for 17 times. All infection experiments started from this P17 cryostabilate.

Infection of tsetse flies

Teneral flies, 8–32 h after emergence, were fed their first meal on immune-suppressed NMRI mice (cyclophosphamide (Endoxan®), 80 mg/kg, given intraperitoneally the day before trypanosome inoculation) from day 5 to day 7 post-inoculation. At this time, mice showed a parasitaemia of $10^{8·4}$–$10^{8·7}$ parasites/ml of a pleomorphic trypanosome population containing at least 70 % short stumpy forms. Only fully engorged flies were retained. After the infective meal, flies were fed on uninfected rabbits 5 days a week until 48 h before dissection.

Collection of trypanosomes from the tsetse

To study the progression of trypanosome development within the fly, microdissection was carried out at a 2-day interval starting from day 2 after the infective meal until day 24. For each experimental point, the parasite content of at least 20 flies was examined. Trypanosomes were collected from the different sites of the alimentary tract of the tsetse fly by dissection in the following sequence: (i) salivary glands, (ii) posterior midgut, (iii) anterior midgut near proventriculus (part of the gut between mycetome and the proventriculus), (iv) foregut and (v) proboscis. The dissected organ was immediately transferred into a drop of phosphate-buffered saline (PBS, 10 mM, pH 7·4 at 20 °C) on a glass-slide and prepared for direct microscopical examination (phase contrast, × 400), counting and smear. To monitor the time of invasion of the foregut, a separate batch of flies was forced to produce a drop of fluid by gentle squeezing of the fly thorax (forced regurgitation). This method allowed us to observe the presence of parasites in the anterior alimentary canal (foregut and proboscis) avoiding contamination with ectoperitrophic midgut trypanosomes. To quantify the midgut trypanosome population during development, entire tsetse) midguts were placed individually into an Eppendorf tube containing 150 μl of PBS. Guts were gently crushed with a Teflon pestle and released parasites were counted in a Thoma haemocytometer.

Infectivity test for mouse

As soon as trypanosomes were observed in the salivary glands, the resulting salivary gland homogenate was inoculated intravenously into a immune-suppressed mouse. Infectivity tests were performed from day 8 to day 18 by daily individual dissection of 10–15 flies.

Morphology and morphometric study

Parasites were air-dried on a glass-slide and fixed for 5 min in methanol. Trypanosomes were stained with Giemsa solution during 30 min and examined by light microscopy (× 1000 magnification). Photographs of the parasites at this magnification compared to that of a graticule allowed manual measurement of the length of the parasite's body + flagellum with an accuracy of ± 0·7 μm.

Quantitative DNA analysis (*ploidy*)

For both flow and image cytometry as well as for *in vitro* cultivation (see below), trypanosomes were collected in Cunningham's medium (Cunningham, 1977) by aseptic dissection: (i) from posterior midgut, a pooled trypanosome population of mainly procyclic trypomastigotes ($n = 1 \times 10^6$ parasites), (ii) from anterior midgut, a pooled trypanosome population of mesocyclic trypomastigotes ($n = 1·5 \times 10^6$ parasites), (iii) from foregut and proboscis, a pool of 5×10^4 parasites could be obtained by forced regurgitation. Metacyclic trypanosomes were purified from minced salivary glands, dissected 21 days after

infection, by elution on a DEAE-52 column (Lanham & Godfrey, 1970) and 3×10^5 metacyclic trypomastigotes were collected from 41 pairs of infected salivary glands.

Bloodstream forms harvested from an infected, immune-suppressed mouse at day 8 after infection served as the trypanosome population of reference. This bloodstream population consisted of 98 % short stumpy trypomastigotes. These forms have been described as diploid cells arrested in the G1-phase of the cell cycle (Shapiro *et al*. 1984).

Flow cytometry

Prior to fixation, trypanosomes were washed 3 times in ice-cold Hanks buffered salt solution HBSS (without Ca^{2+} or Mg^{2+}, addition of 1 mM EDTA). After the last centrifugation, trypanosomes were resuspended in 30 μl of HBSS, and ice-cold 70 % ethanol/5 % glycerol (v/v) was added stepwise under continuous vortexing. Final concentrations of fixed parasites ranged between 1×10^5 and 1×10^6 parasites/ml. Fixed parasites were stored at −20 °C until DNA measurement. Parasites were stained using the DNA stain YOYO-1 iodide (Molecular Probes Inc.) which is a very sensitive and highly specific stain for DNA, and which is very stable with negligible bleaching. Its fluorescence yield is proportional to the DNA concentration. It has already been used for flow cytometric analysis of mammalian cells, suspension of chromosomes and marine prokaryotes (Hirons *et al*. 1994; Marie *et al*. 1996). To our knowledge this is the first time YOYO-1 iodide has been applied to protist cells. Fixed parasites were centrifuged and incubated in 200 μl of Tris-EDTA buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7·2)/ 0·1 % Nonidet P-40 containing 30 μg/ml trypsin during 10 min at room temperature. Afterwards, 150 μl of TE buffer with 500 μg/ml trypsin-inhibitor and 50 μg/ml ribonuclease A were added. After 10 min at room temperature, 150 μl of TE-buffer were added containing the YOYO-1 iodide. For the latter, a final concentration of 200 nM was obtained in a total sample volume of 500 μl. Parasites were incubated at 37 °C during 20 min. Afterwards, stained samples were stored at 4 °C and protected from the light until measurement. Fluorescence intensity from 2000–20000 cells was measured, digitized and stored after excitation at 488 nm using a Becton-Dickinson FACScan flow cytometer. Fluorescence signals from debris and aggregated cells were gated out using pulse shape analysis.

Image cytometry

Trypanosomes were collected in a drop of saline on a slide and air dried for 30 min. Then, parasites were fixed with ice-cold acetone during 15 min. After fixation, slides were stored at −20 °C until staining. The DNA of the parasites was stained with Hoechst 33258 (Sigma) (Loontiens *et al*. 1991). Slides were incubated during 30 min at 4 °C in a 25 μg/ml dye solution in PBS, washed 6 times with PBS, and covered with a slip mounted with 50 % glycerol. They were kept in the dark at 4 °C until analysis. Fluorescence microscopy was performed with a Leitz Ortholux II at a magnification of ×600. To start analysis, nucleus and kinetoplast were set on focus within 2 sec and the excitation light was turned off. After 1 min, excitation light was again turned on and the fluorescent nucleus and kinetoplast were immediately photographed (Kodak professional black & white, 400 ASA, 4″ illumination). All negative films were developed in the same developing bath to avoid intensity differences due to variable environmental conditions.

Negative images of nucleus and kinetoplast (seen as dark grey/black spots on a pale background) were digitized by scanning at a resolution of 1400 dpi (Sharp JX-330) and then analysed using the Pharmacia Software Imagemaster Elite 1D programme. This analysis allowed quantification of the fluorescence intensity of nucleus + kinetoplast as a 'volume', calculated as the summation of pixels defining the spot multiplied by their respective optical density. This fluorescence intensity is proportional to the amount of DNA present in the nucleus + kinetoplast.

Incubation in culture medium

In an attempt to cultivate vitro different trypanosome stages from the fly and to test their capacity for multiplication with reference to the procyclic trypomastigote stage, the following experiment was carried out. Trypanosomes ($n = 3 \times 10^4$) collected from the posterior midgut, anterior midgut and foregut + proboscis respectively, were transferred into a macrophage culture plate well containing 700 μl of Cunningham's medium supplemented with 20 % heat-inactivated FCS, and 140 units penicillin and 140 μg streptomycin. The plate was kept at 26 °C and growth and motility were daily examined during a 10-day period. This experiment was performed twice on trypanosomes collected from separate batches of infected flies.

RESULTS

Development of T. brucei *in the tsetse fly*

On the average, 80 % of the flies from the various experimental batches (Fig. 1) developed an established midgut population of *T. brucei* within the first 6 days after taking the infective feed. In 68 % of the flies, trypanosome development progressed through the proventriculus towards foregut and proboscis

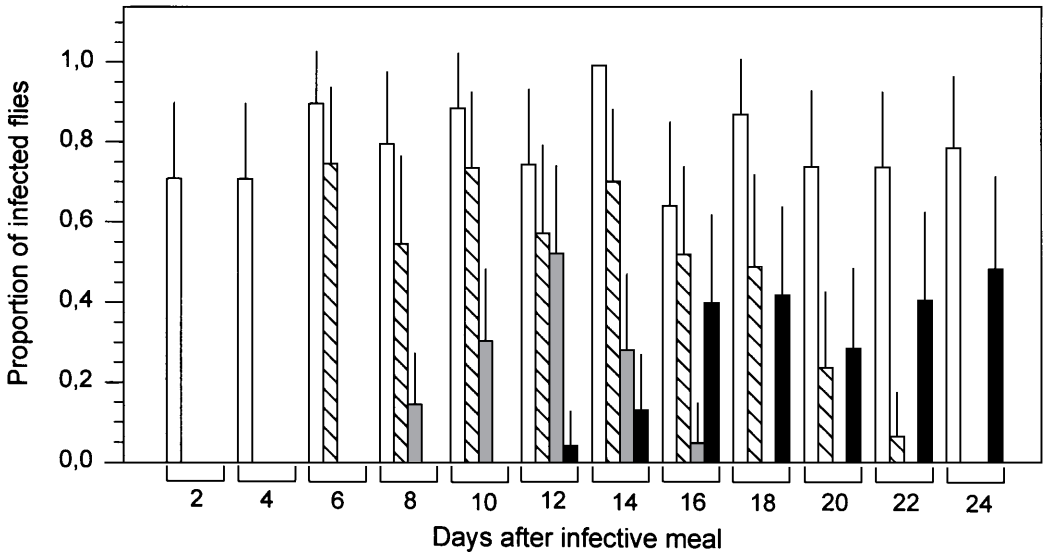

Fig. 1. Kinetics of *Trypanosoma brucei* development within the alimentary tract and salivary glands of *Glossina morsitans*. The major sites of the developmental pathway were examined at 2-day intervals after the infective meal: midgut (□), foregut/proboscis (▨), salivary gland–only attached epimastigotes (▨), salivary gland + metacyclic trypomastigotes (■). Bars are expressing the proportion of flies (±95% c.i.) with a trypanosome population at a specific site; for each experimental point, at least 20 flies were dissected.

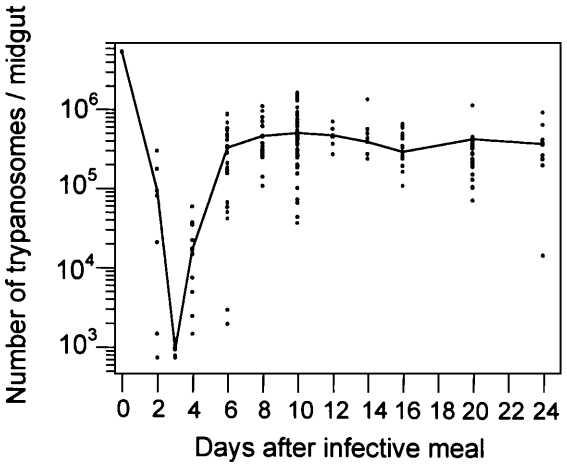

Fig. 2. Course of absolute number of *Trypanosoma brucei* in the midgut of the tsetse fly *Glossina morsitans*. Teneral flies were given a single infective meal on mice showing a parasitaemia of $10^{8.4}$–$10^{8.7}$ trypanosomes/ml (with at least 70% short stumpy forms) and were afterwards maintained on clean rabbits. The solid line is drawn through the median values calculated from the individual experimental points.

from day 6 onwards. Colonization of the salivary glands was observed from day 8 on. Eventually, 44–50% of the flies displayed an infection with infective, metacyclic trypomastigotes in the salivary glands at day 24, the end of our observation period.

Colonization of the tsetse midgut

Considering the parasite level in the host–mouse ($10^{8.4}$–$10^{8.7}$ trypanosomes/ml) and the average quantity of blood ingested by a teneral fly (20 μl), approximately $7{\cdot}5 \times 10^6$ bloodstream trypanosomes were initially taken up by the tsetse flies. During the first 3 days following the infective bloodmeal, parasite number in tsetse midgut drastically dropped to $1–2 \times 10^3$ individuals (Fig. 2). By day 4, the remaining parasite population – i.e. transformed procyclic trypomastigotes – started to expand vigorously to reach a maximum density of $2–5 \times 10^5$ parasites/midgut from day 6 onwards. After this colonization phase, the population of trypanosomes balanced around this level all through the observation period of 24 days.

Transit through the foregut and proboscis

Before day 6, no trypanosomes could be detected in the foregut and proboscis although the trypanosome density in the ectoperitrophic midgut was already high. Presence of trypanosomes was consistently observed from day 6 onwards (Fig. 1) and their number ranged from < 312 to 7500 parasites per fly. These flagellates remained present at similar density until day 18 after which they disappeared progressively between day 18 and day 24. None of these trypanosomes could be detected anymore at day 24 post-infection.

Microscopical examination of foregut and proboscis flagellates showed drastic changes in motility and morphology as compared with the trypomastigotes from the ectoperitrophic anterior midgut. Under phase contrast, most trypanosomes appeared to be long forms with very high motility comparable to *T. vivax* bloodstream forms, alongside with some short, sluggish flagellates. Following Giemsa staining, 4 main morphological forms could be dis-

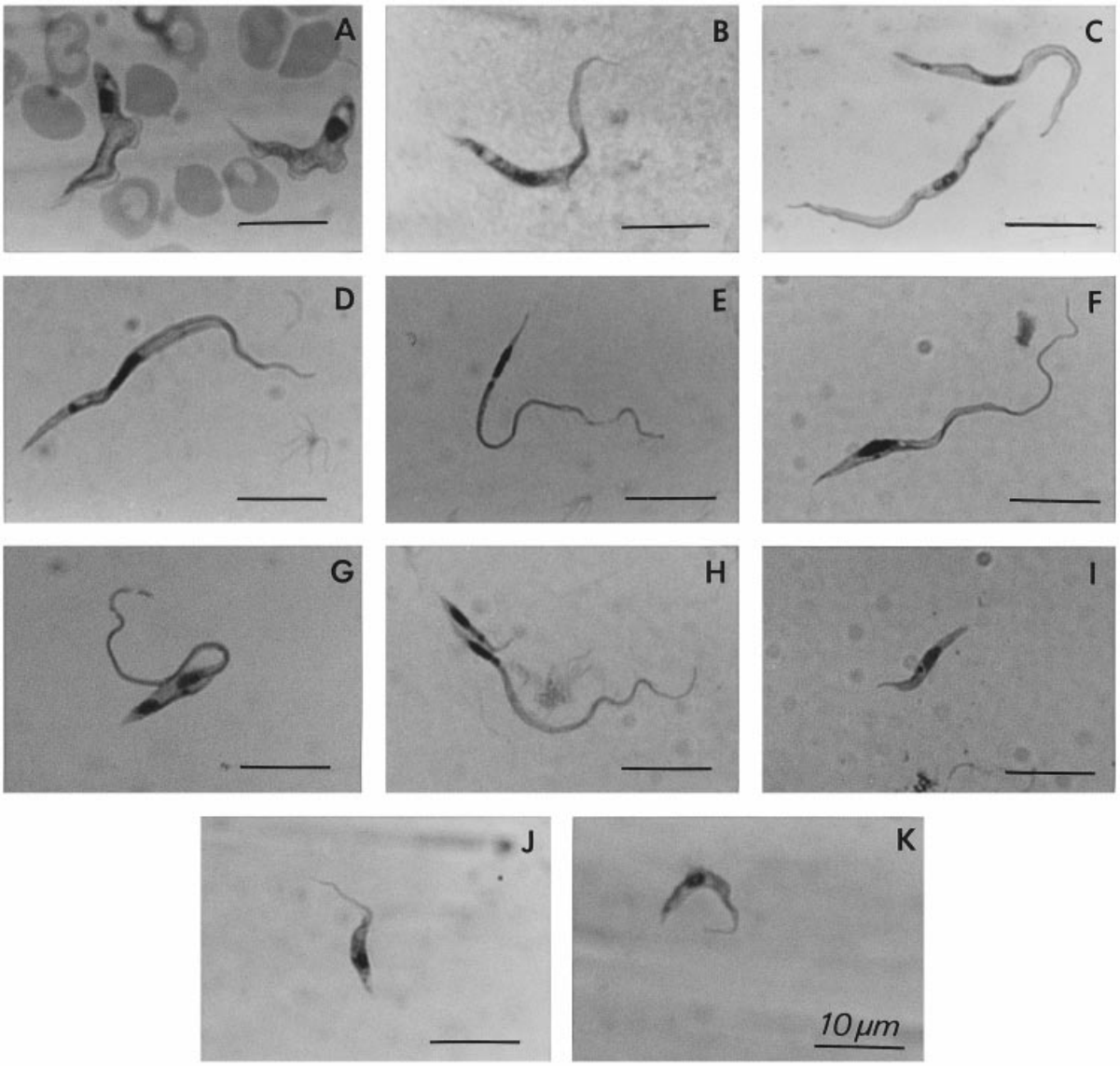

Fig. 3. Light microscopy photographs of the different morphological stages of *Trypanosoma brucei* from the major sites of the developmental pathway. Different stages: bloodstream short stumpy trypomastigote (A); posterior midgut procyclic trypomastigote (B); anterior midgut mesocyclic trypomastigote (C); post-mesocyclic stages in foregut and proboscis: long trypomastigote (D), long epimastigote (E–F); asymmetric dividing epimastigote (G–H) and short epimastigote (I); salivary gland stages: attached epimastigote (J) and metacyclic trypomastigote (K).

tinguished (Fig. 3) at various proportions: long trypomastigotes (Fig. 3D) representing 55–75% of the total population, 22–35% of long epimastigotes (Fig. 3E–F), 2–8% of asymmetric dividing long epimastigotes (Fig. 3G–H) and 0·5–6% short epimastigotes (Fig. 3I). All 4 forms were already present at day 6 and their proportion remained similar throughout their limited period of presence in the foregut and proboscis, between day 6 and day 22 (Fig. 4).

Colonization of the salivary glands

From day 8 onwards, trypanosomes were observed in the salivary glands (Fig. 1). During the first days of colonization of the glands (day 8–day 10), long trypanosomes were detected in the narrow, paired ducts between the hypopharynx and the beginning of the secretory gland epithelium. These parasites were identified by Giemsa staining as long trypomastigotes, long epimastigotes and asymmetric dividing epimastigotes. They were morphologically identical to those described above for the foregut and proboscis. Observation of these forms in the salivary glands was difficult since they were only observed in about 10% of the flies at low density (1–10 parasites/duct) and only within a very narrow time lapse (day 8–day 10 post-infection). In the other flies, salivary glands were either negative or they already showed a typical view of short epimastigotes attached to the gland epithelium. At day 12, the proportion of flies with positive salivary glands reached its maximum average value of 0·55. On this day (day 12), salivary gland homogenates became

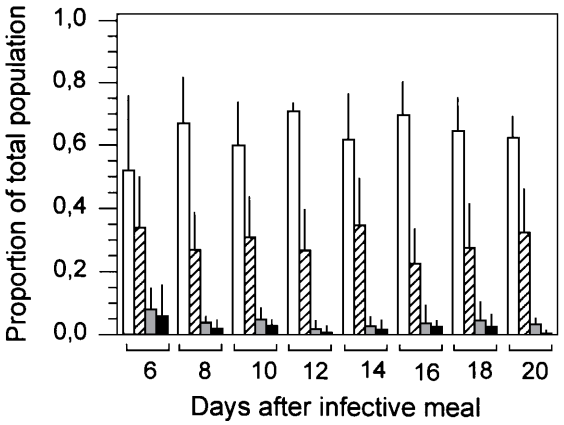

Fig. 4. Proportion of the different morphological stages of *Trypanosoma brucei* present in the foregut and proboscis (post-mesocyclic stages) of infected *Glossina morsitans* males: long trypomastigote (□), long epimastigote (▨), asymmetric dividing epimastigote (▨), and short epimastigote (■). The proportion (±S.D.) of each stage was determined by scoring Giemsa-stained parasites from preparations of individual flies ($n = 8$), dissected at a 2-day interval after the infective meal.

infectious to mice demonstrating the presence of mature metacyclics which was already suggested by Giemsa staining. At day 18, all positive salivary glands proved to harbour mature, infective metacyclic trypomastigotes.

Morphometry

In an attempt to reconstruct the sequence of morphological events during morphogenesis of the trypanosomes in the foregut and proboscis, total length (body + flagellum) of different parasite stages in the fly was measured (Fig. 5). Trypanosomes elongated progressively during their development in the tsetse midgut. Their total length evolved from 18·8 ± 1·5 μm, incoming short stumpy trypomastigote forms, to 25·8 ± 3·6 μm, the procyclic trypomastigotes in the posterior midgut, to 34·6 ± 3·1 μm, the mesocyclic trypomastigotes in the anterior midgut near the proventriculus. Parasite length continued to increase significantly in the foregut and proboscis: long trypomastigote (39·5 ± 1·9 μm), long epimastigote (40·7 ± 2·0 μm) and long dividing epimastigotes (42·4 ± 1·9 μm). In contrast, the short epimastigotes at this site were 3 times smaller, having a length of 13·1 ± 1·3 μm. The attached epimastigote forms, the first stage in the salivary glands, were of a comparable size, i.e. 14·8 ± 2·9 μm. Mature metacyclic forms already appeared to be longer, measuring 17·4 ± 2·0 μm.

Quantitative DNA-analysis (ploidy) of different trypanosome populations

DNA content (nuclear + kinetoplast DNA) of the different trypanosome populations was determined by flow cytometry (Fig. 6) and by image cytometry (Fig. 7).

Flow cytometry

As could be expected, the short stumpy trypomastigote population appeared to be homogenous for DNA content since 94% of the cells were covered by the first peak of fluorescence intensity and 6% in a second peak at double intensity (Fig. 6A). This major peak corresponded to cells with a 2N DNA content. Mesocyclic trypomastigotes collected from the anterior midgut showed a comparable flow cytometric pattern (Fig. 6B): 91% of the cells showed a 2N DNA content, 7% contained 4N DNA and the remaining 2% had a DNA content between 2N and 4N. In sharp contrast with this trypanosome population, the parasite population collected from the foregut and proboscis showed a remarkable shift to the double intensity peak representing a 4N DNA content (Fig. 6C). This peak contained 67% of the analysed cells. Of the remaining cells, 13% of them had a 2N DNA content and 20% showed a DNA level between 2N and 4N. Flow cytometrical analysis of the metacyclic trypomastigote population (Fig. 6D), purified from infected glands, again showed a pattern that was more like that described for the short stumpy bloodstream forms and mesocyclic trypomastigotes from the anterior midgut. The majority of cells (83%) contained 2N DNA, 5% contained between 2N and 4N DNA, and 12% of the cells had a 4N DNA content.

Image cytometry

Both bloodstream short stumpy trypomastigotes and anterior midgut mesocyclic trypomastigote forms revealed a single fluorescence intensity signal which represents a 2N DNA content (Fig. 7). These results corresponded completely with the data obtained by flow cytometry as described above. For the post-mesocyclic parasite population collected from the foregut and proboscis, this individual cytometrical technique permitted us to determine the relative DNA content of the different morphological stages. The major part of the long trypomastigotes (75%) had a 4N DNA whereas the remaining part showed fluorescence intensity values between 2N and 4N DNA. The asymmetric dividing epimastigotes invariably had a 4N DNA content. For the long epimastigotes, 2 subpopulations could be clearly distinguished: 1 subpopulation with a fluorescence signal corresponding to 4N DNA and 1 subpopulation showing a fluorescence intensity which was significantly lower than that for the 2N DNA reference. The latter situation was also observed for the short epimastigotes from the foregut and proboscis and for the epimastigotes and the metacyclics

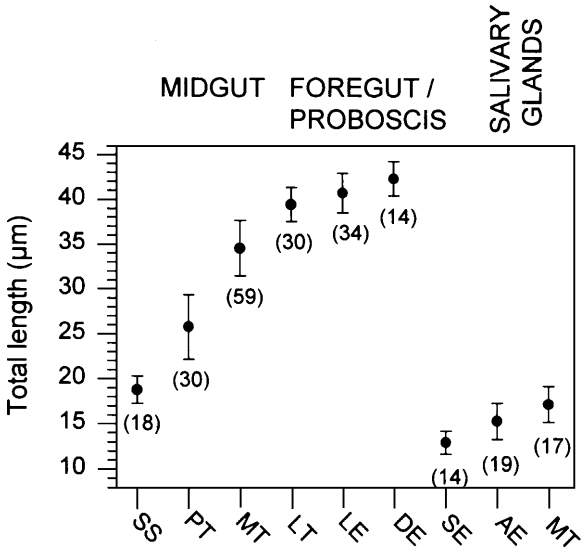

Fig. 5. Morphometric sequence of the development of *Trypanosoma brucei* in *Glossina morsitans* according to the 3 main sites of its life-cycle in the fly. In the foregut and proboscis (post-mesocyclic stages), it is proposed that the most natural sequence starts with the trypomastigote stage – closest to the ectoperitrophic anterior midgut trypomastigotes – and ends with the short epimastigote – as its size is closest to the salivary gland epimastigotes. Total length (body + flagellum) is expressed in μm $\pm$ S.D.; (n) = number of trypanosomes measured. Different stages: short stumpy trypomastigote (SS), posterior midgut procyclic trypomastigote (PT), anterior midgut mesocyclic trypomastigote (MESO), long trypomastigote (LT), long epimastigote (LE), asymmetric dividing epimastigote (DE), short epimastigote (SE), attached epimastigote (AE) and metacyclic trypomastigote (META).

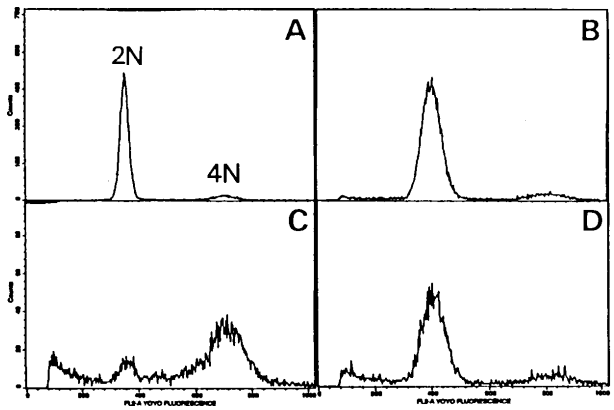

Fig. 6. Measurements by flow cytometry of the DNA content of different developmental stages of *Trypanosoma brucei* in the tsetse fly. Different stages: bloodstream short stumpy trypomastigotes (A) (= reference population), anterior midgut mesocyclic trypomastigotes (B), post-mesocyclic stages in the foregut and proboscis (C), salivary gland metacyclic trypomastigotes (D).

from the salivary glands. However, we strongly believe that this lower fluorescence signal observed for these trypanosome stages was due to the staining

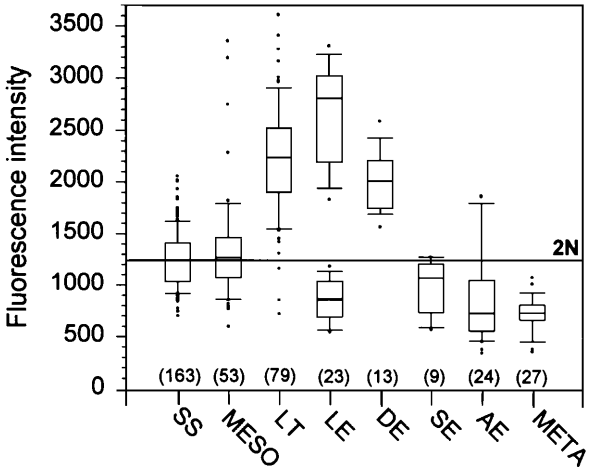

Fig. 7. Measurements by image fluorometry of the DNA-content of different developmental stages of *Trypanosoma brucei* in the tsetse fly. Different stages: bloodstream short stumpy trypomastigote (SS) (= reference population), anterior midgut mesocyclic trypomastigote (MESO), post-mesocyclic stages in foregut/proboscis: long trypomastigote (LT), long epimastigote (LE), asymmetric dividing epimastigote (DE) and short epimastigote (SE), salivary gland stages: attached epimastigote (AE) and metacyclic trypomastigote (META). Data are presented in a box-plot graph indicating the median value and the Q_{90}, Q_{75}, Q_{25} and Q_{10} percentiles.

methodology used for the individual cytometry and consequently that it did not reflect a DNA content lower than 2N. Indeed, data from the flow cytometry described above clearly showed that the metacyclic population had exactly the same DNA content as the 2N-reference short stumpy population and that the minor DNA-peak in the foregut and proboscis population was exactly at this 2N level. However, the lower intensity values obtained by the individual cytofluorometry for these trypanosomes were remarkably reproducible (the same experiment was performed twice and both gave similar results) which suggested that other significant biological differences caused this weaker staining of the nuclear + kinetoplast DNA. Possibly this different accessibility of the Hoechst 33258 dye to the fixed DNA reflects structural changes of the chromatin which occur during trypanosome development in the tsetse fly.

Behaviour in in vitro *culture conditions*

Cultivability and/or morphogenesis *in vitro* of the trypanosomes from the foregut and proboscis were tested for documenting their biological behaviour, in comparison with that of midgut procyclic and mesocyclic trypomastigotes. Procyclics multiplied immediately after transfer *in vitro* and grew heavily. Midgut mesocyclic trypomastigotes reverted to the procyclic stage over the first 120 h *in vitro*, as demonstrated by their regression in length from 34·6 $\pm$ 3·1 μm (n = 59) at the time of transfer to

$24{\cdot}5 \pm 4{\cdot}3\ \mu$m ($n = 17$). Moreover, this reverted population started to multiply as actively as the original procyclic population. In contrast, the post-mesocyclic trypanosomes from the foregut and proboscis disappeared progressively during the 10-day *in vitro* observation period. Moreover, these initially vigorous moving trypanosomes lost their high motility rate within the first 48 h *in vitro*.

DISCUSSION

Bloodstream trypanosomes of *T. brucei* are ingested by the tsetse fly and transform to the procyclic trypomastigotes in the midgut. Our experiments suggest that more than 99% of these ingested parasites are eliminated in the initial phase of development. This means that the majority of the bloodstream forms including the stumpy forms, although they are pre-adapted to life in tsetse (Vickerman, 1985), will simply not survive this early period in the tsetse midgut. Turner, Barry & Vickerman (1988) reported that most bloodstream forms are rapidly killed in both the anterior and posterior portions of the tsetse fly midgut and that slender forms were killed more rapidly than stumpy forms. They stated that this differential death rate supports the view that surviving stumpy forms are primarily responsible for transformation to procyclics. Trypanosome establishment in the tsetse midgut starts from a highly reduced population of transformed procyclic cells. Growth of the latter procyclic population in the tsetse midgut is vigorous to a maximum density of 5×10^5 parasites per midgut. From this moment on, parasite density in the midgut is maintained on a level of equilibrium. Recently, Welburn & Maudlin (1997) have suggested that at this point, parasite density would be regulated by a mechanism of programmed cell death.

During midgut colonization, the procyclic trypomastigote population expands in the ectoperitrophic space from the posterior towards the anterior midgut. A progressive elongation of the parasites accompanies this upward colonization. Long trypomastigote forms found in the ectoperitrophic space in the anterior midgut near the proventriculus, are the mesocyclic forms described in Vickerman's model of reference of *T. brucei* development (1985). These mesocyclic trypomastigotes, as endpoint of trypanosome establishment in the tsetse midgut, are in the G0/G1-phase of their cell cycle which confirms Vickerman's view about the non-dividing nature of these cells. However, they immediately regain growth activity when isolated in culture medium, accompanied by a regression of length i.e. they easily revert to the procyclic trypomastigote status in *in vitro* culture conditions. This suggests that the non-dividing status of the mesocyclic population is controlled by extracellular factors present in the anterior midgut in the fly and that it does not represent an irreversible blocked developmental stage of the parasite. However, this arrestment at the G0/G1 phase is a prerequisite for the cells to enter a stage of differentiation, which is the following step in the trypanosome development. Indeed, these mesocyclic midgut trypomastigotes enter the proventriculus lumen to move into the foregut and proboscis where they undergo a complex differentiation to the short epimastigote. The most obvious sequence of these different post-mesocyclic stages during this differentiation, strongly supported by the morphometrical and DNA cytometrical data, is summarized in Fig. 8. Incoming mesocyclic trypomastigotes (2N) turn into longer trypomastigotes which start to replicate their DNA to a 4N level. However, at the latter point, the long trypomastigotes do not enter mitosis immediately to generate 2 identical 2N daughter cells as this would be the case in a normal cell cycle. On the contrary, the 4N long trypomastigote differentiates into a 4N long epimastigote, i.e. the kinetoplast repositions to an anteronuclear position, after which the cell enters mitosis. This cell divides asymmetrically and generates 2 morphologically different daughter cells, each with a diploid amount of DNA: a long epimastigote and a short epimastigote. The fate of the long epimastigote is unknown and may be to die without further development. The short epimastigote form strongly resembles the attached epimastigote stage in the salivary glands. Each stage is of a similar size, the position of the kinetoplast is anteronuclear and their reduced motility due to the short flagellum makes them adapted to settle down. However, the latter property makes the short epimastigote unsuitable to swim from the proboscis, through the hypopharynx, and reach the salivary glands autonomously. Therefore, the highly motile, asymmetrically dividing epimastigote is the most appropriate candidate to make the journey from the proboscis, through the hypopharynx into the paired ducts of the salivary glands. There, it will complete division and short epimastigotes are now at the right place to settle down. Our observation of long dividing epimastigotes and long epimastigotes present in the gland ducts in the early stage of salivary gland colonization strengthens this hypothesis.

This series of differentiation events appears to be irreversible since these cells do not regain growth activity nor procyclic morphology after isolation in culture medium. Moreover, the asymmetric dividing activity is clearly not aimed for parasite multiplication as trypanosome numbers at this stage remain low and at no point are foregut and proboscis of the tsetse fly permanently colonized by the parasite.

Study of parasite progression within the fly reveals that only during the time-limited occurrence of this population in the foregut and proboscis, are the salivary glands colonized. This observation empha-

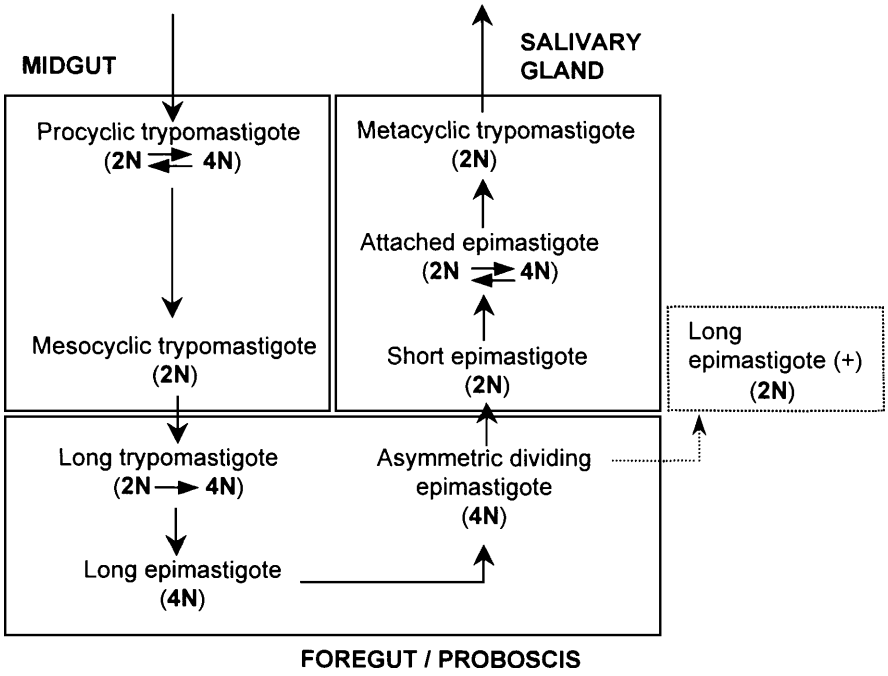

Fig. 8. *Trypanosoma brucei* development in the tsetse fly: proposed sequence of differentiation events in the foregut and proboscis based on morphometric and DNA cytofluorometric data described above.

sizes the key-role of this post-mesocyclic trypanosome population in the parasite life-cycle as a transient, but essential bridge between the established midgut population and metacyclogenesis in the salivary glands. The timing of this transient population coincides with the critical period for maturation of most *T. brucei* spp. in the tsetse fly, i.e. between 8 and 11 days following infection (Dale *et al*. 1995). Midgut parasites which do not continue development during this limited time-frame have effectively missed the opportunity for transmission and remain as procyclic immature infections for the life of the tsetse fly (Welburn & Maudlin, 1997). Our results suggest that at this point, the mesocyclic trypomastigotes from the anterior midgut play a pivotal role. Either these non-dividing cells stay in the anterior midgut where they will be subject to a self-regulating density mechanism (Welburn & Maudlin, 1997) or they proceed development and undergo differentiation in the foregut and proboscis. The latter is only possible during the limited critical period described above. Which environmental factors determine this critical period is at the moment speculative. Possibly, appropriate extracellular conditions/signals which trigger differentiation of the mesocyclic trypomastigotes are optimal only during this time-period.

In conclusion, the experimental data presented in this study demonstrate a transient post-mesocyclic trypanosome population in the foregut and proboscis of the tsetse fly that is essential to proceed development in the salivary glands. This population is the result of a differentiation of the mesocyclic trypomastigotes, the endpoint of trypanosome establishment in the tsetse midgut, to the short epimastigote form which is the starting point of metacyclogenesis in the salivary gland. Therefore, this developmental phase presents the 'missing link' in the *T. brucei* life-cycle in the tsetse fly.

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