The bloodstream differentiation-division of *Trypanosoma brucei* studied using mitochondrial markers

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SUMMARY

In the bloodstream of its mammalian host, the African trypanosome *Trypanosoma brucei* undergoes a life cycle stage differentiation from a long, slender form to a short, stumpy form. This involves three known major events: exit from a proliferative cell cycle, morphological change and mitochondrial biogenesis. Previously, models have been proposed accounting for these events (Matthews & Gull 1994*a*). Refinement of, and discrimination between, these models has been hindered by a lack of stage-regulated antigens useful as markers at the single-cell level. We have now evaluated a variety of cytological markers and applied them to investigate the coordination of phenotypic differentiation and cell cycle arrest. Our studies have focused on the differential expression of the mitochondrial enzyme dihydrolipoamide dehydrogenase relative to the differentiation-division of bloodstream trypanosomes. The results implicate a temporal order of events: commitment, division, phenotypic differentiation.

1. INTRODUCTION

Where cellular differentiation is irreversible, a point of commitment to production of the new cell type must occur. This commitment is likely to be made at a point when the cellular phenotype has not yet changed substantially, but the molecules necessary for coordinating the early differentiation have been synthesized. The African trypanosome *Trypanosoma brucei* is a protozoan parasite of medical, veterinary and economic importance, with a life cycle that alternates between its mammalian hosts and its vector, the tsetse fly. During this life cycle, *T. brucei* undergoes a series of irreversible differentiation events (reviewed in Vickerman 1985).

In the bloodstream of a mammalian host, multiple, morphologically distinct forms of the trypanosome coexist during an infection, a phenomenon described as pleomorphism. The proliferative long, slender form, predominant at low parasite density early in the parasitaemia, differentiates via morphologically heterogeneous intermediate stages to the short, stumpy form, which is the predominant form shortly after each peak of parasitaemia. The slender-to-stumpy differentiation is characterized by the changes in morphology for which the two cell types were originally defined (Bruce *et al.* 1912; Robertson 1912; Hoare 1972), by initiation of mitochondrial biogenesis (Vickerman 1965; Brown *et al.* 1973; Priest & Hadjuk 1994), and by exit from the proliferative cell cycle in G1 (Shapiro *et al.* 1984; Czichos *et al.* 1986; Zeigelbauer *et al.* 1990; Pays *et al.* 1993; Matthews & Gull 1994*b*). These characteristics make *T. brucei* an interesting system in which to study the coordination of three fundamental and biochemically distinct processes. Furthermore, as stumpy forms do not divide, this differentiation has a role in the modulation of parasitaemia, which affects both the virulence and pathogenicity of the disease.

We suggested previously models that may describe the slender-to-stumpy differentiation (Matthews & Gull 1994*a*). These address the potential temporal relationship between the final division of an individual trypanosome in the bloodstream, the differentiation-division and the commitment of that trypanosome to the formation of a stumpy form (figure 1). The decision to become a stumpy form might occur before commitment to division. Alternatively, the decision could be made and then be followed by the differentiation-division.

We have reasoned that by looking at expression of stumpy-specific markers in trypanosomes that were actually in the process of division, it might be possible to distinguish between these two models. If stumpy-specific markers were expressed at the differentiation-division this would indicate that the cell was already committed to the production of a stumpy form. Alternatively, if many such markers are shown to be expressed only after the differentiation-division, this would indicate that commitment occurs after the final division early in G_1.

To date, work on the slender-to-stumpy differentiation has been hindered by the absence of such clearly regulated markers of differentiation. Previous cellular studies (Balber 1972; Seed & Sechelski 1989; McLintock *et al.* 1990, 1993; Turner *et al.* 1995) have

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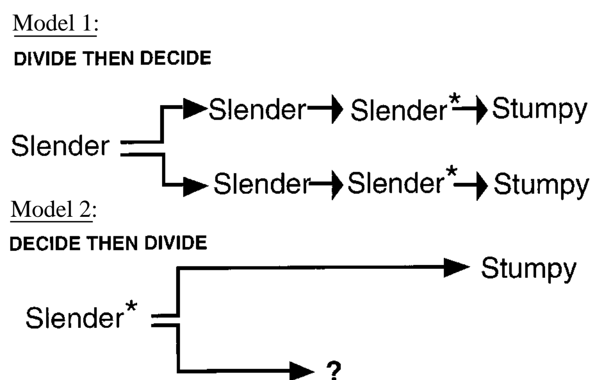

Figure 1. Models of the long slender to short stumpy differentiation. As the stumpy form is non-dividing, the decision (denoted by an asterix) to differentiate may be made after (Model 1) or prior to (Model 2) the differentiation-division. Model 2 produces two cells, at least one of which becomes stumpy without further division; the possible fates of the sibling cell (denoted by '?') cannot be encompassed by a single model. After Matthews & Gull (1994*a*).

relied on morphological markers, the use of which is complicated by the presence of morphologically intermediate forms in a differentiating population. The cytochemical diaphorase test has been used widely in studies to assay the slender-to-stumpy differentiation (Vickerman 1965; Hamm *et al.* 1990; Bass & Wang 1991; Vassella & Boshart 1996). Use of this cytochemical stain, however, is not ideal, as it has not proven readily compatible with the use of other markers in fluorescence studies, and it is unclear whether it represents solely the activity of one enzyme, dihydrolipoamide dehydrogenase (DHLADH).

As acquisition of new mitochondrial enzyme activities is an acknowledged property of the slender-to-stumpy differentiation, we have investigated potential mitochondrial markers of the slender-to-stumpy differentiation. We have then used these markers in cytological studies to investigate commitment to stumpy form production and coordination of morphological change, mitochondrial biogenesis and cell cycle arrest during differentiation.

2. MATERIALS AND METHODS

(*a*) *Trypanosomes*

For all bloodstream parasitaemias a pleomorphic line of *T. b. rhodesiense* EATRO 2340 was used. 1×10^6 trypanosomes were inoculated into immunocompetent BALB/c mice. The morphology of the trypanosome infection was subsequently monitored, and the parasites harvested when an appropriate population was reached. Typically >90% slender form (as defined by morphology) populations were obtained on day 3 post-infection at parasitaemias of less than $5 \times 10^7\ ml^{-1}$. Mixed populations with substantial numbers of slender, stumpy and intermediate forms were obtained on day 4 post-infection at parasitaemias of more than 1×10^8 trypanosomes ml^{-1}. Populations containing >90% stumpy forms were typically obtained on day 5 post-infection at parasitaemias of more than 1×10^8 trypanosomes ml^{-1}. Bloodstream trypanosomes were purified over a DEAE cellulose column (as described by Lanham (1968)). Procyclic trypanosomes were derived from long-term cultures of *T. b. brucei* strain 427.

(*b*) *Western blotting*

Trypanosome samples were prepared by harvesting trypanosomes, pelleting them at 1000 *g* for 10 min; washing them with PBS (200 mg ml^{-1} of KCl; 8 g l^{-1} of NaCl; 114 mg l^{-1} of NaH_2PO_4; 900 mg l^{-1} of Na_2HPO_4 pH 7.2) and pelleting them again at 1000 *g*.

Protein samples were then obtained by lysing the trypanosome pellets in lysis buffer (1% SDS, 0.25 M sucrose, 0.125 M tris-HCl pH 6.8) containing protease inhibitors (50 μg ml^{-1} chymostatin, 5 μg ml^{-1} pepstatin A, 50 μg ml^{-1} leupeptin). Protein concentration was assayed using the Bradford assay (BioRad protein assay; Bradford 1976). Extracts were then separated by SDS-PAGE, electroblotted on to nitrocellulose and visualized as previously described (Matthews *et al.* 1995).

(*c*) *Immunofluorescence and NAD diaphorase assay*

Purified, washed trypanosomes were prepared for immunofluorescence by settling them for 10 min on organosilane-treated slides, rinsing in PBS and fixing in methanol at −20 °C or in 4% paraformaldehyde. Immunofluorescence assay was performed as previously described (Sherwin *et al.* 1987). The NAD diaphorase assay was modified from a previous description by Vickerman (1965). Samples were fixed with 2.5% gluteraldehyde buffered in PBS and then washed briefly in PBS; the reaction buffer, containing 0.3 mg ml^{-1} of nitrotetrazolium blue and 1.3 mg ml^{-1} $NADH_2$ dissolved in PBS, was added to the sample and incubated for 1 h prior to mounting as for immunofluorescence. Microscopy was performed using a Leica DMRXA fluorescence microscope via a ×100 oil immersion planar fluorotar objective lens. Resultant images were either captured on Ilford XP-2 film, or using a cooled charge coupled device (CCD) (Photometrics series 200, Munich, Germany) with IPlab spectrum software; images were processed using Adobe Photoshop.

3. RESULTS

(*a*) *Defining markers of the slender-to-stumpy differentiation*

A cytological localization for each of these antigens in bloodstream and culture-form procyclic cell types was performed using immunofluorescence microscopy, to determine whether there was a clear difference in localization or expression of any of these putative markers between cell types (figure 2).

(i) *CB1*

This antigen is associated with the lysosomal protein gp57/42 of *T. brucei* (Brickman & Balber 1993, 1994). As the structure stained was not a tightly defined single

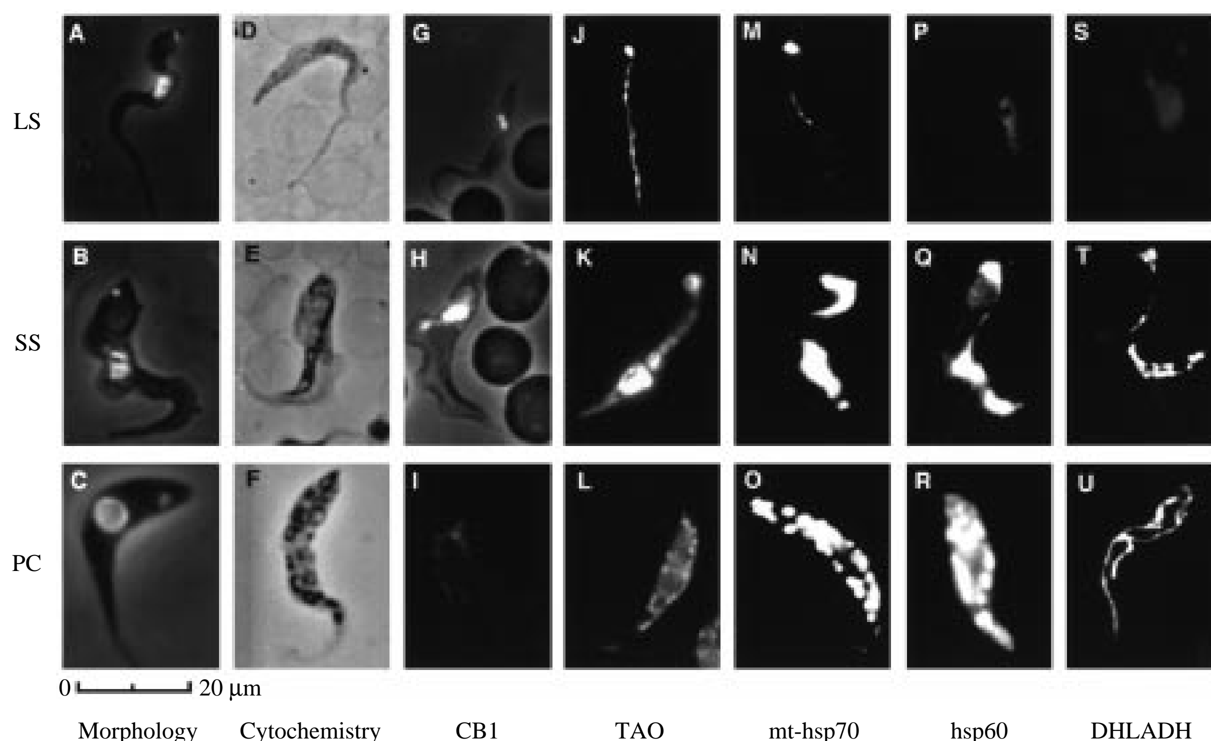

Figure 2. Life cycle stage regulation of established and putative markers for the long, slender (LS) to short, stumpy (SS) differentiation. The upper row shows images of slender-form trypanosomes; the middle row shows stumpy-form trypanosomes; and the bottom row procyclic trypanosomes. (A), (B) and (C) Differentiated cell types can be distinguished by morphological criteria such as length-to-width ratio, cell volume and length of free flagellum using phase-contrast microscopy; this discrimination is further facilitated by fluorescent localization of the nucleus and kinetoplast using DAPI, which allows visualization of shape, size, and relative positions of these organelles. (D), (E) and (F) The NAD diaphorase assay is a cytochemical assay used as a benchmark test to discriminate slender from stumpy; cell morphology and localization of formazan deposits are visualized by phase-contrast microscopy. (G)–(U) These show the results of immunofluorescence microscopy; (G), (H) and (I) are stained for CB1, which localizes to the lysosomes and endosomes of some bloodstream forms; (J), (K) and (L) for TAO, which localizes throughout the mitochondrion of all forms tested, albeit less strongly in the procyclic form; (M), (N) and (O) for mhsp70, which stains the mitochondrion of procyclic, stumpy and some long slender forms. Staining of other long slender forms is, however, restricted to the region of the kinetoplast; (P), (Q) and (R) for hsp60 specifically stains the mitochondrion of the stumpy and procyclic forms; (S), (T) and (U) DHLADH also stains specifically the mitochondrion of the stumpy and procyclic forms. The DHLADH antibody gave good immunofluorescence with a paraformaldehyde fix preserving mitochondrial integrity (U) compared with methanol fixation used for other markers (L), (O) and (R), which disrupted the mitochondrial network giving a distinctive punctate appearence. In (G) and (H), an immunofluorescence/phase-contrast image is shown to facilitate the localization of staining; (I) is fluorescence only to highlight the very low level of fluorescence in the procyclic form by immunofluorescence microscopy.

organelle, figures 2G and 2H show the immunofluorescence localization of the epitope merged with the phase image of the cell. To demonstrate the very low level of fluorescence of the procyclic stage the fluorescence-only image is shown (figure 2I). As can be seen from figures 2G and 2H, by an immunofluorescence criterion CBl does show increased expression in the stumpy form.

(ii) *Trypanosome alternative oxidase (TAO)*

This *T. brucei* gene has been cloned and monoclonal antibodies raised to the expressed protein (Chaudhuri *et al*. 1995*a*,*b*). The slender- and stumpy-form mitochondria were clearly visualized by the immunolocalization of TAO (figures 2J and 2K), which precludes the use of TAO as a stumpy-specific marker. The procyclic form showed relatively weak staining with the antibody to TAO compared to that seen for the other mitochondrial markers assessed in the procyclic form; however, the localization was similar (figure 2L).

(iii) *Mitochondrial hsp70 (mhsp70)*

T. cruzi and *T. brucei* genes for some mhsp70 proteins have been identified (Engman *et al*. 1989; Bangs *et al*. 1993). The polyclonal antibody raised to *T. cruzi* mtp70 was used in these studies. The mhsp70 localized to the mitochondrion of intermediate, stumpy and procyclic trypanosomes (figures 2N and 2O). Slender forms were apparently subdivided into two different populations. One population showed strong staining of the thread-like mitochondrion with a 'bright point' at the kinetoplast; the other population (as in figure 2M) showed either staining at the kinetoplast only, or comparatively strong kinetoplast staining, with only very weak staining at the rest of the mitochondrion.

(iv) *Hsp60*

The *T. cruzi* and *T. brucei* genes have been cloned and sequenced (Giambiagi de Marval *et al*. 1993; Bringaud *et al*. 1995) and a polyclonal antibody raised against the

T. cruzi protein (Sullivan *et al.* 1994), which, in the absence of an available *T. brucei* antibody, was used in these studies. The hsp60 localization pattern was similar to mhsp70 in the stumpy (figure 2Q) and procyclic forms (figure 2R), clearly staining the mitochondrion, but staining of the slender-form mitochondria was scarcely detectable (figure 2P).

(v) *Dihydrolipoamide dehydrogenase (DHLADH)*

The *T. brucei* gene for this nuclear-encoded enzyme, which is thought to be responsible for the NAD diaphorase activity, has been isolated and sequenced (Else *et al.* 1993). The *T. cruzi* protein is also well characterized; we used a polyclonal antibody raised to this protein (Lohrer & Krauth-Siegel 1990). When used for immunofluorescence, a similar staining pattern to the diaphorase assay was obtained, localizing to the mitochondrion of the stumpy and procyclic forms (figures 2T and 2U), but failing to detect the protein in the slender form (figure 2S). The use of paraformaldehyde fixation resulted in far superior mitochondrial integrity (figure 2U). Methanol fixation disrupted the mitochondrial network giving it the distinctive punctate appearance seen with the other mitochondrial markers (figures 2L, 2O and 2R).

(*b*) *Regulated expression of antigens and cellular morphology*

To determine the specificity of these antigens as markers of the slender-to-stumpy differentiation, individual cells were first considered in morphological terms: by size and shape, by relative position of the nucleus and kinetoplast, and by the length of free flagellum. Using these criteria, cells were categorized (from photographs of fields of cells) as slender, intermediate or stumpy, with the expectation that the intermediate category would show some degree of overlap with the slender and stumpy categories, but that slender and stumpy forms would seldom be confused.

Having been categorized morphologically, each cell was subsequently assessed qualitatively by immunofluorescence for the expression of each putative marker. This was done by scoring fields of cells of 'fluorescence only' photographs corresponding to those phase-contrast images used for scoring the cells for morphology. Two of the authors assessed each criterion independently and in this way a 'double blind' regime was established. Only after morphology (by phase-contrast) and expression (by immunofluorescence) counts were completed were the results compared. Expression was assessed for 100 slender, 100 stumpy and 100 intermediate forms.

Representative results from one of three independent experiments are summarized in table 1, to give an indication of the potential of each antigen as a cytological marker. CB1 staining was predominant in stumpy forms. However, there were exceptions and some slender forms also showed clear staining. This lack of specificity for any one cell type meant that we were unable to use CB1 as a marker for the slender-to-stumpy differentiation. TAO was localized to the mitochondrion of all cell types, confirming that TAO expression could not be used as a marker of the slender-to-stumpy differentiation (table 1).

Cytochemical staining from the NAD diaphorase assay and immunofluorescence of DHLADH, hsp60 and mhsp70 was localized to the mitochondrion of all stumpy trypanosomes and the majority of intermediate forms, but only a minority of slender forms. NAD diaphorase activity at the mitochondrion first becomes detectable close to the onset of morphological change (table 1), as is also the case for detection of hsp60 expression by immunofluorescence. By comparison, immunofluorescence shows DHLADH and mhsp70 expression in some slender forms, suggesting that a detectable increase in expression of these markers occurs prior to the onset of morphological change. It is notable that immunofluorescence for DHLADH detects mitochondrial localization of this enzyme in a higher proportion of slender forms than the NAD diaphorase stain, which is thought to reflect the enzymatic activity of DHLADH.

(*c*) *Stage-specific marker expression by western blotting*

Western blotting with the antibodies used for immunofluorescence was conducted to allow a direct comparison of stage-specific expression of each of the putative markers (figure 3). Samples were prepared from predominantly slender trypanosomes taken on day 3 of the primary parasitaemia, from predominantly stumpy trypanosomes (day 5) and from procyclic trypanosomes (from *in vitro* culture). The differences in expression as assessed by western blot were marginal for most of these markers. CB1, TAO and mhsp70 (figures 3C, 3D and 3G) showed only modest increases in stumpy population samples compared with slender population samples. In the procyclic samples the amount of mhsp70 detected

Table 1. *Cell type specificity of putative markers assessed by immunofluorescence*

(100 bloodstream trypanosomes of each morphological form (slender, intermediate and stumpy) were assessed for expression of putative markers by diaphorase assay and immunofluorescence assay for DHLADH, hsp60, mhsp70, CB1 and TAO. The samples used were different for each expression assay and were taken from mixed (day 4) populations.)

	diaphorase assay	DHLADH	Hsp60	mhsp70	CB1	TAO
slender	0	32	3	36	27	100
intermediate	94	99	88	99	74	100
stumpy	100	100	100	100	82	100

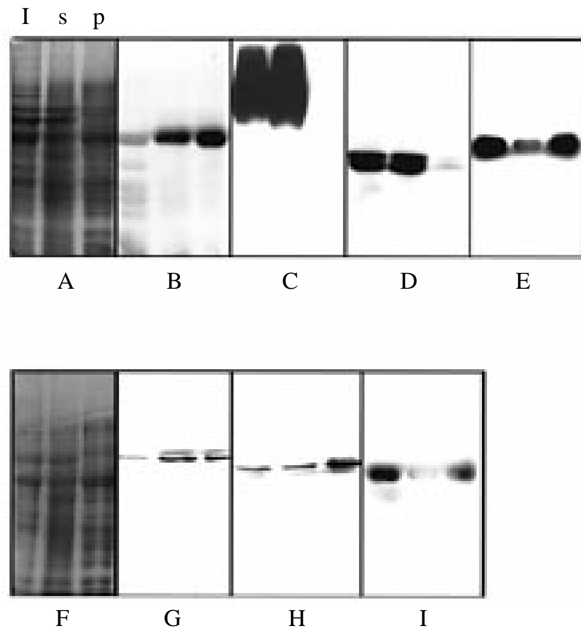

Figure 3. Life cycle stage expression of putative markers of the slender-to-stumpy differentiation. Gels were loaded by equal protein concentration. Duplicate sets of lanes (>90% long slender (labelled **L**), >90% short stumpy (labelled **S**) and procyclic (labelled **P**)) were run in parallel on the same SDS-polyacrylamide gel. One set of lanes was cut off and stained with coomassie, the other sets of lanes were then blotted and probed with antibody to one of the putative markers. In the absence of an obvious loading control coomassie-stained gels are shown (A) and (F). Alpha-tubulin was also included as a control for protein integrity; it was shown to be lower in the stumpy form than in either the slender or procyclic form (E) and (I); (B) shows the immunoblot of DHLADH; (C) of CB1; (D) of TAO; (G) of mhsp70; and (H) of hsp60.

again increased marginally; in comparison, the TAO detected was considerably lower in the procyclic sample than in either bloodstream form, and CB1 expression was not detected. Surprisingly, hsp60 (figure 3H) did not show a reproducible increase in expression between slender and stumpy populations; it is not clear why the immunofluorescence should show such a clear difference in staining for hsp60 between slender and stumpy forms (see figures 2P and 2Q). DHLADH, however, gave a clear increase in expression in the stumpy form relative to the slender form (figure 3B). Alpha-tubulin was also included as a control for protein integrity, and was found to be less expressed in the stumpy form than in either the slender or procyclic forms (figures 3E and 3I).

(*d*) *DHLADH expression increases during the primary parasitaemia*

On the basis of immunofluorescence and western blot, DHLADH was selected as the best marker to study the slender-to-stumpy differentiation; it showed a more pronounced increase in expression than other markers tested by western blotting (figure 3) and was undetectable in the slender-form mitochondrion by an immunofluorescence criterion (compared with the distinctive kinetoplast staining of mhsp70). To confirm that the proportion of cells expressing DHLADH matched the proportion of intermediate and stumpy forms during an infection, blood smears were taken during the time-course of the primary parasitaemia. Figures 4 and 5 demonstrate that the numbers of cells that showed a clear mitochondrial staining for DHLADH increased during the course of the primary parasitaemia, consistent with DHLADH expression being upregulated during differentiation.

(*e*) *The differentiation-division occurs prior to substantial morphological or mitochondrial differentiation*

We used the stage-regulated antigenic markers to address the temporal order of events during the slender-to-stumpy differentiation, and so to distinguish between the models proposed in figure 1. To determine whether cells had initiated differentiation prior to their differentiation-division, cells undergoing this division were sought during days 4 and 5 of the primary parasitaemia. At these time-points most cells showed clear expression of DHLADH by immunofluorescence and had already exited the cell cycle. We reasoned that of

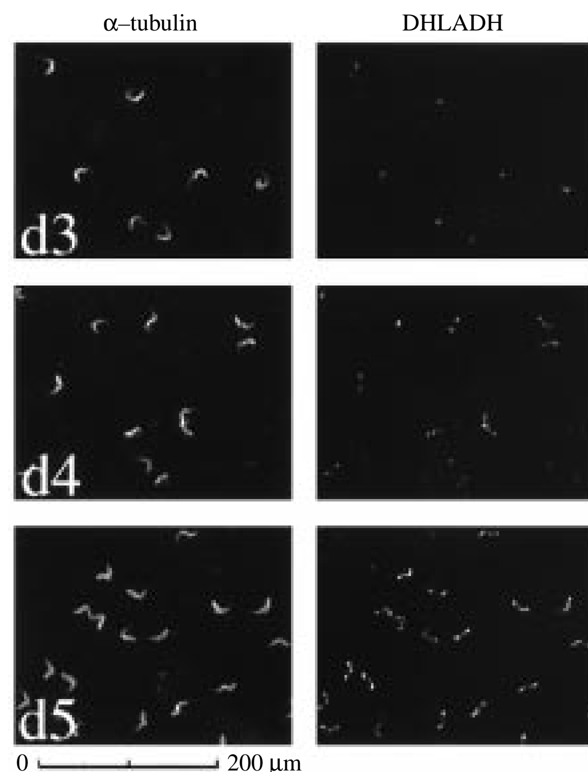

Figure 4. Immunofluorescence demonstrates an increase in DHLADH expression during the course of the primary parasitaemia. Cells were double-labelled with the mouse monoclonal anti-α-tubulin antibody and the rabbit polyclonal antibody to DHLADH. Staining for α-tubulin was visualized in the rhodamine channel and was used to highlight the morphology of the cells. Staining for DHLADH was visualized in the fluorescein channel and increased during the course of the primary parasitaemia. Samples were prepared as blood smears taken from an infected mouse 3 days, 4 days and 5 days after infection.

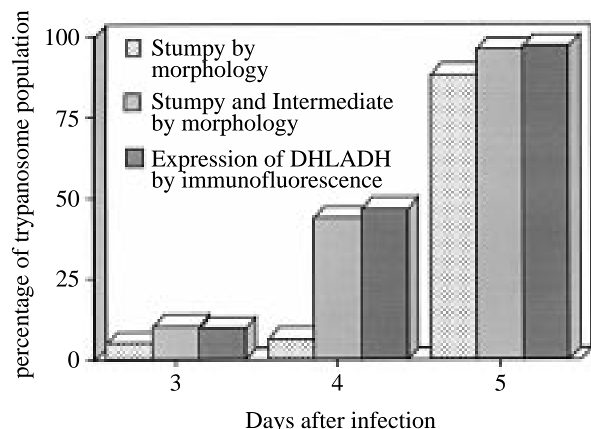

Figure 5. Immunofluorescence shows an increase in the numbers of trypanosomes expressing DHLADH during the primary parasitaemia. This increase parallels the increase in number of stumpy forms and is very similar to the increase seen in the total number of stumpy and intermediate (non-slender) forms. One thousand trypanosomes from populations taken 3 days, 4 days and 5 days after infection were assessed by immunofluorescence assay for the clear mitochondrial expression of DHLADH. One thousand trypanosomes each day, from the same populations, were also assessed by morphology and categorized as slender, intermediate or stumpy.

the few remaining cells in the process of division, many would be undergoing differentiation-division. Trypanosomes were considered to be in the process of division when they possessed two kinetoplasts and one or two nuclei, as viewed by DAPI fluorescence. If such dividing cells express a stumpy-specific marker, that expression is indicative of commitment to the production of a stumpy form taking place prior to or during the differentiation-division.

Using immunofluorescence, dividing forms were assessed for mitochondrial expression of DHLADH, hsp60 and mhsp70. Table 2 shows the results from two time-points late in the parasitaemia. As most cells in these late parasitaemia populations were non-dividing stumpy forms, it was necessary to search through over 10 000 cells at some time-points to locate just 100 dividing cells. We realized that a subpopulation of the dividing cells occurring very late in the primary parasitaemia might actually represent variant surface glycoprotein (VSG) heterotypes beginning to establish the secondary parasitaemia before the immune response had cleared the primary one, and that such cells might not be in their differentiation-division. It was for this reason that dividing forms were also assessed at the earlier time-point on day 4 of the primary parasitaemia.

For the studies with DHLADH and hsp60 it was possible to categorize most cells as being either positive (showing clear mitochondrial staining) or negative (showing no clear mitochondrial staining). For the study with mhsp70, however, many cells were assigned to a ± category of intermediate fluorescence. The results of these studies were then tabulated (table 2). With a few exceptions, dividing cells showed no expression of either DHLADH or hsp60 by immuno-fluorescence. Most of the exceptional cells expressing DHLADH or hsp60 and having two kinetoplasts and one or two nuclei had an unusual morphology in terms of size, shape and nuclear/kinetoplast positioning, which may indicate that they were cells that had failed to segregate in an aberrant division.

DHLADH and hsp60 expression was detected by immunofluorescence in the vast majority of intermediate forms and some slender forms (table 1), but not in the dividing trypanosomes of differentiating populations, most of which are likely to represent cells in their differentiation-division (table 2). Taken together, these observations imply that the majority of mitochondrial and morphological changes that occur during the course of the slender-to-stumpy differentiation take place after the differentiation-division.

Significantly, staining of dividing cells for mhsp70 showed distinct, though weak, staining of the mitochondrion in many such cells (figure 6F). This is indicative of expression beginning to increase during

Table 2. *Trypanosomes in the process of division assayed by immunofluorescence do not generally show staining for DHLADH or hsp60*

(Immunofluorescence for DHLADH, hsp60 and mitochondrial hsp70 was performed on populations of trypanosomes taken from mouse blood four days and five days post-infection. A search was made for dividing cells possessing two kinetoplasts and one or two nuclei visualized by DAPI stain. For each sample, approximately 100 dividing cells, were assessed for staining of the mitochondrion. When assessed for DHLADH and hsp60 very few of these cells showed detectable mitochondrial staining; however, mhsp70 staining was detected in a substantial number of cells.)

antigen assayed	days post-infection	No. of cells counted	percent of population +ve for antigen	dividing cells seen	dividing cells +ve for antigen	dividing cells +ve/−ve for antigen	dividing cells −ve for antigen
DHLADH	4	2700	60%	100	0	0	100
	5	13 200	92%	100	1	0	99
hsp60	4	2000	67%	103	3	3	97
	5	10 000	95%	102	2	4	96
mhsp70	4	2000	95%	100	5	58	37
	5	10 000	99%	106	21	23	62

+ve: Trypanosomes showing strong mitochondrial expression by immunofluorescence.
*−ve: Trypanosomes not showing mitochondrial expression by immunofluorescence.
+ve/−ve: Trypanosomes showing a weak/intermediate mitochondrial expression by immunofluorescence.

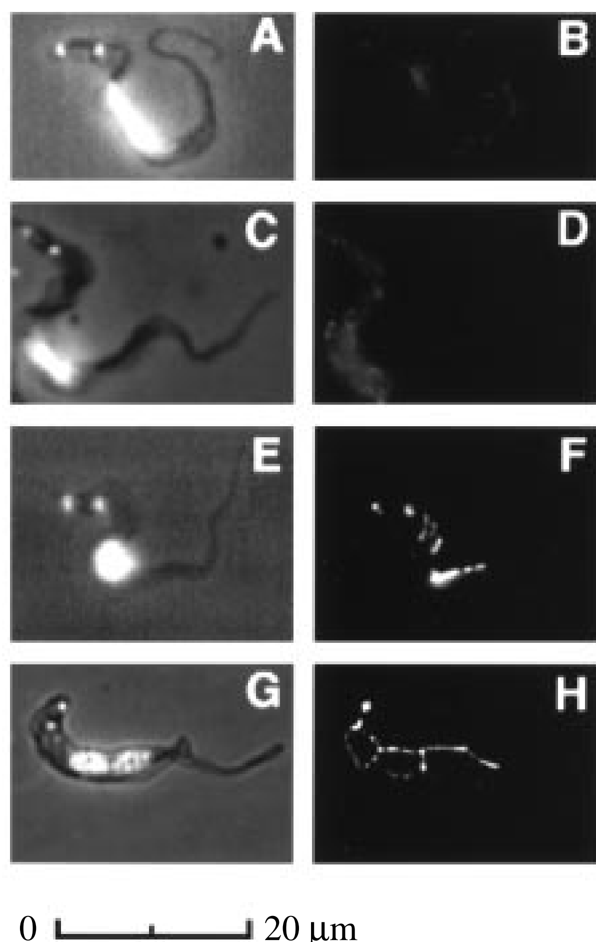

Figure 6. Expression of putative markers, in dividing cells, when assayed by immunofluorescence. The figure shows phase-contrast images merged with DAPI fluorescence to allow the kinetoplasts and nuclei to be used as markers of the cell cycle and to define cells which are in the process of division (i.e. cells containing two kinetoplasts and one or two nuclei) (left column); the corresponding images (right column) are immunofluorescence microscopy. When immunofluorescence was performed for DHLADH ((A) and (B)), the majority of cells showed no significant staining of the mitochondrion; this was also the case for hsp60 immunofluorescence ((C) and (D)). Immunofluorescence for mhsp70 ((E) and (F)) showed an increase in expression in some but not all dividing forms. Immunofluorescence for TAO ((G) and (H)) stained the mitochondrion in all such cells revealing its branched appearance during cellular division.

the differentiation-division. Figure 6 compares this with the corresponding lack of staining seen for DHLADH (figure 6B) and hsp60 (figure 6D), and with the localization of the dividing mitochondrion visualized using immunofluorescence for TAO (figure 6H).

(*f*) *Commitment to the production of a stumpy form*

The significant mhsp70 fluorescence in many dividing cells could indicate that the commitment to make a stumpy form occurred before or at the differentiation-division. Anucleate cytoplasts (termed 'zoids') occur naturally in trypanosome populations (Matthews & Gull 1994*b*) and arise by aberrant cytokinesis (Robinson *et al.* 1995). Lacking a nucleus, zoids cannot transcribe mRNA for nuclear-encoded genes *de novo* and must receive mRNAs for any such proteins from the mother cell prior to cytokinesis. Despite this, zoids clearly all possess a mitochondrion, as revealed by immunofluorescence with the antibody to TAO (figures 7F and 7H). We therefore looked at bloodstream trypanosome populations by immunofluorescence microscopy to determine whether zoids express DHLADH (figures 7B and 7D). In predominantly slender populations, most zoids had a slender morphology and did not express levels of DHLADH detectable by immunofluorescence (figure 7B). However, in predominantly stumpy populations most zoids shared some of the morphological characteristics of the stumpy form (figures 7C and 7G), and so have been termed 'stumpy zoids' (Matthews & Gull 1994*b*), and showed clear mitochondrial staining for DHLADH expression by immunofluorescence microscopy (figure 7D). These stumpy zoids were present at levels up to 0.5% of the population. This result indicates that at least some of the nuclear-encoded

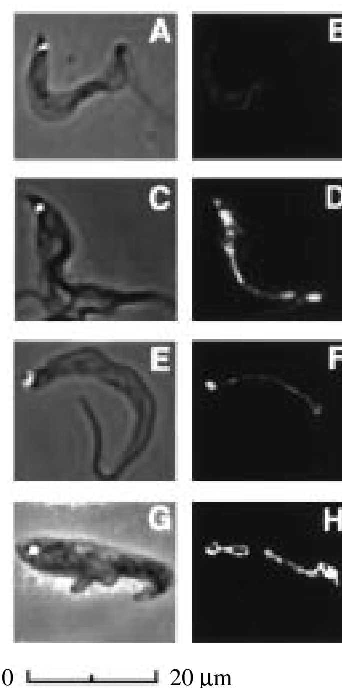

Figure 7. Anucleate forms, 'zoids', can show clear staining for DHLADH by immunofluorescence. These stumpy zoids were present at levels up to 0.5% of the population. Populations of trypanosomes from the bloodstream of mice 3 days and 5 days after infection were assayed by immunofluorescence (right column) with DHLADH ((B) and (D)) and TAO ((F) and (H)); the corresponding images (left column) are phase-contrast merged with DAPI staining to show the cell morphology of these forms and emphasize their lack of a nucleus. Zoids observed in the day 3 population appeared slender and generally showed mitochondrial staining with TAO (F), but not with DHLADH (B). Zoids observed in a day 5 population showed some morphological characteristics of a stumpy trypanosome and showed mitochondrial staining with both TAO (H) and DHLADH (D).

stumpy-specific mRNA, including DHLADH mRNA, is made prior to or at the differentiation-division, as DHLADH mRNA cannot be transcribed *de novo* by an anucleate zoid. The result was confirmed using diaphorase staining and immunofluorescence for hsp60 and mhsp70 and in each case zoids were observed that clearly showed mitochondrial staining with these nuclear-encoded markers (data not shown).

4. DISCUSSION

African trypanosomes undergo a specific differentiation step in the bloodstream of their mammalian host, entailing transformation from a proliferative slender-form cell to a division-arrested cell with stumpy morphology. Whatever the mechanism by which the signal to initiate the slender-to-stumpy differentiation is perceived and transduced, the result is to initiate coordinated cytological change with respect to three key processes: cell-cycle progression, morphogenesis and mitochondrial biogenesis. Here, we have developed useful molecular markers for this differentiation step and have employed the best of these to investigate the interrelation between these fundamental processes in the developmental programme of the trypanosome life-cycle.

(*a*) *Antigenic markers of the slender-to-stumpy differentiation*

We focused our attention on mitochondrial proteins, as elaboration of this organelle is one of the few well-defined characteristics of differentiation to the stumpy-cell form. Of these proteins, dihydrolipoamide dehydrogenase (DHLADH), mhsp70 and hsp60 were found to be useful markers, whereas the trypanosome alternative oxidase was not. TAO represents a poor marker because there is a substantial degree of expression in both slender and stumpy forms. Similarly, the stumpy-enriched non-mitochondrial antigen recognized by the CB1 antibody proved not to be a useful marker because of the heterogeneity of its expression in the stumpy-cell population.

The mitochondrial heat shock proteins hsp60 and mhsp70 both demonstrated elevated expression in stumpy forms, although this demarcation was less clear by western blotting. We do not know the basis for this difference between immunofluoresence and blotting results; it may be that hsp60 epitopes are sterically masked in the slender mitochondrion. Regardless of this, the immunofluoresence with these markers proved useful; mhsp70 was elevated in a proportion of slender forms in division, whereas hsp60 expression was exclusively restricted to non-dividing intermediate and stumpy forms. Previous immunofluorescence studies of mhsp70 have localized the protein exclusively with the kinetoplast (Engman *et al.* 1989; Olson *et al.* 1994; Effron *et al.* 1993; Klein *et al.* 1995), whereas our results using the same antibody localized the protein not only in the kinetoplast, but also throughout the mitochondrion of the intermediate, stumpy and procyclic forms. In line with this observation, mhsp70 of *Leishmania major* was detected throughout the mitochondrion using immunoelectron microscopy studies (Searle *et al.* 1993).

Of all the markers tested, DHLADH gave the clearest discrimination between slender and stumpy forms, by both immunofluoresence and western blotting. Furthermore, the staining observed in slender, stumpy and procyclic forms was consistent with the staining pattern seen cytochemically using the NAD diaphorase assay (Vickerman 1965). Although cell fractionation studies have localized DHLADH to the plasma membrane of the slender form (Danson *et al.* 1987; Jackman *et al.* 1990), this localization was not obvious from our immunofluorescence or indeed from the diaphorase stain. Regardless of this, the clear difference in DHLADH expression and localization between slender and stumpy forms meant we could confidently use DHLADH expression as a marker in studies of commitment and differentiation onset relative to the differentiation-division.

(*b*) *Cell division and commitment during stumpy-form generation*

In figure 1, Model 1 entails a slender trypanosome committing to become stumpy after the completion of cytokinesis, such that differentiation occurs without further cell division. In this scenario, the expectation would be that none of the stumpy-enriched markers would show substantially increased expression in any bloodstream trypanosomes that were in the process of division. In contrast, in Model 2, cells commit to differentiation prior to their terminal division, and stumpy-specific markers may begin to be expressed in cells in this differentiation-division.

To distinguish these models, we investigated the expression of DHLADH, hsp60 and mhsp70 in populations late in the primary parasitaemia. Such populations consist predominantly of intermediate and stumpy forms and we reasoned that many of the dividing cells in these populations would be in flux between the slender and stumpy form. When we examined DHLADH and hsp60 expression, less than 5% (1% for DHLADH) of trypanosomes that were in the process of division showed detectable mitochondrial fluorescence. In contrast to this, the majority, if not all of the cells that were morphologically intermediate or stumpy, demonstrated clear expression of these markers. This indicates first that DHLADH and hsp60 expression are events that occur after the differentiation division. Second, the observation that all intermediate forms express these markers whereas dividing cells never express them, indicates that the intermediate forms do not divide and are a product downstream of the differentiation-division. Together this suggests that the major phenotypic changes associated with production of the stumpy form occurs after the terminal bloodstream division, and that cells in this differentiation-division are morphologically slender.

Although the hsp60 and DHLADH analyses are compatible with both Models 1 and 2 describing the generation of a stumpy cell, analysis of mhsp70 expression distinguishes between them. Expressly, when we examined a differentiating population for dividing

forms, we found that approximately one half of these demonstrated increased mhsp70 expression with respect to the weakly staining slender population. This suggests that early differentiation events may precede or coincide with the differentiation-division, and positions the commitment to differentiation in the cell cycle prior to this terminal division (i.e. Model 2). A model in which commitment occurs prior to the differentiation-division would be consistent with data from growth rates and mathematical modelling (Turner *et al.* 1995). These mathematical models accommodate a final division that is heterogeneous with previous divisions, taking longer to complete, suggesting that the decision to differentiate is made prior to or at the time of this division. These mathematical models have also suggested a substantial time delay in the emergence of stumpy forms. Our data suggest that this time delay could be explained by the time taken for the transition between the morphological extreme of slender and stumpy forms, via the heterogeneous intermediate morphologies, after the differentiation-division. This sequence of events would mean that the differentiation-division would not have to accommodate concomitant structural change associated with the slender-to-stumpy differentiation.

If commitment to the production of a stumpy trypanosome is made prior to the differentiation-division we reasoned that this might be reflected in a peculiarity of trypanosome biology, the zoid. Zoids are motile, anucleate cytoplasts with a flagellum, kinetoplast and basal body,which apparently arise by an aberrant mitosis (Robinson *et al*. 1995). Lacking a nucleus, zoids are unable to transcribe the nuclear gene DHLADH *de novo*. We therefore reasoned that if zoids that expressed this protein did exist, the mRNA must have been transcribed during or prior to the differentiation-division. We therefore searched successfully for zoids that showed clearly detectable DHLADH expression by immunofluorescence. As in dividing cells DHLADH expression was not detectable; and in zoids DHLADH expression is detectable; the mRNA for increased DHLADH expression must be transcribed prior to cytokinesis of the differentiation-division and then translated and processed subsequently by the zoid. Although we are aware that release from a nuclear-encoded negative regulator of differentiation could generate this result, we do not favour this explanation because slender zoids also exist earlier in the parasitaemia and these never demonstrate the expression of stumpy-specific markers. Instead, we believe that the expression of stumpy-specific markers enriched in zoids advocates the interpretation that the commitment to differentiation occurs prior to the terminal division and that this is then followed by morphological transformation.

In conclusion, we have developed an experimental regime for the dissection of the cytology of the differentiation of trypanosomes in their mammalian host. We have identified suitable marker proteins for this differentiation and have employed these to distinguish models describing stumpy-cell generation. Our results implicate a model in which phenotypic differentiation encompassing the classic intermediate forms of the parasite occurs after a final division in the bloodstream, but predicts that commitment occurs at some point prior to this differentiation-division. The tools and methodologies developed are likely to provide a valuable experimental approach to the dissection of differentiation control in the bloodstream-form trypanosome. Unravelling this process provides a means to understanding and potentially controlling trypanosome proliferation, pathogenicity and transmission.

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