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## **Analysis by flow cytometry of DNA synthesis during the life cycle of African trypanosomes**

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### **Summary**

DNA content, at different stages in the life cycle of the hemoprotozoan parasite *Trypanosoma brucei*, has been analysed with a fluorescence activated cell sorter. It was observed that the long slender bloodstream form stage and procyclic culture forms (analogous to the tsetse fly midgut stage) are dividing cell populations with cells in G1, S, G2 and mitosis. Short stumpy bloodstream form and metacyclic fly salivary gland form populations are composed of non-dividing parasites stabilized in G1 or G0 of the cell cycle. Haploids, possible sexual forms, were not detected. In response to transfer to a culture system which mimics the fly midgut, short stumpy bloodstream form parasites were readily able to initiate DNA synthesis and differentiate into dividing procyclic culture forms. This supports the suggested role of the short stumpy form as a transitional stage between the mammalian host and the tsetse fly vector. Analysis of early and late bloodstream populations of another salivarian trypanosome, *Trypanosoma vivax*, revealed a transition from dividing to stationary cell population similar to that observed with *T. brucei*. A hitherto unrecognized morphological form of *T. vivax*, analogous to the *T. brucei* short stumpy form, was detected. It is suggested that the long slender to short stumpy morphological transformation, long known in *T. brucei*, reflects a physiological transition from dividing to nondividing parasite relevant to the life cycle of all the salivarian trypanosomes.

**Key words:** protozoan parasites; cell cycle; fluorescence activated cell sorter; *Trypanosoma brucei*; *Trypanosoma vivax*.

### **Introduction**

Salivarian trypanosomes, protozoan parasites transmitted by tsetse flies, are responsible for widespread disease in Africa. During their life cycle in the

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mammalian host and in the tsetse fly vector these parasites proceed through several morphologically distinguishable stages. Surprisingly, after 80 years of research, there is still disagreement about the significance of some of the stages in the parasite life cycle.

Infections with *Trypanosoma brucei* are characterized by fluctuating parasitemias. The mammalian bloodstream forms of *T. brucei* are pleomorphic, ranging from long slender to short stumpy organisms (Hoare, 1970). Long slender forms are found predominantly in rising parasitemias while short stumpy forms predominate when the parasitemia is falling. Some investigators postulate that the stumpy form is a nondividing cell responsible for initiation of the tsetse fly infection (Vickerman, 1971) while others claim that it is not a necessary stage in the life cycle but simply a degenerating cell (Ormerod et al., 1974). There is also some disagreement about whether short stumpy forms are really nondividing cells (Lavier, 1927; Hoare, 1956). While the bloodstream forms of other salivarian trypanosome species, *Trypanosoma vivax* and *Trypanosoma congolense*, show some morphological variability they are classically described as not demonstrating differences significant enough to be attributed to different life cycle stages (Hoare, 1972; Ormerod et al., 1974).

Another unsettled question about the trypanosome life cycle is whether sexual recombination takes place. Many investigators, in the past, have postulated that mating occurs among trypanosomes, however the mating form has been elusive (Hoare, 1972; Ormerod, 1979). Recent biochemical and genetic observations have rekindled interest in sexuality in trypanosomes (Tait, 1983). If recombination does take place, can a mating form be detected with the more sophisticated technology now available?

Flow cytometry makes possible the measurement of biochemical properties on a cell by cell basis. It has proved to be a useful tool for the analysis of cell cycle kinetics of eukaryotic cells (Horan and Wheelless, 1977). We have applied this technique to the analysis of DNA synthesis, cell division and ploidy in some of the morphologically different forms of *T. brucei*. We have also analysed early and late bloodstream populations of *T. vivax* where significant morphological variation has not been previously noted. Our observations clarify some aspects of the life cycle of African trypanosomes.

## Material and Methods

*Cells.* The *T. brucei* populations analysed were derived from *T. brucei* ILTat 3.3 which had been cloned from an early stabilate of *T. brucei* isolate UHEMBO/64/EATRO/795. Long slender bloodstream forms were collected on the third day following intraperitoneal inoculation of sublethally irradiated (600R) Balb/c mice with  $10^7$  parasites. Nearly homogenous populations of short stumpy form parasites were produced as previously described by Luckins (1972) and Balber (1972). Irradiated mice were inoculated with a low inoculum (20 trypanosomes) and from day eight of infection onward smears of infected blood were examined for the presence of stumpy forms. Short stumpy bloodstream populations containing fewer than 5% long slender and apparently dividing organisms were analysed for DNA content. Long slender and short stumpy form trypanosomes

were isolated from mouse blood by chromatography on DEAE-cellulose (Lanham and Godfrey, 1970). Procyclic culture form trypanosomes, which are analogous to the tsetse fly midgut stage (Brown et al., 1973; Ghiotto et al., 1979; Bienen et al., 1981), were derived from a short stumpy bloodstream form population in vitro and maintained in culture (Brun and Schönenberger, 1979). Metacyclics are the stage passed from the tsetse fly salivary gland to the mammalian host. For production of this stage *Glossina morsitans centralis* were fed through a membrane (Bauer and Wetzel, 1976) on culture medium containing  $2 \times 10^7$  per ml procyclic culture form parasites and then maintained on rabbits. After 35 days metacyclic trypanosomes were purified from minced dissected salivary glands by chromatography on DEAE-cellulose (Lanham and Godfrey, 1970).

The line of *T. vivax* used was cloned from Nigerian isolate Zaria Y486 (Leefflang et al., 1976); it is transmissible by tsetse flies (Moloo, 1981). The early and late parasite populations shown were taken from the same sub-lethally irradiated C57B1/6 mouse, 6 and 10 days respectively after intraperitoneal infection with  $2 \times 10^2$  parasites. Infected blood was obtained from the retroorbital plexus of the mouse under anesthesia. *T. vivax* parasites were isolated from mouse blood by Percoll density gradient centrifugation (Grab and Bwayo, 1982).

*Fixation, staining and flow cytofluorometric analysis.* All parasite stages were suspended at  $10^4$  to  $10^6$  cells per ml in PSG buffer (60 mM sodium phosphate, pH 8.0, 45 mM NaCl, 55 mM glucose) and then fixed by addition of glycerol and ethanol to 5% (v/v) and 70% (v/v) respectively. Fixed cells were stored at  $-20^\circ\text{C}$  until use. Prior to staining, fixed cells were recovered from ethanol by centrifugation for 10 min at  $500 \times g$ . The cell pellet was suspended at  $10^5$  per ml in a buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl and 15 mM  $\text{MgCl}_2$ . Parasite DNA was stained by addition of chromomycin A<sub>3</sub> (Sigma London Chemical Co., Dorset, UK) to 25  $\mu\text{g}$  per ml (Crissman et al., 1976). Samples were allowed to equilibrate for 30 min at room temperature before flow cytometric analysis. Cell fluorescence was measured on a FACS-II cell sorter (Becton Dickinson Co., Mountain View, CA, USA). Stained cells were excited at 458 nm and analysed for fluorescence using a 520 nm long pass barrier filter in the emission photometer. Ten thousand cells were counted from each sample. The data were acquired by a pulse height analyser (ND 100, Nuclear Data Inc., Schaumburg IL, USA) and displayed as DNA histograms on a linear scale on an X-Y recorder (Model 7041A, Hewlett Packard, San Diego, CA, USA). The FACS light scatter signals were displayed with fluorescence pulses as a two parameter dot plot on the FACS-II oscilloscope (Model 603, Tektronix Inc., Beaverton, OR, USA).

## Results and Discussion

Representative histograms of DNA content for populations of different morphological stages in the life cycle of *T. brucei* are shown in Fig. 1. Long slender and short stumpy mammalian bloodstream forms, short stumpy forms in transformation to procyclics, and procyclic and metacyclic fly forms were analysed. Long slender forms, the early stage in the mammalian bloodstream, and procyclic forms, the early fly stage, show a pattern of cell fluorescence typical for unsynchronized dividing eukaryote cell populations with cells in G1, S, G2 and mitosis (Horan and Wheelless, 1977). Metacyclic forms from the fly salivary gland, which are responsible for transmission of infection to the mammalian host, are seen to be nondividing cells stabilized in G1 or G0 of the cell cycle. Short stumpy bloodstream parasites also appear to be nondividing cells. When removed from the mammalian bloodstream and put into a procyclic culture medium under optimal conditions for transformation (Brun and Schönenberger, 1981) more than half of the cells in a stumpy population were

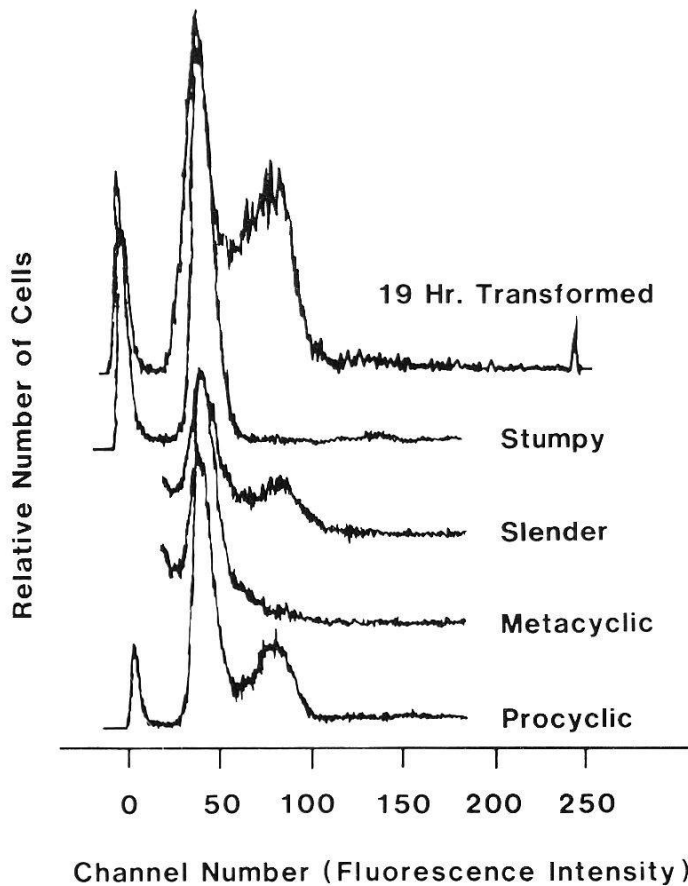


Fig. 1. Comparison of the DNA contents of parasites during different stages in the life cycle of *Trypanosoma brucei*. DNA histograms of short stumpy form parasites transforming to procyclics, of short stumpy and long slender bloodstream forms, of metacyclic tsetse fly salivary gland and procyclic fly midgut forms are shown. Relative DNA content (abscissa) is based on chromomycin A<sub>3</sub> binding. The peak of particles at the origin is nonfluorescent debris in the cell suspension. There is relatively more debris in the cell suspensions of lower concentration.

seen to have entered S phase within 19 hours. By 24 hours the population had doubled.

Available data suggest that bloodstream and procyclic trypanosomes are diploid cells (Tait, 1980; Borst et al., 1982). A sexual stage of the parasite might be expected to be haploid (Tait, 1983). Cells with half the G1 content of DNA were not observed in any of the populations examined. Neither was haploidy detected when purified nuclei (Shapiro and Doxsey, 1982) from bloodstream and procyclic culture populations were analysed (not shown). A sexual stage in the trypanosome life cycle, although not yet found, has long been postulated to exist (reviewed by Hoare, 1972). Recent isoenzyme studies suggest that recombination of genetic markers is indeed occurring in nature (Tait, 1980; Gibson et al., 1980), and there is evidence for genetic recombination in the related trypanosomatid *Crithidia fasciculata* (J. Glassberg and M. R. Rifkin, personal communication). Our data suggest, however, that if mating does occur in the populations we have studied, it is either too rapid or insufficiently frequent to be

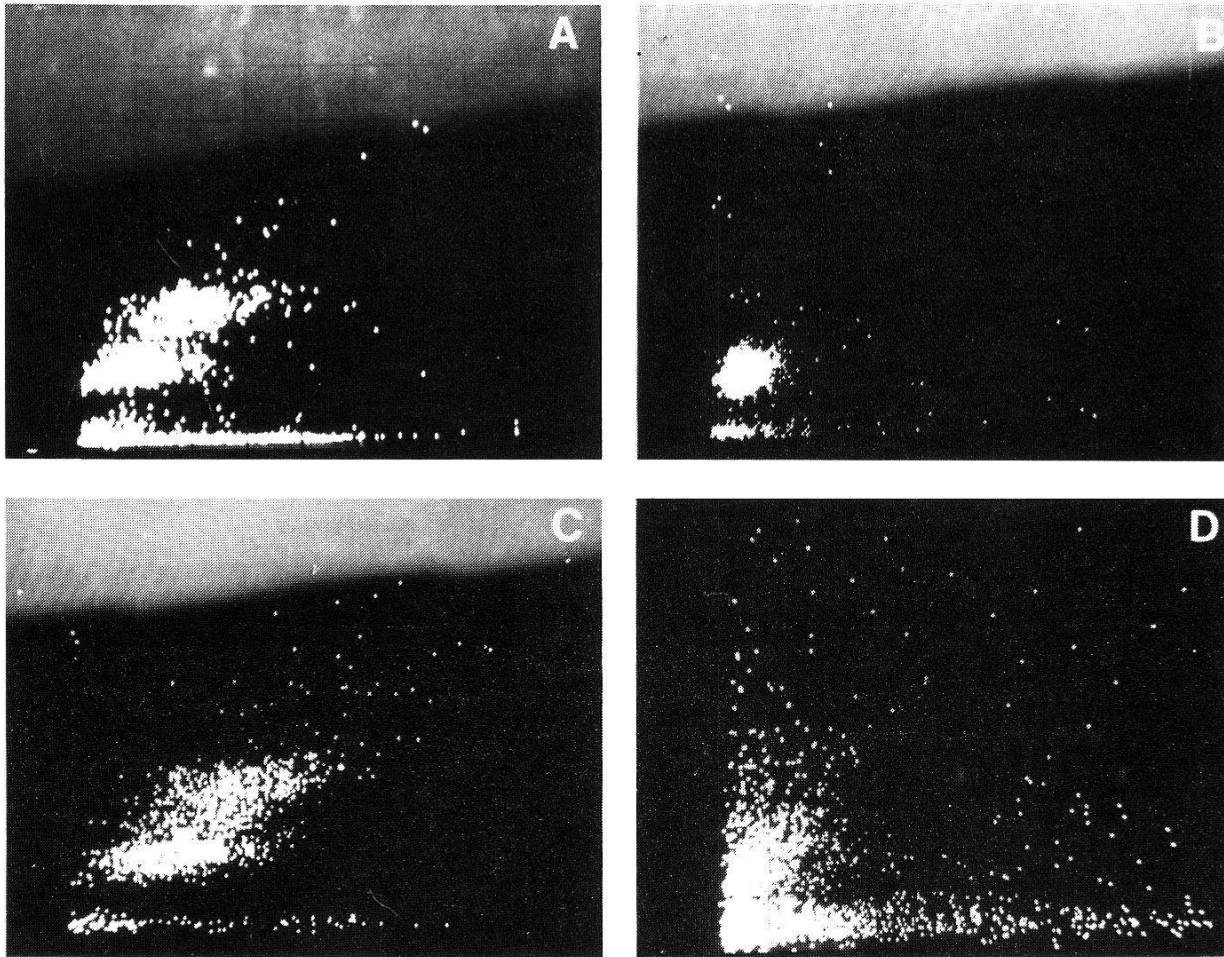


Fig. 2. Analysis of DNA content versus light scatter characteristics for different morphological stages in the life cycle of *T. brucei*. The FACS light scatter signals (horizontal) which give an indication of relative cell shape and size are displayed with fluorescence pulses (vertical) as a two parameter dot plot on the FACS-II oscilloscope. Populations of slender bloodstream (A), stumpy bloodstream (B), procyclic fly (C) and metacyclic fly (D) forms were analysed. Pulses at the base of each plot represent debris in the fixed cell suspensions.

detected by the FACS or it does not involve classical meiotic division. Other investigators using a FACS to study *C. fasciculata* have also failed to detect haploidy (Hughes et al., 1982).

Fig. 2 displays the histograms in the form of two dimensional dot plots of fluorescence intensity versus light scatter pulses. The broader size distribution of the slender (Fig. 2A) and procyclic (Fig. 2C) cells is consistent with their elongated shape. The more compact size distribution of the stumpy (Fig. 2B) and metacyclic (Fig. 2D) forms is consistent with their relatively compact shape. An increase in cell size in the G2/M stage of the cell cycle of the dividing cells is also apparent in these plots.

Fig. 3 shows the DNA contents of different bloodstream populations of *T. vivax*. The parasites in the bloodstream early after inoculation are seen to be proliferating cells similar to *T. brucei* long slender forms. However, later during the infection the DNA content of the parasites resembles that of the nondivid-

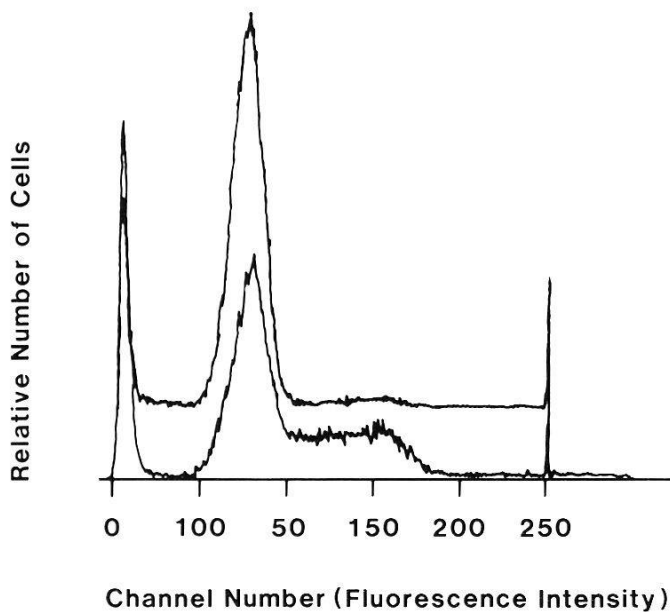


Fig. 3. Comparison of the DNA contents of the bloodstream population of *T. vivax* at different times in the course of infection. The early (lower line) and late (upper line) parasite populations shown were taken from the same mouse, 6 and 10 days respectively after infection.

ing *T. brucei* short stumpy population. When the data are displayed as two dimensional dot plots of fluorescence intensity versus light scatter signals (Fig. 4) the respective similarity of the early and late *T. vivax* bloodstream populations to slender and stumpy *T. brucei* populations in DNA content is also apparent. However, the similarity in light scatter signals of the two *T. vivax* populations suggests that there is not as much difference in morphology as is observed when slender and stumpy *T. brucei* parasites are compared.

When the parasites in the two *T. vivax* populations were examined by light microscopy a hitherto unrecognised bloodstream parasite dimorphism was observed (Fig. 5). Early during infection only the classically described bloodstream form of *T. vivax* was detected (Fig. 5A). This cell has a club shaped body tapering toward the anterior end; its kinetoplast occupies a terminal position. The form prevalent later during the course of infection (Fig. 5B), when mostly nondividing cells predominate, was slightly longer and its kinetoplast was sub-terminal. Also, this form's undulating membrane was less developed and it had an irregular and densely staining nucleus. On the basis of recent *in vitro* studies it has been suggested that this form, like the *T. brucei* stumpy, may be responsible for initiating development in the tsetse fly vector (H. Hirumi, personal communication). This form looks like a form which previously had been described as a "degenerating trypanosome in (the fly) midgut" (Hoare, 1970, p. 26). Our observation suggests this designation was incorrect.

Much of the controversy about the salivarian trypanosome life cycle centers on the characterization and role of the *T. brucei* short stumpy bloodstream form. We believe this nondividing form is important for transmission of the

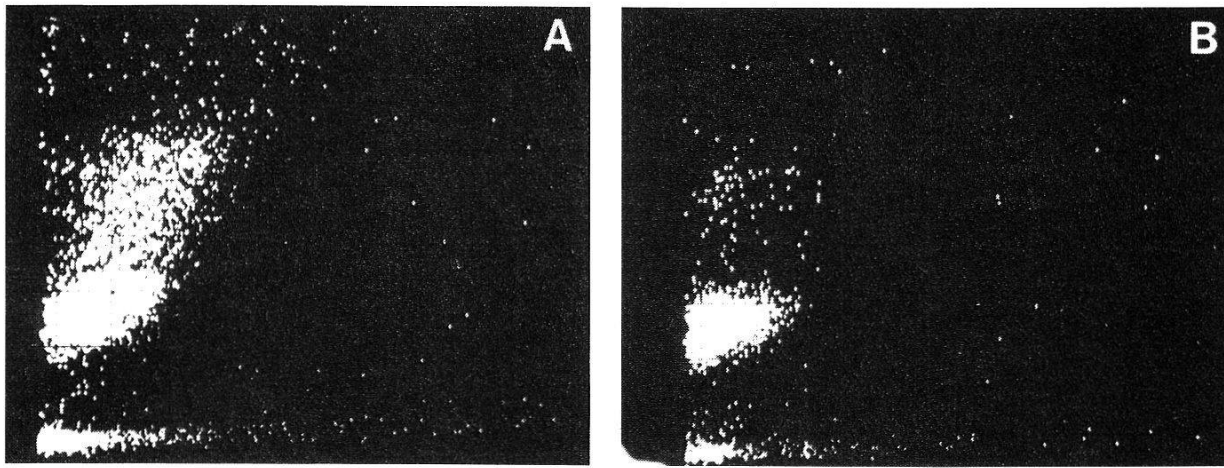
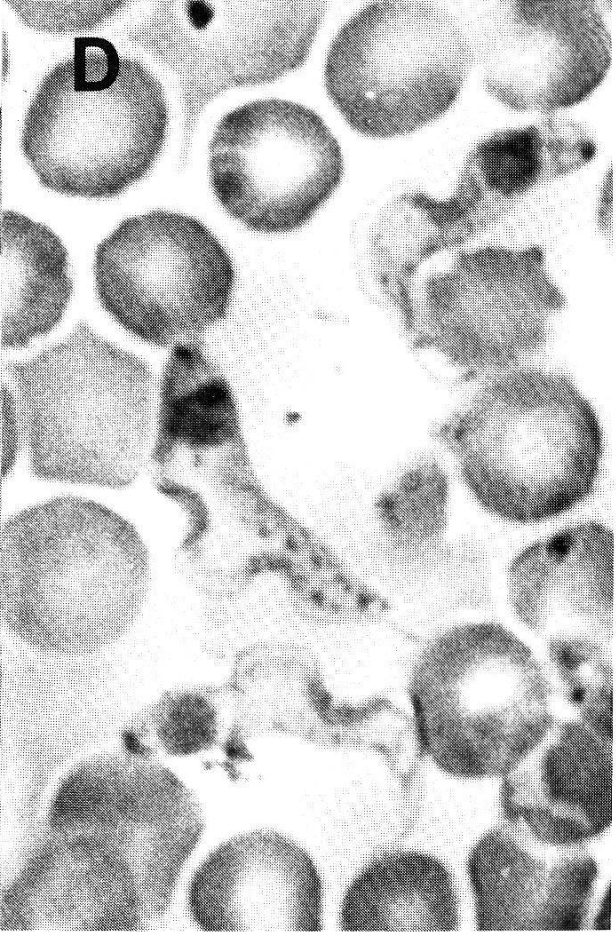
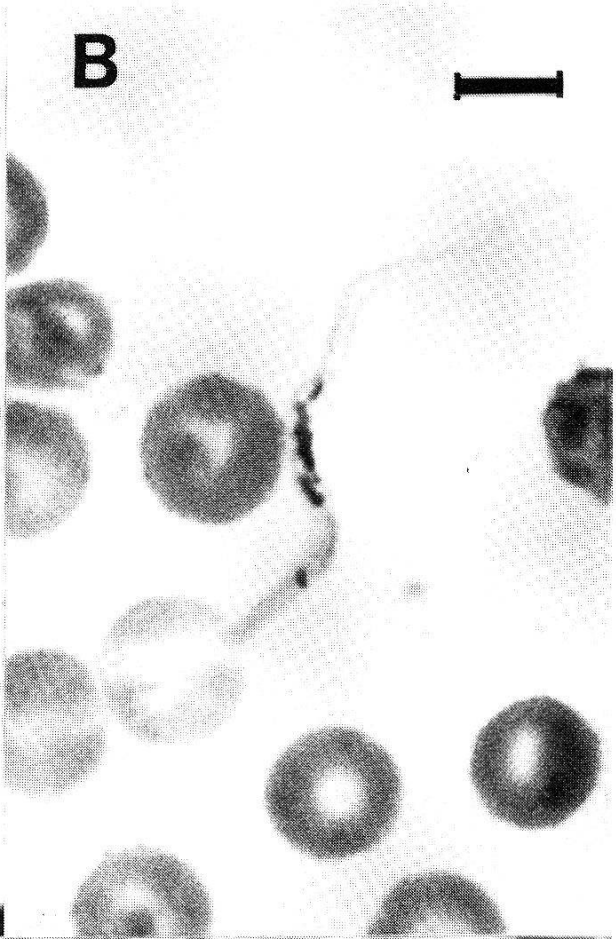
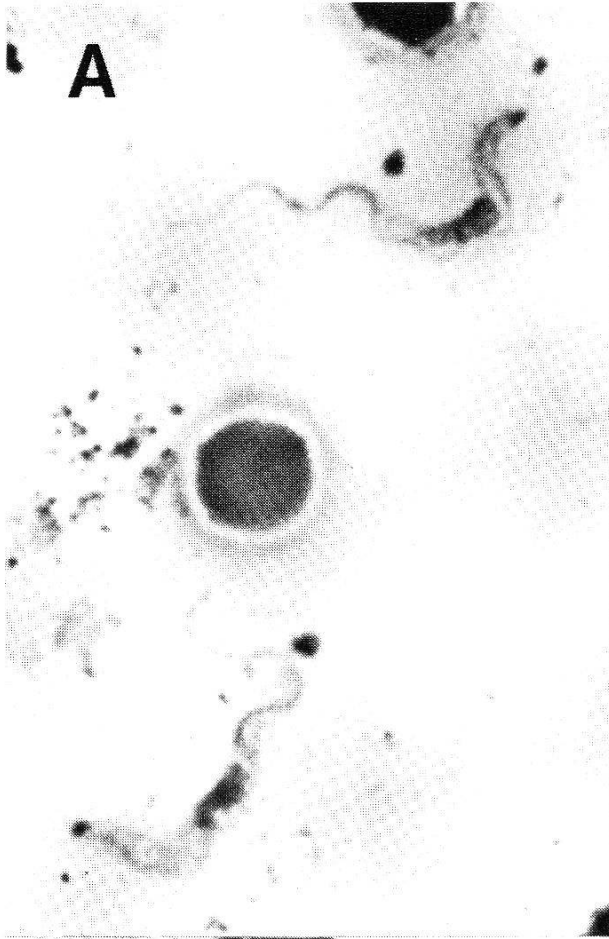


Fig. 4. Analysis of DNA content versus light scatter characteristics for different bloodstream populations of *T. vivax*. The FACS light scatter signals (horizontal) are displayed with fluorescence pulses (vertical) for the same early (A) and late (B) parasite populations analysed in Fig. 4.

parasite to the tsetse fly vector. Some investigators have reported observation of short stumpy parasites in the process of division and therefore have challenged its status as a nondividing cell (Lavier, 1927; Hoare, 1956). Previous evidence for nondivision of short stumpy forms was the rarity of observation of dividing short stumpy forms (Robertson, 1913), the predominance of short stumpy forms during falling parasitemias (Wijers, 1960; Luckins, 1972; Balber, 1972) and their relative or complete lack of infectivity for the mammal (Cunningham et al., 1963; Black et al., 1982). Our cytofluorometric study clearly confirms the generally accepted characterization of this form as a nondividing cell. In the most widely accepted model of the *T. brucei* life cycle, proliferating long slender parasites differentiate into nondividing short stumpy parasites. The stumpy forms can then transform into proliferating procyclic forms when taken into the fly midgut (Vickerman, 1971). The parasite population in the mammalian host's bloodstream is replenished by trypanosomes that have escaped the transition in the bloodstream by growing in the tissue spaces of the host's lymphoid organs (Tanner et al., 1980). In contrast, some investigators believe that since short stumpy organisms rapidly die in the mammalian bloodstream they are not a life cycle stage but are simply degenerating organisms (Ormerod et al., 1974). Failure to observe an analogous stage in the life cycle of other salivarian trypanosomes (Hoare, 1972; Ormerod, 1979) further weakens the argument for the *T. brucei* short stumpy form as a life cycle stage. Our analysis of DNA content in early procyclic cultures shows that most short stumpy organisms can respond to a stimulus mimicking transfer to the fly midgut with renewed DNA synthesis (Fig. 1). This supports the view that the short stumpy form is a stage in differentiation. An analogous situation is exhibited by the nondividing virgin B lymphocyte which also lives only a few days unless stimulated by antigen to differentiate into an antibody producing cell (Strober, 1977). Furthermore, our





detection of a nondividing late bloodstream form of *T. vivax*, with the recent report of such a stage in *T. congolense* (Nantulya et al., 1978), indicates that the morphological transformation of long slender to short stumpy parasite (Fig. 5C, D), long known in *T. brucei*, reflects a physiological transition from dividing to stationary phase parasite occurring in the life cycle of all species of salivarian trypanosomes.

Cessation of division in the stumpy form parasite may be a requirement of the process of differentiation into the stage specialized for transmission to the alternate host. Such a cessation of DNA synthesis and division prior to differentiation is seen frequently in metazoan systems (Tsanev, 1975). However, this change may be of additional importance to the African trypanosome because of the limitation it places on parasite population growth (Sendashonga and Black, 1982; Black et al., 1983b). Such limitation would clearly be of evolutionary value to the parasite by limiting the virulence of the infection (Herbert and Parratt, 1979). The fact that the transition to stumpy form occurs more readily with some parasite lines in their natural bovid hosts than in laboratory rodents (Black et al., 1983a) supports the suggestion that this stage of the parasite life cycle may represent an evolutionary adaptation of the parasite to its natural hosts. Furthermore this host specificity suggests that a specific host-parasite interaction may play a role in this differentiation event. An understanding of the molecular mechanism(s) underlying this transition to nondividing cell may suggest specific ways to enhance the transition and thus moderate the virulence of African trypanosome infections in humans and domestic animals.

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Fig. 5. Bloodstream forms of *T. vivax* and *T. brucei*. Smears of infected mouse tailblood on glass slides were fixed in methanol and then stained with Giemsa's solution. Typical early (A) and late (B) bloodstream forms of *T. vivax* and long slender (C) and short stumpy (D) bloodstream forms of *T. brucei* are shown. Scale bar represents 5  $\mu\text{m}$ .

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