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Evidence for diploidy in metacyclic forms of African trypanosomes

(parasitology/microfluorometry/flow cytometry)

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ABSTRACT **The DNA contents of bloodstream form trypanosomes (life cycle stages circulating in the blood of the vertebrate host) of four African *Trypanosoma* species and of metacyclic forms (the life cycle stage that is injected into the vertebrate by the tsetse fly during its bite) of the same four species were measured by cytofluorometry of individual cells or nuclei. The results showed unambiguously that the metacyclic forms cannot be considered to be products of meiosis containing only half of the DNA of bloodstream forms, in contrast to what was previously reported for *Trypanosoma brucei* [Zampetti-Bosseler, F., Schweizer, J., Pays, E., Jenni, L. & Steinert, M. (1986) *Proc. Natl. Acad. Sci. USA* 83, 6063–6064] during an attempt to localize the gametes in the life cycle after experimental evidence of sexual gene exchange in this parasite was reported.**

Three years ago direct experimental evidence was presented (1) of genetic exchange in *Trypanosoma brucei*, a trypanosome species complex of parasites infective for humans and livestock and transmitted in Africa by tsetse flies. The data appeared to fit a simple Mendelian model of eukaryotic chromosomal recombination through mating and meiosis, as suggested earlier by isoenzyme polymorphism in *T. brucei* isolates from East Africa (2). The hybridization experiments (1) indicated that the metacyclic stages (MET), found in the salivary glands of tsetse flies infected with *T. brucei*, could not be haploid, as has also been concluded from flow cytometric studies (3) and from the observation of hybrid genotypes in clones derived from single MET (4). Almost simultaneously, however, contradictory results were published, based on direct microfluorometry of total nuclear DNA of *T. brucei* life cycle stages, which suggested that MET were haploid and that they should be considered as gametes (5). In an attempt to bridge this apparent contradiction between the genetic and microfluorometric results, it was postulated that the hybrids might be tetraploid (6). It was subsequently reported (7) that bloodstream forms (BSF) of hybrids were temporarily subtetraploid, presumably as a result of somatic cell fusion in the tsetse fly. This would then temporarily generate subdiploid MET gametes, which should explain the heterozygous character of some of the clones derived from hybrid MET in spite of the gamete character of these forms.

To verify the gamete character of MET, the DNA contents of BSF and MET of different trypanosome isolates, including one of the two parent *T. brucei* clones used in the initial genetic studies (1), were measured accurately by fluorometry, enabling us to distinguish differences of a few femtograms (fg; 10^{-15} g) per nucleus. In all four *Trypanosoma* species, representing the three subgenera of African trypanosomes, *Duttonella*, *Nannomonas*, and *Trypanozoon*, MET did have the same ploidy as their corresponding BSF, and their gamete character, therefore, must be refuted.

MATERIALS AND METHODS

Microfluorometry. Animal blood or culture medium containing trypanosomes was spun (7 min, 1000 × *g*), and the trypanosomes were resuspended in phosphate-buffered saline (PBS, 142 mM NaCl/9.07 mM Na_2HPO_4/1.92 mM NaH_2PO_4, pH 7.3) and spun again (7 min, 1000 × *g*). PBS was removed and slides were made from the trypanosomes. *T. brucei* STIB 247 MET were derived by allowing tsetse flies (*Glossina morsitans centralis*) to probe directly on warm glass slides. Histograms were obtained by microfluorometry of individual nuclei after Feulgen/pararosaniline staining, as described earlier (8, 9). To avoid premature photodecomposition of the dye, cells were searched for and brought into focus under low-intensity long-wavelength light. Excitation time was 1 sec exactly. For each individual measurement the background value (5–10% of total fluorescence) was subtracted. Histograms were analyzed as indicated in the legend of Fig. 1.

Flow Cytometry. Measurements were performed with a FACS analyzer as described earlier (10). For each recording, trypanosome suspensions were mixed and then fixed in 70% methanol/30% PBS (vol/vol) for 70 hr at 4°C, stained in 1 μM Hoechst 33258 in PBS for 1 hr at 37°C, allowed to cool to room temperature, and measured immediately thereafter. Filter settings optimal for this dye were used. For each recording, 10,000 cells were counted.

Trypanosomes. *T. brucei brucei* EATRO 1066 (Lugala I) was isolated in 1958 in Uganda (11). *T. brucei brucei* TC 221 is a clone from strain 427 (12), isolated in 1960 from a sheep in Uganda and axenically cultured at 37°C (13); it shows long, slender BSF morphology and remains infective for mice and goats, as was regularly tested. *T. brucei brucei* STIB 247 is a 1971 isolate from a hartebeest in the Serengeti National Park, Tanzania (14). *Trypanosoma simiae* GMOS/GAM/85/008A/ITCE is from an experimental culture in Bristol; it was isolated in November 1985 in Keneba, The Gambia (P.D., unpublished data). *Trypanosoma congolense* IIU 100 was isolated in December 1986 in Utrecht from a dog that had recently traveled through East and Central Africa (Tanzania and Zaire). *T. congolense* Kilifi 82/IL/45/1 (15) and Transmara I (16) are from two different areas in Kenya. *Trypanosoma vivax* Zaria Y 58 was isolated in 1972 in north Nigeria

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Abbreviations: BSF, bloodstream form of trypanosomes; MET, metacyclic trypanosomes; FU, fluorescence units.

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from N'dama cattle (17). *T. vivax* IL 1392 is a derivative from stock Zaria Y 486, isolated in 1973 from a zebu in north Nigeria (17). *T. vivax* IL 2005 is from Uganda (18). These *T. vivax* isolates are all infective for mice. All BSF were obtained from mice, except *T. vivax* Y 58 from a goat, *T. simiae* from a pig, *T. brucei* 427 from a rat, and its derivative TC 221 from culture. MET were from tsetse flies (*T. brucei* STIB 247) or culture. Cultured MET were infective for mammals.

RESULTS AND DISCUSSION

The DNA content of BSF and MET of four Trypanosoma species, *T. brucei, T. congolense, T. simiae,* and *T. vivax,* was measured by direct microfluorometry of the nuclei of individual parasites after staining with Feulgen/pararosaniline (Table 1 and Fig. 1). This method has been used for decades for quantitative studies on a wide range of cell types (22). It is based on the covalent binding of the fluorescent dye to the aldehyde groups generated on the backbone of the DNA by hydrolytic removal of the purine bases. Under defined hydrolysis and staining conditions, binding is stoichiometric and the staining reaction is highly specific for DNA (23). Histograms were analyzed as indicated in the legend of Fig. 1 (21).

The DNA content of BSF of one *T. congolense* isolate (IIU 100) and of two *T. brucei* lines (EATRO 1066 and TC 221) that showed the largest difference after Feulgen staining was also measured by flow fluorometry after staining with Hoechst 33258 (Fig. 2), to see whether the small difference observed with the former method would also appear in measuring a large number of cells with a different technique and using a different reference cell. Hoechst 33258 binds preferentially to A+T-rich regions of double-stranded DNA in an equilibrium reaction (26).

The fluorescence intensity histograms for the BSF, obtained either by microfluorometry or by flow cytometry, were typical for mitotically dividing cell populations: they all showed a main peak of cells in G_1 phase of the cell cycle and a tail to the right representing cells during and after DNA replication, with or without a distinct second peak of cells in (G_2 + M) phase (Figs. 1*A* and 2). By contrast, histograms for MET, obtained in the same way, invariably showed a Gaussian distribution of fluorescence intensities, confirming that these stages are nonreplicating (Fig. 1*B*).

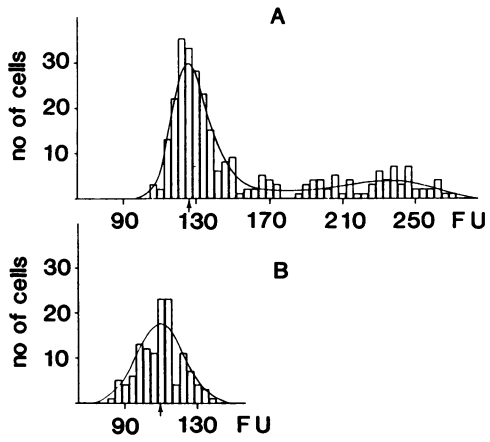

FIG. 1. Histograms of nuclear DNA content, expressed in fluorescence units (FU) obtained by microfluorometry of BSF (*A*) and MET (*B*) of *T. brucei* STIB 247. BSF are from blood smears of infected mice and MET are from saliva probes of infected tsetse flies. Bars represent the number of cells in each channel; the lines represent the best-fitting curve calculated by a model based on an iterative method (21). FU are equal to those in Table 1 (1 FU ≈ 1 fg, calibrated with *P. berghei* ringforms).

Table 1 shows that the three trypanosome subgenera, *Nannomomas, Trypanozoon,* and *Duttonella,* differed significantly with regard to the fluorescence values for their BSF (G_1 peak) as well as for their MET, while the values for the *T. congolense* isolates were nearly identical and were close to the values for the related *Nannomonas* species *T. simiae.*

Table 1 also shows that the fluorescence values for the MET of three of the four species agreed perfectly with the fluorescence value of the G_1 peak of the BSF of the corresponding *Trypanosoma* species. Not a single MET with only half the fluorescence intensity of BSF of the same species was detected. This also applies to the MET of *T. brucei* STIB 247 [a clone of which was used as parent in the initial genetic study (1)], reported in a previous study (5) to be haploid. Thus, none of the MET of the four trypanosome species can

Table 1. Nuclear DNA contents, expressed in Feulgen fluorescence values, of BSF and MET of various *Trypanosoma* species

Subgenus	Species	Isolate	Fluorescence value, FU	
			BSF in G_1 (*n*, % in G_1)	MET (*n*)
Trypanozoon	*T. brucei brucei*	EATRO 1066	130 ± 0.6 (223, 70%)	
		STIB 247	126 ± 0.8 (282, 62%)	111 ± 1.4 (128)
		TC 221	114 ± 0.8 (292, 52%)*	
		427	120 ± 1.7 (163, 64%)	
Nannomonas	*T. simiae*	GMOS/GAM/85/008A	90 ± 1.3 (119, 73%)	91 ± 1.1 (86)*
	T. congolense	IIU 100	94 ± 1.4 (246, 58%)	
		Kilifi 82/IL/45/1	96 ± 1.8 (67, 72%)	
		Transmara I		94 ± 1.0 (161)*
Duttonella	*T. vivax*	Zaria Y 58	134 ± 0.8 (134, 86%)	
		IL 2005	100 ± 0.7 (121, 77%)†	
		IL 1392		100 ± 0.6 (157)*†

The fluorescence value of the top of the best-fitting curve (see legend on Fig. 1) is considered to be the mean value of the cells in G_1 phase. Standardization is reached through measuring *Plasmodium berghei* (ANKA strain) ringforms (19) from a synchronized culture (20) along with the trypanosomes. These intra-erythrocytic ringforms do not synthesize DNA during the first 15 hr of their development and give narrow symmetrical fluorescence histograms (10, 19). The mean of 25 ringforms was set to 27 fluorescence units (FU) [≈27 fg (9)]. Results are given ± SEM, with the number of parasites and percentage in G_1 given in parentheses. From numerous measurements on different stages of trypanosomes, malaria parasites, and other protozoan parasites with genome sizes of the same order of magnitude (data not shown), it appeared that the coefficient of variation of the means of successive measurements is about 3% in our hands, so that differences larger than about 6% will be statistically significant at an acceptable level ($P \leq 0.05$).

*Values are for cultured forms.

†These *T. vivax* BSF and MET were compared directly. The mean of the MET was arbitrarily set to 100 FU. These units, therefore, are not comparable with those in all preceding lines of the table.

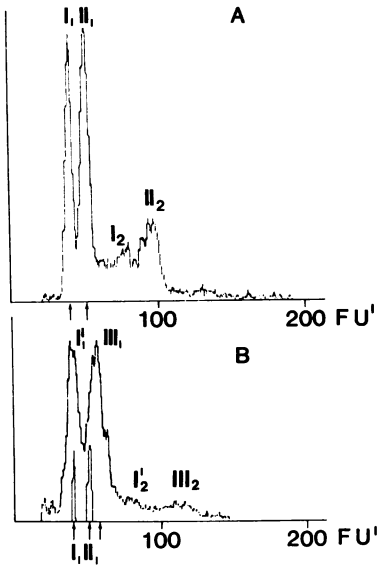

FIG. 2. Histograms of DNA content obtained by flow cytometry of *Trypanosoma* BSF. The abscissa represents fluorescence intensity in arbitrary units (FU') on a linear scale; the ordinate, the relative number of cells. (*A*) I_1 and I_2, G_1 and G_2 peaks, respectively, of *T. congolense* IIU 100; II_1 and II_2, G_1 and G_2 peaks, respectively, of *T. brucei* TC 221. (*B*) I'_1 and I'_2, G_1 and G_2 peaks, respectively, of *T. congolense* IIU 100; III_1 and III_2, G_1 and G_2 peaks, respectively, of *T. brucei* EATRO 1066; I_1 and II_1, tops of G_1 peaks from *A*. *T. congolense* was separated from erythrocytes by differential centrifugation (24); *T. brucei* EATRO 1066 was separated by column chromatography (25). *T. brucei* TC221 was derived directly from cell-free cultures. Trypanosome populations of the two species were mixed and then further processed as indicated in *Materials and Methods*.

be considered haploid, if BSF are diploid, as previous reports indicate (1, 2, 4, 27, 28).

Recently the same conclusion, as far as *T. congolense* is concerned, has been reached from Feulgen microfluorometry measurements on two clones (29), in spite of the fact that in one of these clones a difference between MET and BSF was found of the same order as that on which it was previously concluded that *T. brucei* MET are haploid (5).

We cannot explain why MET of the same *T. brucei* strain were reported to be haploid in an earlier study (5). Essentially the same technique was used. There are, however, a few differences which may be crucial. (*i*) We measure the cells without a preexposure time of 7 sec to the excitatory light (5), thus improving the resolution of the technique. During 7 sec of excitation the fluorescence emission decreases by 30–40% (9). (*ii*) Time of excitation during the measurement itself is crucial. We illuminate each parasite for 1 sec, which is the optimal time for cells with small genome sizes (W.A.L.D. and R.F.K., unpublished observation). The excitation time is not given in ref. 5. (*iii*) We always remove serum components from BSF preparations before fixation to reduce background fluorescence to 5–10% (percentage not given in ref. 5). This appears not necessary for MET derived from tsetse flies by direct probing on glass slides. Although the background fluorescence is subtracted from the nuclear fluorescence, too high levels can lead to systematically elevated estimates of nuclear fluorescence of BSF (C.J.J. & R.F.K., unpublished observation). The absence of a reference value in the contradicting paper (5) makes it impossible to distinguish whether the reported difference between MET and BSF arose as a result of increased BSF values or of decreased MET values. (*iv*) The histogram representing the BSF (and proventricular) DNA content distribution in ref. 5 does not give a distinct G_2 peak where it is expected, at the double G_1 peak value, in contrast to our histograms (Fig. 1).

The only difference we found was a 13% difference between MET and BSF of *T. brucei* STIB 247. Though far too small to attribute to haploidy in these MET, this difference needs to be considered. The fluorescence value of these MET corresponded with the G_1 peak value for cultured BSF of TC 221 (a clone of stock 427), while that of the BSF of STIB 247 corresponded with the value of BSF of another *T. brucei brucei* isolate, EATRO 1066. The BSF populations examined from the latter two stocks were polymorphic, with the short, stumpy, nondividing forms by far exceeding the long, slender, dividing forms; hence, the low percentage of total cells in (G_2 + M) phase in the histograms of these samples (Figs. 1 and 2*B*). By contrast, the percentage of cells in (G_2 + M) phase for TC 221 BSF, consisting of monomorphic long, slender, and dividing trypanosomes collected from culture during the logarithmic phase of growth, was much larger, resulting in a distinct second peak in the histogram (Fig. 2*A*). The small but significant (see legend of Table 1) difference between the G_1 peak values of the monomorphic cultured TC 221 BSF and that of the polymorphic EATRO 1066 BSF, detected by microfluorometry using *P. berghei* ringforms as reference cells, was confirmed when both were compared by flow fluorometry with the same *T. congolense* BSF (Fig. 2). We assume that this difference is related to cell differentiation to short, stumpy forms in the BSF of the polymorphic stocks. Using pulsed-field gel electrophoresis, a technique suitable to separate whole chromosomes of trypanosomes (30), we have indeed obtained indications of slightly increased DNA in short, stumpy forms as compared to long, slender forms. Whatever the explanation for the difference, it is reasonable to consider the G_1 peak value for long slender, actively dividing BSF of *T. brucei* as being closest to the diploid genome size of this stage. We conclude that the MET of *T. brucei* and those of the other trypanosome species examined are diploid and that it remains to be determined where sexual stages occur in the life cycles of these parasites.

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