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Production of metacyclic forms by cyclical transmission of West African *Trypanosoma (T.) brucei* isolates from man and animals

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Summary

Fifteen West African *Trypanosoma (T.) brucei* isolates from man and animals were cyclically transmitted. Five stocks, belonging to the *non-gambiense* group, could easily be transmitted through *Glossina morsitans morsitans* or *Glossina m. centralis* infected on mice, whereas successful transmission of the 10 isolates, identified as *Trypanosoma brucei gambiense*, was performed using *G. palpalis gambiensis* as vector. *Glossina p. gambiensis* was infected with culture-derived procyclic trypanosomes by repeated membrane feeding. In both cases, metacyclic forms could normally be detected in saliva samples of positive flies 3 to 4 weeks after first infection. These forms of major interest were subsequently characterized relative to their resistance/sensitivity against normal human serum in vitro and their antigenic properties, using indirect immunofluorescence: Metacyclic forms of all the *T. b. gambiense* isolates were determined by a stable human serum resistance and a restricted metacyclic variable antigen type (mVAT) repertoire, whereas representatives of the *non-gambiense* group (including TH162/78E 021) were sensitive against the trypa-nolytic factors of normal human serum and expressed a heterogeneous meta-cyclic antigen profile.

Key words: *Glossina*; *Trypanosoma brucei gambiense*; cyclical transmission; metacyclic forms.

Introduction

Limited experimental work has been done concerning cyclical transmission of *Trypanosoma brucei gambiense* in the laboratory (Moloo et al., 1986).

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However, field data from West Africa indicate a clear preference of *T. b. gambiense* for tsetse flies of the riverine *Glossina palpalis* group as their natural vectors (Hoare, 1972).

New technologies, like isoenzyme analysis and DNA hybridization, revealed the existence of 2 different types of human pathogenic trypanosomes in West Africa, a *T. b. gambiense* group, distinguishable from *T. b. rhodesiense* and *T. b. brucei*, and a *non-gambiense* group (Paindavoine et al., 1986).

In vivo and in vitro metacyclogenesis of *T. b. gambiense* has been a major goal in trypanosomiasis research for a long time, since the vertebrate infective forms of this subspecies, generated by the fly, are characterized by an extremely stable and restricted metacyclic variable antigen type (mVAT) repertoire compared to that of *T. b. rhodesiense* or *T. b. brucei* (Gray, 1972; Jones et al., 1981; Turner, 1985).

In this paper, we present a possible approach to obtain *T. b. gambiense* metacyclic forms by membrane feeding of *Glossina palpalis gambiensis* with culture-derived procyclic forms and subsequent cyclical transmission. The resulting metacyclic forms represent a highly interesting and challenging test material for diverse phenotypic and genotypic investigations with regard to immunization against *gambiense* sleeping sickness (Steinert and Pays, 1986).

Material and Methods

Trypanosome stocks

A complete list of cyclically transmitted West African *Trypanosoma (T.) brucei* stocks is presented in Table 1. They were isolated from human patients and animals in highly endemic areas of the Ivory Coast and Liberia (Mehlitz et al., 1981, 1982; Zillmann et al., 1984).

Glossina species

Puparia of *Glossina morsitans morsitans* were obtained from the Tsetse Research Laboratory, Department of Veterinary Medicine, Langford House, Langford, Bristol; those of *Glossina morsitans centralis* were received from the International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya. *Glossina palpalis gambiensis* originated from the colony of the Institut d'Élevage et de Médecine Vétérinaire des Pays Tropicaux (IEMVT), Maisons-Alfort, France.

Transformation of bloodstream parasites to procyclic culture forms

Bloodstream trypanosomes, propagated in immunosuppressed *Mastomys natalensis* (Richner et al., in prep.) were used either to infect *G. m. morsitans* and *G. m. centralis* or were directly transformed to procyclic forms in vitro (Fig. 1). In vitro transformation of bloodstream forms from the vertebrate host or isolation of trypanosomes as midgut forms from the vector was performed according to Brun and Schönemberger (1979, 1981) with some modifications: The medium initially used for in vitro transformation was Minimum Essential Medium (MEM) with 25 mM HEPES, 1 g/l additional glucose, 1% MEM nonessential amino acid concentrate (100×) supplemented with 15–20% heat-inactivated foetal bovine serum (in-FBS) and the TCA-cycle intermediates, citrate and cis-aconitate at 3 mM concentrations (Brun and Schönemberger, 1981). After one week, MEM was replaced by a semidefined medium (SDM-79, Brun and Schönemberger, 1979) and 10% inactivated fetal bovine serum (in-FBS). Gentamycin (10 µg/ml) was used as an antibiotic throughout the experiment. Midgut forms isolated from infected tsetse flies could be directly adapted to SDM-79 containing 15% in-FBS and 50–100 µg/ml gentamycin depending on secondary contamination.

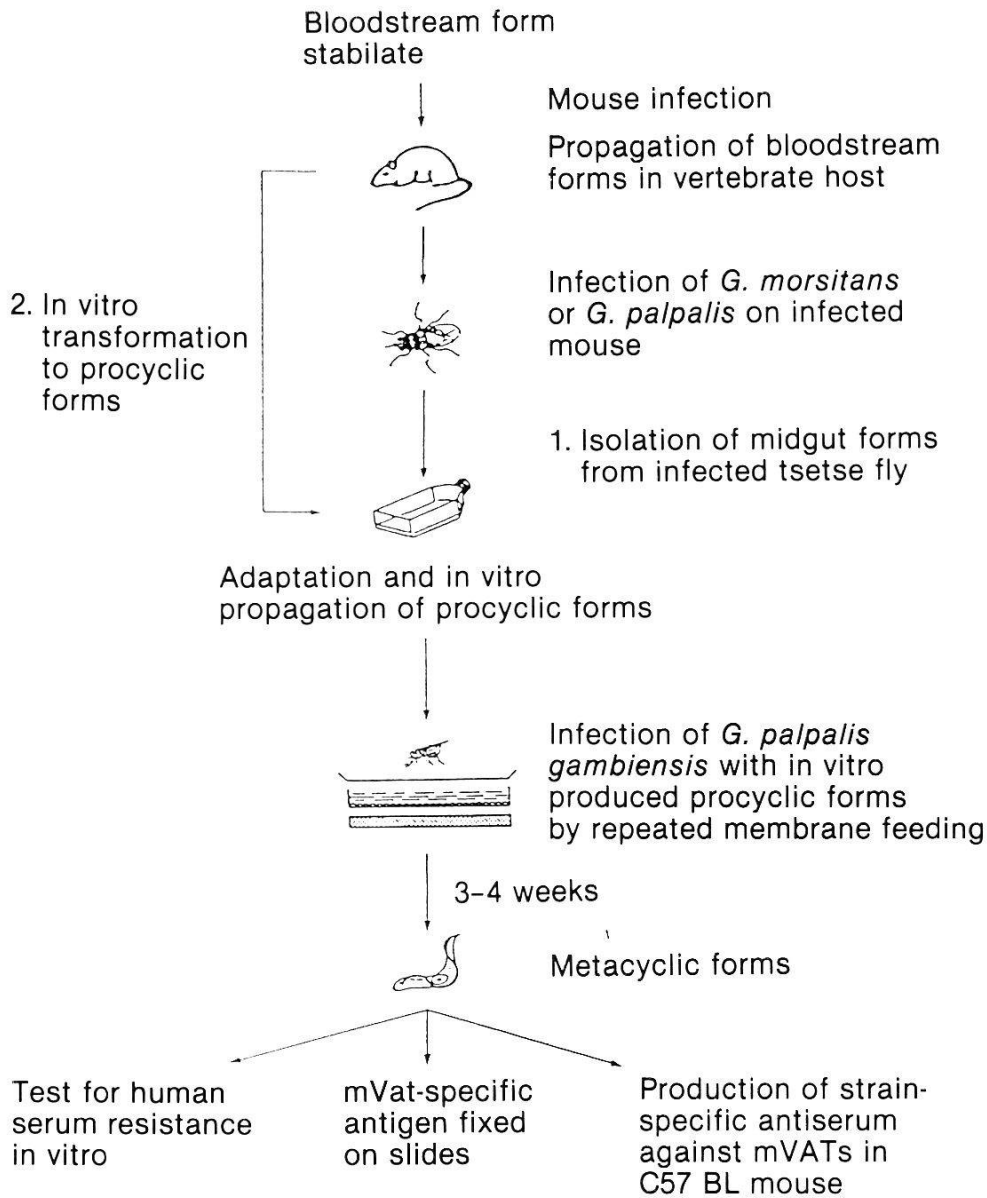


Fig. 1. Schematic illustration of cyclical transmission experiments with *Trypanosoma brucei gambiense*.

Transformation and adaptation were carried out at 27°C in 10 ml plastic tubes (Falcon) or T-25 tissue culture flasks (Sterilin, Teddington, UK).

Preparation of washed human red blood cells

Human whole blood (group A, Rhesus +) was centrifuged under sterile conditions for 20 min at 800 g and 7°C. The supernatant plasma was discarded and the blood cell pellet was resuspended in an equal volume of SDM-79. The procedure was repeated twice and the washed human red blood cells were stored at 7°C for up to one week.

Rehydration of freeze-dried pig blood

Lyophilised pig blood from the International Atomic Energy Agency (IAEA), Vienna, was reconstituted under sterile conditions with sterile distilled water (1:4.32 w/w). Gentamycin (0.5 µg/ml) was added and the blood was stored frozen in batches of 20 ml at -20°C until use.

Preparation of infective mixtures

Procyclic forms used for infection of *G. p. gambiensis* by membrane feeding technique were taken from well adapted cultures in the logarithmic growth phase. The procyclic cultures were counted just before use in a Coulter Counter (model ZBI). The cell densities were in the range 2×10^6 – 2×10^7 /ml of medium. Procyclic culture forms were offered to the tsetse flies by membrane feeding technique (Bauer and Wetzel, 1976) in 3 different mixtures B, C and D (compare with Gingrich et al., 1985):

- B Procyclic culture forms in SDM-79 with 10% in-FBS and gentamycin (10 µg/ml)/rehydrated lyophilized pig blood at a ratio of 1:1 (v/v).
 - C Procyclic culture forms in SDM-79 with 10% in-FBS and gentamycin (10 µg/ml)/human red blood cells at a ratio of 7:3 (v/v).
 - D Procyclic culture forms in SDM-79 with 10% in-FBS and gentamycin (10 µg/ml)/human red blood cells at a ratio of 1:1 (v/v).
- B, C, D, see Table 1, mode of infection and maintenance of flies.

Infection of Glossina to transform bloodstream trypanosomes to procyclic midgut forms

Teneral *Glossina m. morsitans* or *G. m. centralis* were allowed to feed on an infected *M. natalensis* for 15–20 min under conditions described by Richner and Jenni (1986).

Infection of Glossina palpalis gambiensis with procyclic culture forms by membrane feeding technique

The first infective meal was offered to *G. p. gambiensis* within 24 h of hatching. Subsequent artificial infective bloodmeals (1 to 12) were given to the same flies at intervals of one to two days. The duration of feeding procedure was normally restricted to 30 min and the viability of the trypanosomes was checked by phase contrast microscopy before and after every blood-meal.

Maintenance of infective tsetse flies

Infected tsetse flies were fed on freeze-dried or fresh-defibrinized pig blood mixed with SDM-79 in 1:1 ratio for *G. p. gambiensis* or fed undiluted to tsetse flies of the *G. morsitans* group (Richner and Jenni, 1986).

Analysis of metacyclic forms

1. Living metacyclic forms were harvested from infected flies by allowing them to salivate into a drop of warmed medium (MEM containing 20% heat-inactivated horse serum) on a lymphocyte migration plate (Richner and Jenni, 1986). Extruded metacyclic forms were tested for human serum resistance in vitro according to Jenni and Brun (1983).
2. Alternatively, saliva samples containing metacyclic trypanosomes were fixed on slides (Hölzel) using acetone (10 min) and stored at -70°C in desiccated plastic bags for subsequent indirect fluorescent antibody test (IFAT). Fixed metacyclic forms of *T. b. brucei* STIB 247-L were used as a control.

Preparation of antisera and subsequent indirect immunofluorescent antibody test (IFAT)

Stock specific antisera against metacyclic VATs were raised in C57BL mice (Richner and Jenni, 1986) and the specificity for surface glycoproteins was checked on fixed procyclic culture forms used as antigen. The indirect fluorescent antibody test (IFAT) was performed in homologous and heterologous reactions according to Ambroise-Thomas (1969).

Results

Cyclical transmission

The main results of the cyclical transmission experiments are listed in Table 1. TH162/78E (021) was the only West African human isolate transmis-

sible through both *G. m. centralis* and *G. p. gambiensis*. Extensive attempts to transmit *T. b. gambiense* TH3/78E (020) and TH152/78E (026) through *G. m. centralis* have failed so far (Table 1). Mature, vertebrate infective forms could never be obtained with these two stocks, which was proved by several unsuccessful trials of cyclical infection in mice. For all the other *T. b. gambiense* stocks tested here, only midgut infections could be established in tsetse flies of the *morsitans* group; in the salivary glands only epimastigotes occurred. In *G. p. gambiensis*, however, the same stocks reached maximal rates of mature salivary gland infections of more than 10% e.g. TH152/78E (026).

No significant difference between the artificial infective diets (B, C, D) could be observed with respect to salivary gland infection rate or survival of the flies. With diet C, an accelerated digestion was evident so that the ratio between liquid and solid components was altered to 1:1 (diet D).

The two *non-gambiense* isolates TSW180/78E (028) and TD52/78E (021) could be transmitted through *G. m. morsitans*, by feeding on an infected mouse, and through *G. p. gambiensis* by the membrane feeding technique. Conversely, *non-gambiense* TSW73/78E (022) and TGP2/80 Lib isolates have only been transmitted through *G. p. gambiensis* (membrane feeding).

Human serum resistance of metacyclic forms (Table 2)

TH162/78E (021) was the only human isolate showing sensitivity against the lytic factors of normal human serum in metacyclic forms as well as in the subsequent bloodstream form population. All the other isolates from West African human patients expressed stable resistance to human serum. The animal isolates were human serum sensitive.

Indirect immunofluorescent antibody test (IFAT)

Heat-inactivated antisera were applied in a reciprocal serum dilution of 80 and 40 in the case of TH 170/78E (027) and TH1/78E (031). Controls on procyclic culture forms revealed a high degree of coat-specificity (negative reactions).

Metacyclic forms of the different *T. b. gambiense* stocks tested in heterologous reactions showed an extreme homogeneity concerning their surface antigens (> 90% of positive trypanosomes). Antisera against *T. b. gambiense* metacyclics did not react with *T. b. brucei* STIB 247-L metacyclic antigen. Antiserum TH162/78E (021) revealed negative reactions with metacyclic forms of all *T. b. gambiense* isolates.

Discussion

The principal aim of the present study was to produce metacyclic forms of *T. b. gambiense*, comparative data are still lacking for most of the stocks with respect to their vector preference or vector specificity. Negative control experi-

Table 1. List of cyclically transmitted *T. b. gambiense* and *non-gambiense* stocks, isolated in the Ivory Coast and Liberia containing stabilate designation, tsetse fly species, infection and maintenance of flies, salivation and infection rates

Stabilate designation	Tsetse species (source)	Mode of infection, number of infectious meals	Maintenance of flies	Saliva analysis of individual surviving flies neg / met / epi	% meta-infections of originally infected flies
<i>T. b. gambiense</i>					
TH1/78E (020)	G.p.g. (N)	B (1)	B	48 / 1 / -	1.01
	G.p.g. (P)	B (2-8)	B	100 / - / 1	0.00
TH3/78E (020)	G.p.g. (N)	B (2)	R	259 / 1 / -	0.34
	G.m.c. (N)	B (2)	R	84 / - / 1	0.00
	G.m.c. (N)	D (6)	C	30 / - / 1	0.00
TH141/78E (022)	G.p.g. (P)	D (3-6)	B/D	201 / 14 / 1	3.88
TH31/78E (025)	G.p.g. (P)	D (4-6)	D	100 / 4 / -	2.72
TH152/78E (026)	G.p.g. (P)	B (7-12)	B	93 / 11 / -	3.80
	G.m.c. (N)	D (7)	C	34 / - / 9	0.00
	G.p.g. (P)	D (3-8)	D	218 / 2 / 1	0.55
TH115/78E (027)	G.p.g. (P)	D (3-7)	D	126 / 10 / -	4.13
TH170/78E (027)	G.p.g. (P)	B (7-9)	B	118 / 4 / -	1.15
TH1/78E (031)	G.p.g. (P)	D (2-3)	D	131 / 3 / -	1.14
TH2/78E (031)	G.p.g. (P)	D (1-4)	D	152 / 4 / 1	1.12
TH Gamey Dolo/80 Lib	G.p.g. (P)	D (4-9)	D	111 / 4 / -	1.08
<i>Non-gambiense</i>					
TH162/78E (021)	G.p.g. (P)	B (4-7)	B	174 / 10 / 26	3.38
	G.m.c. (N)	C (3-5)	A	214 / 18 / 18	5.20
TH162/78E (021) clone 2	G.m.c. (N)	D (2)	C	40 / 2 / 3	2.63

Table 1. (continued)

Stabilate designation	Tsetse species (source)	Mode of infection, number of infectious meals	Maintenance of flies	Saliva analysis of individual surviving flies neg / met / epi	% meta-infections of originally infected flies
TSW73/78E (022)	G.p.g. (P)	D (1-4)	D	122 / 3 / 2	0.08
TSW180/78E (028)	G.p.g. (P)	D (3-5)	D	30 / 18 / 1	14.40
	G.m.m. (L)	A (2)	C	25 / 5 / -	6.25
TD52/78E (021)	G.m.m. (L)	A (1-2)	C	56 / 11 / 5	7.28
TGP 2/80 Lib	G.p.g. (P)	D (1-4)	D	87 / 2 / 1	0.91
G.p.g. = <i>Glossina palpalis gambiensis</i>					
G.m.c. = <i>Glossina morsitans centralis</i>					
G.m.m. = <i>Glossina morsitans morsitans</i>					
(N) = International Laboratory for Research on Animal Diseases (ILRAD), Nairobi, Kenya					
(P) = Institut d'Élevage et de Médecine Vétérinaire des Pays Tropicaux (IEMVT), Maisons-Alfort, France					
(L) = Tsetse Research Laboratory, University of Bristol, Langford, Bristol, UK					
Lib. = Liberia					
<i>Mode of infection</i>					
In vivo feeding: A = feeding fly on infected mouse					
Membrane feeding technique: B = Procyclic culture forms/rehydrated lyophilized pig blood (RLPB), 1:1					
C = Procyclic culture forms/human red blood cells (HRBC), 7: 3					
D = Procyclic culture forms/HRBC, 1:1					
<i>Maintenance of infected flies</i>					
Saliva analysis					
A	= Rehydrated lyophilized pig blood (RLPB)	neg	= negative		
B	= RLPB/semi-defined medium SDM-79, 1:1	meta	= metacyclic forms		
C	= Fresh defibrinized pig blood (FDPB)	epi	= epimastigote forms		
D	= FDPB/SDM-79, 1:1				
R	= Feeding on rabbit				

Table 2. Resistance to normal human serum of metacyclic forms tested in vitro

Stabilate designation	Result of in vitro test for human serum resistance
TH1/78E (020)	+
TH3/78E (020)	+
TH141/78E (022)	+
TH31/78E (025)	+
TH152/78E (026)	+
TH115/78E (027)	+
TH170/78E (027)	+
TH1/78E (031)	+
TH2/78E (031)	+
TH Gamey Dolo/80 Lib	+
TH162/78E (021) (including clone 2)	-
TSW73/78E (022)	-
TSW180/78E (028)	-
TD52/78E (021)	-
TGP2/80 Lib	nd

+ = Human serum resistant

- = Human serum sensitive

nd =Test not done

ments with *T. b. gambiense* TH3/78E (20) and TH152/78E (026) stocks in combination with *G. m. centralis* demonstrated that a preference for *G. p. gambiensis* was evident, as infective metacyclic forms could just be produced in this *Glossina* subspecies (Table 1). In *G. m. centralis* the cyclical development of *T. b. gambiense* (Table 1) was blocked at the epimastigote stage, indicating a possible species-specific stimulating or inhibiting factor within the salivary gland, which could be responsible for maturation to metacyclics. Contrary to these observations, other authors were able to transmit certain *T. b. gambiense* stocks through *G. m. centralis*, suggesting that there is no absolute restriction of *T. b. gambiense* for one *Glossina* subspecies (Brun and Jenni, 1983; Mooloo et al., 1986).

Membrane infection of *G. p. gambiensis* with procyclic culture forms proved to be a very suitable method for the transmission of *T. b. gambiense*. The main advantages are as follows: Bloodstream forms of this avirulent subspecies often occur in a subpatent quantity within their vertebrate hosts. This characteristic feature renders a successful infection of a large number of flies at a given time (within 24 h after hatching) rather difficult. However, in vitro produced procyclic *T. b. gambiense* are available at any time and in large quantities. Hundreds of flies can be infected at the same time, under identical experimental conditions. The use of laboratory animals can be reduced. Procyclic culture forms are preadapted to the ecological environment of the tsetse midgut, resulting in higher midgut infections rates (Evans, 1979; Gingrich et al., 1985).

Best results were obtained with freshly adapted procyclic forms which had been in the culture system for about one month, compatible with the observations made by Schöni et al. (1982). Evans (1979), however, was able to transmit trypanosome stocks, which have been cultured as procyclic forms for 14 months previous to infection.

The results from the *in vitro* human serum resistance tests of metacyclic forms confirmed previous observations in that *T. b. gambiense*, even in its metacyclic form, is highly resistant against the trypanolytic factors of normal human serum (Paindavoine et al., 1986). Conversely, *non-gambiense* stocks were characterized by a complete loss of human serum resistance in both metacyclic and subsequent bloodstream form populations (Jenni and Brun, 1984; Brun and Jenni, 1987), indicating that there was no human-pathogenic stock among the 3 animal isolates (compare Gibson et al., 1978; Mehlitz et al., 1982; Zillmann et al., 1984; Paindavoine et al., 1986).

The results from the indirect immunofluorescent antibody test (IFAT) revealed a high degree of antigenic similarity among the different *T. b. gambiense* isolates (Broom and Brown, 1940; Gray, 1965, 1972). All the heterologous reactions between the *T. b. gambiense* stocks proved to be highly positive, independent of their geographical origin within West Africa. Nonreactive forms were most probably extruded pre-metacyclic (coat-less) trypanosomes (Steiger, 1973; Le Ray et al., 1978; Vickerman, 1985). Crossreactions between *T. b. gambiense* and *non-gambiense* stocks turned out to be negative. The East African isolate STIB 247-L, used as a control, showed about 50% of positive forms in the reaction with *non-gambiense* stock TH 162/78E (021), but reacted negatively with *T. b. gambiense*. These results clearly indicate a great homogeneity among *T. b. gambiense* mVATs, whereas *non-gambiense* stocks seem to be rather heterogenous. Because its antigen repertoire is remarkably stable compared to that of *T. b. brucei*, the *T. b. gambiense* mVATs could be a major target for future immunization trials (Steiner and Pays, 1986). The method of cyclical transmission of *T. b. gambiense* in the laboratory described here, could be of principal interest because of its relative simplicity in supplying material for e.g. molecular investigation of *T. b. gambiense* metacyclic forms.

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