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Studies on *Trypanosoma (nannomonas) congolense*

III. Antigenic variation in three cyclically transmitted stocks

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SUMMARY

Cyclical transmission of different variable antigen types of *Trypanosoma congolense* STIB 228 resulted in the development of metacyclic trypanosome populations which were similar in their variable antigen composition as judged by immunofluorescence and neutralization assays. The variable antigen types present in the ingested bloodstream populations were not found in the metacyclic populations. The bloodstream populations which were obtained from cyclically infected, irradiated (900 rad.) mice contained variable antigen types which were not present in the corresponding metacyclic populations. When derivatives of 2 other stocks of *T. congolense*, isolated in a different area of Tanzania, underwent cyclical development in the tsetse fly, the metacyclic populations of each stock also had a characteristic variable antigen composition. The metacyclic populations of the 3 stocks were, however, completely dissimilar in variable antigen composition. Simultaneous infection of tsetse flies with a mixture of different stocks resulted in the concurrent production of metacyclic trypanosomes which contained the characteristic variable antigen types of each stock. The effect of cyclical transmission on the process of antigenic variation in *T. congolense* infections is therefore similar to that in *T. brucei* infections.

INTRODUCTION

It was originally observed by Broom & Brown (1940) that if different variable antigen types of the same stock of *Trypanosoma brucei* were cyclically transmitted to new hosts the antigenic composition of the first detectable trypanosome population in each was similar. The antigenic compositon of this first population also appeared to be characteristic of each stock since cyclical transmission of different stocks gave rise to early populations which contained different variable antigen types.

This phenomenon was then investigated by several workers and the results have been the subject of several reviews (Vickerman, 1974; Gray & Luckins, 1976; Doyle, 1977). More recently, however, it has been demonstrated that the variable antigen composition of the metacyclic trypanosome population is itself

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different from that of the bloodstream trypanosomes ingested by the tsetse fly but that the antigenic composition of metacyclic populations which arise following cyclical transmission of different bloodstream variable antigen types of the same stock is, however, similar (Jenni, 1977*a*, *b*; Le Ray, Barry & Vickerman, 1978). The metacyclic populations derived from different stocks of *T. brucei* apparently also consist of different characteristic variable antigen types (Jenni, 1977*a*, *b*).

As the work that has been carried out to investigate the occurrence of a similar phenomenon in cyclically transmitted *T. congolense* infections has yielded conflicting results (Wilson & Cunningham, 1970; Uilenberg & Giret, 1972*a*, *b*; Uilenberg, Maillot & Giret, 1973; Schlappi & Jenni, 1977) it was decided to re-investigate this situation by analysing the antigenic composition of stocks and clones of *T. congolense* before and after cyclical transmission.

MATERIALS AND METHODS

Trypanosomes

The trypanosome populations used in this study were derived from 3 stocks of *T. congolense*, STIB 228, STIB 212 and STIB 249, all isolated from individual lions in the Serengeti National Park, Tanzania (Geigy & Kauffmann, 1973).

Three derivatives of STIB 228 were used in this study. STIB 68-F was obtained from lethally irradiated mice 26 days following cyclical transmission of an extensively syringe-passaged line (31 passages in mice) of the original isolate STIB 228. STIB 68-F was subsequently inoculated into lethally irradiated mice and the population cloned, also in irradiated mice, to provide population STIB 68-O. STIB 68-O was also extensively passaged in normal and irradiated mice before finally being cloned on day 24 of an infection in a normal mouse to provide population 68-M-AA. For convenience, these populations will subsequently be referred to as 228A (STIB 68-F), 228B (STIB 68-O) and 228C (STIB 68-M-AA).

STIB 212 was originally isolated 12 days after infection of a normal rat with blood taken from an infected lion. STIB 212C is a derivative of STIB 212 obtained on day 7 following cyclical infection of a normal mouse with STIB 212.

STIB 249 was originally isolated 14 days after infection of a normal rat with blood taken from another infected lion and STIB 249B was obtained on day 7 following cyclical infection of a normal mouse with STIB 249.

Laboratory animals

ICR white female mice and male C57Bl/6 mice were used. In preliminary experiments both mouse strains were found to be equally susceptible to infection with the stocks of *T. congolense* used in this study.

Tsetse flies

Pupae of *Glossina m. morsitans* were provided by the Tsetse Research Laboratory, Langford, Bristol, U.K., and maintained as described by Nantulya, Doyle & Jenni (1978). Teneral flies were allowed to feed once on a mouse infected with the try-

panosome population under study. Infected flies were then identified by examination of saliva probes and maintained in separate cages as the source of metacyclics.

Metacyclic trypanosomes

Metacyclic trypanosomes were obtained for the neutralization of infectivity test by either allowing infected flies to probe into a warm drop of phosphate-buffered saline glucose (PSG), pH 8 (Taylor, Lanham & Williams, 1974), or by gently holding the fly round the thorax, which stimulated the formation of a drop of saliva at the tip of the proboscis. The tip of the proboscis was then rinsed in a drop of PSG.

Immunofluorescence was carried out on the trypanosome population present in saliva probes obtained when infected flies were allowed to probe on marked areas of warm microscope slides.

Experimental design

The first experiment was designed to investigate the effect of cyclical development on one stock (228A) and 2 clones (228B, 228C) derived from this stock, in respect of the antigenic composition of (*a*) the original ingested bloodstream populations, (*b*) the metacyclic populations and (*c*) the first bloodstream forms appearing after cyclical transmission.

The second experiment was designed to compare the antigenic composition of the metacyclic trypanosome populations obtained after cyclical development of the 3 stocks of *T. congolense* isolated in different areas of the Serengeti National Park.

Production of antisera

Antiserum to each metacyclic population was obtained as follows. Three flies, infected with a given trypanosome population, were fed on a single normal mouse on 4 occasions at 2-day intervals and the mouse bled 10 days after the initial feed. Trypanosomes were first found in the peripheral blood of such mice 10 days after the first fly bite, by the haematocrit centrifuge technique (Woo, 1971). Antisera to the cloned bloodstream populations 228B and C were obtained by infecting normal mice with 10^5 trypanosomes from a cryopreserved stabilate of either clone and bleeding 8 days later.

Serological tests

(*a*) *The indirect immunofluorescent-antibody test* (*IFAT*)

This was performed on formalin-fixed bloodstream trypanosomes as described by Nantulya & Doyle (1977). The IFAT on metacyclic trypanosomes was carried out on saliva probe material without prior fixation.

Antisera to metacyclic populations were routinely titrated at doubling dilutions from 1/5 to 1/80 against homologous and heterologous populations and used at dilutions of 1/20 and 1/40 which represented the mid-points of the plateau titres. Similarly, antisera to bloodstream populations were titrated at doubling dilutions from 1/10–1/1280 and again used at dilutions of 1/160 and 1/320 which represented the mid-point of the plateau titres. Dilutions of normal mouse serum and buffer alone were included as specificity controls in each assay.

The fluorescent conjugate used was an FITC-conjugated IgG fraction of a rabbit anti-mouse immunoglobulin (Lot 8864, Capel Laboratories Inc., Downington, Penn.). It had a molar fluorescein to protein ratio of 3:1 and after titration was routinely used at a dilution of 1/80.

Microscopic examination was carried out using phase/fluorescence objectives ($\times$ 40 and $\times$ 63) on a Zeiss fluorescent microscope equipped with epi-illumination, a 100 W halogen lamp together with KP 490/500 exciting filter and LP 455 barrier filter.

(*b*) *The neutralization of infectivity tests* (*NIT*)

The neutralization assay for metacyclic trypanosomes was performed as described by Jenni (1977*a*). Three replicates each of 50–100 metacyclic trypanosomes (saliva probes from 2 flies) were incubated in immune serum for 45 min on ice and then separately inoculated intraperitoneally into mice. The test was repeated at least 3 times for each dilution (1/2 and 1/5) of immune serum. The immune serum was replaced by normal mouse serum at similar dilutions in the control populations. Each mouse was tested for the appearance of bloodstream trypanosomes for up to 30 days after inoculation. Control mice routinely showed detectable parasitaemia 10–12 days after inoculation.

RESULTS

Comparative analysis of the variable antigen types present in 3 *populations of STIB* 228 *before and after cyclical transmission*

Immunofluorescent analysis of trypanosome populations 228A, 228B and 228C using antisera raised to clones 228B and 228C was carried out prior to their cyclical transmission and the results are given in Table 1. Antiserum to clone 228B did not recognize any trypanosomes present in clone 228C and vice versa, but each recognized more than 99·9 % of its homologous population. These cloned populations were, therefore, different in their composition of variable antigen types. Antiserum to clone 228B recognized 80 % of the trypanosomes present in stock 228A but no trypanosomes in this population were recognized by antiserum to clone 228C.

When these antisera were tested against the metacyclic populations derived from 228A, 228B and 228C no positive reactions were detected at any of the serum dilutions used (1/80, 1/160 and 1/320).

Antiserum to each of the metacyclic populations, however, uniformly stained all the metacyclic trypanosomes both in its homologous infection and also in the other 2 infections (Table 2), when tested by immunofluorescence on probes obtained at 72-h intervals from flies infected with each bloodstream population. Immunofluorescence on saliva probes taken from flies with early infections (< 4 weeks) or obtained by probing flies with mature infections at 24-h intervals, showed heterogeneity of immunofluorescence staining. Some metacyclic trypanosomes stained brightly while others displayed weak staining and epimastigote forms were unstained. Such populations were, however, completely neutralized by all 3 metacyclic antisera at all dilutions tested (1/2, 1/5) (Table 3).

Table 1. *Comparison of the variable antigen types present in 3 populations of a stock of* Trypanosoma congolense *(STIB 228) before and after cyclical transmission as determined by immunofluorescence*

	Percentage of parasites showing positive immunofluorescence staining											
	228A			228B			228C					
Antisera to	Population ingested by tsetse flies	Metacyclics	Population from cyclically infected mice	Population ingested by tsetse flies	Metacyclics	Population from cyclically infected mice	Population ingested by tsetse flies	Metacyclics	Population from cyclically infected mice
228B Bloodforms	80	0	50	> 99·9	0	65	0	0	60
228B Metacyclics	0	> 99·9	0	0	> 99·9	0	0	> 99·9	0
228C Bloodforms	0	0	0	0	0	0	> 99·9	0	0
228C Metacyclics	0	> 99·9	0	0	> 99·9	0	0	> 99·9	0

Table 2. *The variable antigen types of* Trypanosoma congolense *metacyclics*

(Results of serotyping using indirect immunofluorescence. For each point in the table, 2 serum dilutions (1/20 and 1/40) were tested at least 3 times using 50–100 metacyclics on each occasion.)

Antisera to metacyclics of	Percentage of metacyclics showing positive immunofluorescence staining						
	STIB 228A	STIB 228B	STIB 228C	STIB 249	STIB 249B	STIB 212	STIB 212C
STIB 228A	> 99·9	> 99·9	> 99·9	0	0	0	0
STIB 228B	> 99·9	> 99·9	> 99·9	0	0	0	0
STIB 228C	> 99·9	> 99·9	> 99·9	0	0	0	0
STIB 249B	0	0	0	> 99·9	> 99·9	0	0
STIB 212C	0	0	0	0	0	10–20	60–80

Table 3. *The variable antigen types of* Trypanosoma congolense *metacyclics*

(Results of serotyping of different stocks and their derivatives using the neutralization of infectivity test. Each point in the table represents the results of the test using 18 mice. Neutralization was considered positive (+) if all the 18 mice did not show parasitaemia over a period of 30 days after inoculation, and negative (−) when all or some of the mice became infected.)

Antisera to metacyclics of	Antigens (metacyclics)							
	STIB 228A	STIB 228B	STIB 228C	STIB 249	STIB 249B	STIB 212	STIB 212C	22A and 249B
STIB 228A	+	+	+	−	−	−	−	−
STIB 228B	+	+	+	−	−	−	−	−
STIB 228C	+	+	+	−	−	−	−	−
STIB 249B	−	−	−	+	+	−	−	−
STIB 212C	−	−	−	−	−	−	−	−
STIB 228C and STIB 249B	+	+	+	+	+	−	−	+

Following cyclical transmission of each population, trypanosomes were first detected in the blood of infected, irradiated mice 5 days after infection. The trypanosome populations from these mice were subsequently sub-passaged twice into other irradiated mice and then were analysed by immunofluorescence using antisera to the metacyclic populations and the original bloodstream clones 228B and 228C. The results are given in Table 1. Antisera to the 3 metacyclic populations and clone 228C did not recognize any trypanosome present in the initial populations at the limit of sensitivity of the immunofluorescent technique (1/1000). Antiserum to clone 228B did, however, recognize 50–65 % of the populations from 228A, B and C.

Comparative analysis of the variable antigen types present in the metacyclic populations derived from STIB 228, *STIB* 249 *and STIB* 212

Antisera were prepared against the variable antigen types present in the metacyclic populations derived from STIB 228A, B and C, STIB 249B and STIB 212C. Each antiserum was subsequently tested by IFAT and NIT against all 7 metacyclic

populations which appeared following cyclical development of STIB 228A, B and C, STIB 249 and 249B and STIB 212 and 212C.

The results presented in Tables 2 and 3 show that whereas antisera raised to the metacyclic populations derived from 228A, B and C cross-reacted completely by IFAT and NIT; such antisera did not, however, recognize any of the metacyclic populations obtained from STIB 249 and 249B or STIB 212 and 212C.

Antisera to the metacyclic populations derived from STIB 249 and STIB 249B cross-reacted completely both by IFAT and NIT (Tables 2 and 3) but did not recognize the metacyclic populations derived from STIB 228 or STIB 212.

Antiserum to the metacyclic population of STIB 212C did not, however, recognize all of the metacyclic trypanosomes present following cyclical development of STIB 212 and 212C by IFAT (Table 2) and was consequently not capable of neutralizing these populations (Table 3). It was considered possible that such a result could be obtained if the stock ingested by tsetse flies contained mixtures of different genetic lines, each capable of producing metacyclic populations of different variable antigen composition. This hypothesis was briefly examined by infecting a mouse with both 228A and 249B and then analysing the metacyclic population which developed in tsetse flies infected by feeding on such a mouse. Antiserum to the metacyclic populations of each stock do not cross-react as demonstrated above. The metacyclic population which developed in such flies when examined by IFAT showed that each metacyclic population was present but that their relative proportions varied in each probe. Each antiserum on its own was unable to neutralize such a mixed metacyclic population but when combined they could (Table 3).

The original population of STIB 212 is currently being cloned and transmission studies carried out to see how many possible metacyclic populations of different variable antigen composition can be obtained.

DISCUSSION

It would appear from the results of these experiments that the effects of cyclical transmission on the process of antigenic variation in *T. congolense* infections are similar to those reported in *T. brucei* infections.

Cloned and uncloned drivatives of *T. congolense* STIB 228 were shown by IFAT to be composed of different variable antigen types prior to ingestion by tsetse flies, yet the metacyclic populations which subsequently developed cross-reacted completely by immunofluorescence and neutralization tests. This corresponds to the observation (Jenni, 1977*a*, *b*) on cyclical transmission of different variable antigen types of individual stocks of *T. brucei*.

The results of these experiments, however, do not allow any conclusive statement to be made concerning the possible number of variable antigen types present in the metacyclic populations which appear following cyclical development of derivatives of this stock of *T. congolense*. The monospecificity of antisera raised by infection is always open to question so we are approaching the problem of obtaining defined antisera to *T. congolense* metacyclic variable antigen types by means of

monoclonal antibody production as the very small number (50–100) of metacyclic trypanosomes produced by *T. congolense*-infected tsetse flies precludes isolation of the variable antigens by conventional biochemical techniques.

The observation that antiserum to clone 228B recognized, by IFAT, the majority of trypanosomes present in the bloodstream populations obtained after cyclical transmission of all 3 populations 228A, B and C is in agreement with the one by Gray (1965) that there are characteristic 'predominant' variable antigen types which tend to appear early in the mammalian host following cyclical transmission of different variable antigen types of the same stock of *T. brucei*. In view of this finding that by IFAT the 'predominant' variable antigen types represented only a proportion of the early trypanosome population, it is perhaps, not surprising that previous attempts to demonstrate the occurrence of 'predominant' variable antigen types following cyclical transmission of *T. congolense* populations, by neutralization assays rather than by immunofluorescence techniques gave conflicting results (Wilson & Cunningham, 1970; Uilenberg & Giret, 1972*a*, *b*; Uilenberg *et al.* 1973).

In our studies, antiserum to each of the metacyclic populations from *T. congolense* STIB 228, STIB 249 or STIB 212 did not recognize variable antigen types present in the metacyclic populations of the other 2 stocks. Similar development of metacyclic populations where variable antigen composition is different for different stocks has also been reported in *T. brucei* infections (Jenni, 1977*a*, *b*).

Whereas antisera prepared against the metacyclics of STIB 228 and STIB 249 were capable of recognizing all the variable antigen types present in the corresponding metacyclic populations both by immunofluorescence and by neutralization, an antiserum raised in the same manner to the metacyclics of STIB 212C recognized only 60–80% of the homologous metacyclic populations and 10–20% of metacyclics derived from the parent STIB 212 by immunofluorescence, and did not completely neutralize such populations. It may be that STIB 212 contains more than 1 genetic line of trypanosomes and that each genetic line produces its own characteristic metacyclic variable antigen types. This is at present under investigation. The observation that simultaneous infection of flies with STIB 228A and STIB 249 resulted in the concurrent development of metacyclic trypanosomes which contained the characteristic variable antigen types of each stock, however, shows that this is indeed a possibility.

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