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## Studies on antigenic variation of cyclically transmitted *Trypanosoma congolense*

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### Summary

Antigenic variants of a strain of *T. congolense* transmitted by *G. m. morsitans* through normal and X-irradiated mice were investigated by means of the neutralization test and IFAT. Clones of a cyclically passaged derivative strain were isolated from irradiated and normal mice. The IFAT revealed cross immunofluorescent reactions between most of the stabilates, whereas only the two clones obtained from irradiated mice were totally neutralized by their homologous antisera. These two antisera showed no cross neutralizing activity. The results indicate a possible antigenic heterogeneity of the extruded metacyclic forms.

### Key words

*T. congolense* – *G. m. morsitans* – antigenic types – clones.

### Introduction

The occurrence of antigenic variation in salivarian trypanosomes during the course of infection in the mammalian host is of great immunological interest. These immunological phenomena have recently been reviewed by Doyle (1976) and Gray and Luckins (1976). The information compiled in these reviews demonstrate the increase of interest in the immunology of trypanosomes during the last two decades. Problems involved in trypanosomiasis control by immunological methods as well as the role of antigenic variation as a factor influencing the host/parasite relationship are pointed out. Most of the work has been done on trypanosomes of the subgenus *Trypanozoon*, whereas comparatively little information exists of the variations in antigenicity of *T. congolense*, one of the causative agents of bovine trypanosomiasis. The number

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and temporal occurrence of antigenic types in animals infected with *T. congolense* was studied by Wilson and Cunningham (1972) and by Uilenberg and Giret (1972).

The antigenic reversion to a basic strain antigen during cyclical development, as it occurs in *T. brucei* (Gray, 1965), was also investigated in *T. congolense*. Wilson and Cunningham (1970) had no definite evidence for the formation of a similar antigen when trypanosome populations from mice were tested 17–20 days after the inoculation of metacyclic forms. On the other hand, Uilenberg and Giret (1972) quoted that they had identified trypanosomes with a basic antigen of a cyclically transmitted *T. congolense* strain. These workers tested trypanosome populations from mice 2–3 days after the injection with parasites from cyclically infected sheep which had carried an infection for 7–10 days in their turn.

Wilson et al. (1973) in extending the laboratory investigations on antigenic variation to the field, detected numerous different antigenic types in «primary» parasite populations which developed in mice or cattle after the inoculation of metacyclic forms isolated from infected wild tsetse flies.

One of the main difficulties involved in the work with *T. congolense* is the low virulence of this trypanosome species in most laboratory animals. This leads to a prolonged prepatent period and therefore antigenic variation may have occurred before the number of parasites is high enough for immunological examinations.

In this paper we describe an attempt to serotype a *T. congolense* strain and its cloned derivatives cyclically transmitted by *Glossina m. morsitans*.

## Material and methods

### a) Trypanosomes

The parent strain, *T. congolense* STIB 228, was isolated from a lion in the Serengeti in 1971 (Geigy and Kauffmann, 1973). Infected blood from the lion was inoculated into a rat and the developing parasite population stabilized as STIB 228 after 28 days. All derivatives of STIB 228 including the clones are shown in Fig. 1. The stabilates were kept at  $-70^{\circ}\text{C}$  or at  $-196^{\circ}\text{C}$ .

The clones were prepared by picking out single cells with a fine capillary which was drawn out from a haematocrit tube. After a first direct microscopical examination of the capillary content, the drop of solution was blown onto a glass slide and once more checked for single cells. Finally, the single parasite was drawn up in 0,1 ml of phosphate buffered saline glucose (PSG) 6:4, pH 8, into a 1 ml syringe and injected intraperitoneally (i.p.) into an irradiated or normal mouse. The resulting parasitaemia stayed at a submicroscopical level and could only be detected and stabilized after 3 blood passages in irradiated mice (Fig. 1).

### b) Laboratory animals

White mice (ICR, 25–30 g) and white rats (SIV, 150–200 g) were used throughout all the experiments.

Mice were irradiated with a Philips RT 305 X-ray tube. A dose of 600 rad per total body weight was administered. Challenged with this dose, the mice normally died after 7 days. This survival period was not decreased by a following trypanosome infection.

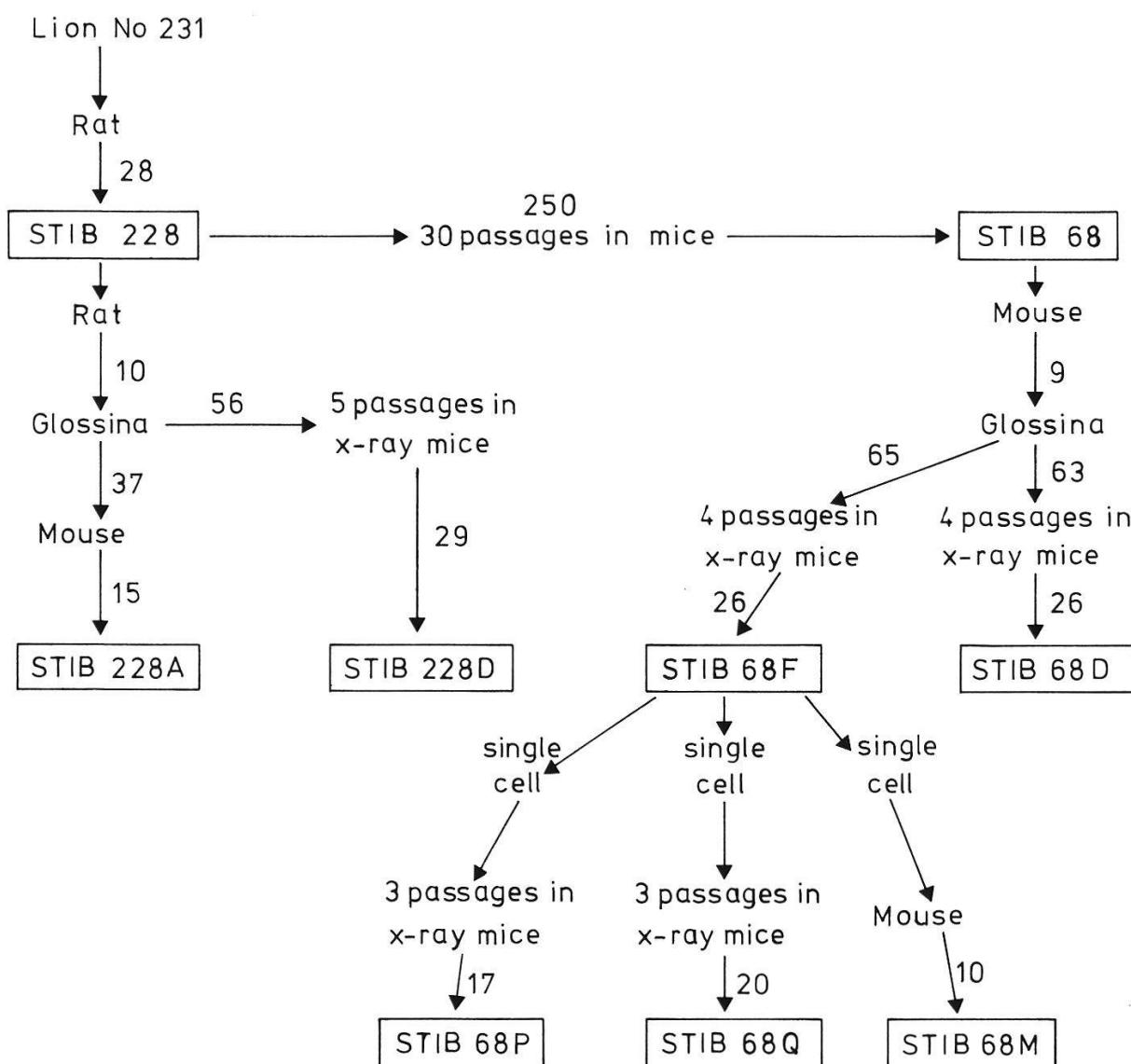


Fig. 1. Pedigree of *T. congolense* stabilates. The numbers indicate the number of days during which the different hosts were infected before the parasite populations were cryopreserved or passed to the next host.

The mice were infected by syringe injection or Glossina feeding 24 h after the exposure to the X-rays. Parallel with trypanosomes the mice were treated with 70 U of Penicillin and 70 µg of Streptomycin.

Some of the sera from infected irradiated mice were examined to confirm the absence of significant antibody titers against the corresponding trypanosome populations.

### c) Tsetse flies

Puparia of *G. m. morsitans* were obtained from Dr. A. M. Jordan from Langford, Bristol. The pupae were kept in sterile sand at 25°C and 80% r.h. in the dark. Newly hatched flies were fed once on an infected rat or mouse which showed a first peak parasitaemia. The flies (both sexes together) were grouped in batches of 20 and transferred into Geigy cages. The flies were kept at the same conditions as the pupae.

After the infective bloodmeal, the flies were fed on uninfected mice every second day. The ventral surface of any mouse or rat used for fly-feeding was cleaned with a 70% alcohol solution

before the flies were offered a bloodmeal. This cleaning was done in order to protect the midgut of the flies from a microfloral contamination. We have some evidence that certain bacteria do interfere with the development of trypanosomes in the vector.

Infected flies were detected by examining unstained saliva probes microscopically using phase contrast. Single infected flies were fed on irradiated and non-irradiated mice. Blood of irradiated animals had to be passaged 4–5 times in immunosuppressed animals until the parasitaemia was suitable for cryopreservation or cloning (Fig. 1).

*d) Antisera*

Antisera against the different stabilates were prepared in rats. The rats were inoculated (i. p.) with  $10^5$  thawed trypanosomes from stabilates and the sera collected 11 days later. The infected blood was left to coagulate for 4 h at 0–2 °C and the sera were obtained by centrifugation at the same low temperature. All antisera were inactivated at 56 °C for 30 min and tested with the neutralization and immunofluorescent test.

*e) Antigens*

Trypanosomes from infected mouse blood were serologically compared using the neutralization and the indirect fluorescent antibody test (IFAT). Infected blood was withdrawn from mice with heparin and immediately cooled to 0–2 °C. One part of blood was frozen down and stabilized at –70 °C whereas trypanosomes of the other part were isolated from blood and washed (4 times) in PSG by several centrifugation steps at 0–2 °C. 5  $\mu$ l drops of 500 washed trypanosomes in PSG containing 1% bovine serum albumin were dried on glass slides and then fixed with acetone of analytical grade. The slides were stored in air tight polyvinyl bags at –70 °C until the trypanosome samples were tested with the IFAT.

The cryopreserved trypanosome populations were later used for neutralization tests.

*f) Serological tests*

The neutralization test we used was similar to that described by Wilson and Cunningham (1970) and Uilenberg and Giret (1972).  $10^4$  trypanosomes from thawed stabilates in 0.1 ml of PSG were mixed with 0.1 ml of test or control sera (diluted 1:5 with PSG or undiluted) from rats and then incubated at 0–2 °C for 45 min. After this time, the 0.2 ml of trypanosome/sera/PSG mixture was inoculated (i. p.) into one mouse. At one time, 6 parallel incubations with the same antigen and antiserum were carried out. Each test combination was done twice. The blood of negative mice was examined up to 6 weeks.

Parallel tests with the addition of 20  $\mu$ l complement (pooled guinea pig serum, Miles Lab.) to the incubation mixture revealed the same results as tests without additional complement.

IFAT were carried out with antisera and normal sera from rats. Phosphate buffered saline (PBS), pH 7.3 was used as diluent. Drops of suitable serum dilutions were allowed to react with the fixed trypanosome samples at 37 °C for 25 min. The unreacted serum was then removed and the slides washed 3 times in PBS. The trypanosomes were incubated in a 1:80 dilution of the respective fluorescein conjugated antiserum at 37 °C for 15 min (rabbit antirat IgG, Cappel Lab.). Thereafter, the slides were washed as above and mounted in Tris buffered glycerol, pH 7.7.

Control tests included incubation in normal serum and then in conjugated serum or incubation in conjugated serum alone. Readings were made by phase contrast and Ploem illumination using a Zeiss fluorescent microscope. The microscope was equipped with a halogen lamp (100 W), exciting filters KP 490/KP 500, barrier filter LP 455 and the phase objectives  $\times 40$  and  $\times 63/1.25$ . A number of 100 trypanosomes was examined per test sample. Trypanosomes exhibiting brilliant green fluorescence were regarded as positive, whereas a red coloured or mat green appearance was marked as negative. Reactions were graded as negative, minimum positive (+) or maximum positive (++) and the results presented in Table 3.

## Results

Out of all the flies infected with STIB 228, 5.4% developed a mature infection, whereas the corresponding number for STIB 68 was 10%. We observed that infective flies did not extrude metacyclic forms infective for mice during every bloodmeal. Some flies even lost their infectivity after an infective period of 4–5 weeks. When these flies were dissected, no trypanosomes were found in their midgut and mouthpart. In addition, we have found that flies which were infected with other *T. congolense* strains (not mentioned above) were unable to infect mice, although some flies carried trypomastigote forms in their hypopharynx.

Trials to obtain clones directly from single metacyclic forms in irradiated or normal mice failed. Clones could only be grown from parasites which had developed in mice (STIB 68F) after an infectious bite (Fig. 1). Clones grown in normal mice (e.g. STIB 68M) could be cryopreserved after 10 days, whereas those clones which were obtained through massive blood passages in irradiated mice were suitable for cryopreservation not before 17 days (STIB 68P).

The neutralization test series yielded neutralization effects of only two antisera against their respective parasite populations (Table 1). Thawed parasites of STIB 68P and STIB 68Q were each totally neutralized by their corresponding antiserum but not cross neutralized. Both stabilates were clones which were grown by means of 3 passages in irradiated mice. These results indicate that specific neutralizing antibodies had developed in the rats within 11 days and that these two stabilized populations were each most certainly antigenically homogenous. In additional tests, the parasite population which had developed in these rats within 11 days were antigenically different from the initially inoculated trypanosomes for they were not neutralized by the homologous 11 days sera.

Table 1. Neutralizing activity of rat antisera against different trypanosome stabilates (final serum dilution 1:10)

Antigens (stabilates)	Antisera 11 days postinoculation (prepatent periods in days)					Control serum
	STIB 228	STIB 228 D	STIB 68 P	STIB 68 Q	STIB 68 M	
STIB 228	6	5	5	4	5	4
STIB 228 D	6	6	5	7	7	4
STIB 68 D	5	5	6	6	6	3
STIB 68 P	5	5	∞	8	5	2
STIB 68 Q	5	6	8	∞	5	3
STIB 68 M	6	6	7	5	6	3

∞ = No trypanosomes were detected during the period of the experiments (6 weeks).

Table 2. Neutralizing activity of rat antisera against different trypanosome stabilates (final serum dilution 1:2)

Antigens (stabilates)	Antisera 11 days postinoculation (prepatent periods in days)			Control serum
	STIB 228	STIB 228 D	STIB 68 M	
STIB 228 .....	6	5	6	4
STIB 228 D .....	6	6	7	4
STIB 68 D .....	4	5	6	2
STIB 68 M .....	6	6	6	3

Table 3. Immunofluorescent activity of rat antisera against different trypanosome stabilates

Antigens (stabilates)	Antisera 11 days postinoculation				
	STIB 228	STIB 228 D	STIB 68 P	STIB 68 Q	STIB 68 M
STIB 228 .....	+ <sup>Δ</sup> + <sup>ΔΔ</sup>	+	+ / - *	+	+
STIB 228 A .....	- -	- -	- -	- -	- -
STIB 228 D .....	+	++ +	- -	+	+
STIB 68 D .....	+	+	+ / - ***	- -	- -
STIB 68 F .....	+	+	+	+	-
STIB 68 P .....	+	+	++ +	+	+
STIB 68 Q .....	+	- -	+	++ +	+
STIB 68 M .....	- -	- -	- -	- -	- -

Δ serum dilution 1:320

ΔΔ serum dilution 1:480

\* 80% +, 20% -

\*\* 60% +, 40% -

\*\*\* 95% +, 5% -

Trypanosomes of a third clone (STIB 68M) which was grown in an immunocompetent mouse were not neutralized. This indicates that STIB 68M was antigenically heterogenous. Minor antigenic types in this population were not sufficiently recognized by the immune system of the rat within 11 days. Therefore, specific neutralizing antibodies were absent or only present in too low amount for a complete neutralization of trypanosomes incubated in our test

combination. The cloning experiments suggest that single parasites can lead to a parasitaemia with a prepatent period of only 5 days.

Neutralization tests with 1:2 diluted sera (Table 2) showed the same results as tests with the corresponding 1:10 dilutions.

The IFAT results are shown in Table 3. Normal rat sera in control incubations did sometimes positively react up to a titer of 1:160. Therefore, the baseline was set at a serum dilution of 1:320. In Table 3, the readings of serum dilutions 1:320 and 1:480 are given. All sera were negative at a titer of 1:640. In contrast to the neutralization tests, the IFAT revealed cross reactions between different stabilates and antisera. In results denoted positive, fluorescence was exhibited uniformly by all members of the population. But in three tests, only a certain proportion of parasites showed a positive immunofluorescent staining whereas the remaining parasites were negative (e.g. antiserum against STIB 68P tested against STIB 228 and STIB 68D). STIB 228A, a trypanosome population which was cryopreserved 15 days after the infective bite showed no positive cross reaction with any of the sera tested. No positive immunofluorescence was found with STIB 68M even when tested with the respective antiserum against STIB 68M. In addition, it is interesting to note that although the homologous antisera against STIB 228D showed a maximum fluorescent staining the corresponding neutralization tests were negative (Tables 1 and 2). The strongest cross reactions occurred between STIB 68P and 68Q.

## Discussion

Whereas clones from single metacyclic forms of *T. (T.) brucei* could be obtained in irradiated and normal mice (Jenni, 1977) the same experiments with single metacyclines of *T. congolense* STIB 228 and its derivatives failed. In addition, all cloning attempts with metacyclic forms obtained from other *T. congolense* strains isolated in the field and not mentioned in this paper were also unsuccessful. On the other hand, cloned populations could be obtained from single bloodstream forms which were derived from first peak parasitaemia populations after cyclic transmission.

While other workers (Wilson and Cunningham, 1970; Wilson et al., 1973; Uilenberg, 1974) used immunocompetent hosts in their immunological experiments with *T. congolense*, X-irradiated mice were used in our experiments, in order to suppress the immune-response in the mice and by that avoid interactions between specific antibodies and parasites. This may abolish antigenic variation and may lead to an enhancement of the virulence of the parasite (Walker, 1968). Yet, the irradiation did not decrease the prepatent periods of *T. congolense* infections in mice. On the other hand, no specific antibody response could be detected in our irradiated mice.

Whether antigenic variants did develop in immunosuppressed mice could not be stated with certainty. Minor variant types could not be detected with the

IFAT. There is some evidence on the basis of the neutralization results of the two clones STIB 68P and STIB 68Q which were grown in irradiated mice that antigenic variation did not occur during cloning. These two clones were not cross neutralized by their mutual antisera, but slight neutralizing cross reactions may be indicated by the prolonged pre-patent periods which were both 8 days. The comparison of the respective IFAT values also shows strong immunofluorescent activities between the two stabilates with the two related antisera.

The immunofluorescent activity of one antiserum to different stabilates can be explained by the fact that the IFAT detects common antigens (Wilson and Cunningham, 1971; Schindler, 1972) beside specific variant type antigens (Van Meirvenne et al., 1975). On the other hand the neutralization test is exclusively capable of detecting variant antigenic types (Wilson and Cunningham, 1972).

From the IFAT results it can be concluded that STIB 228A is antigenically different from all the other stabilized antigens tested. STIB 68M as well showed no immunofluorescent activity and was not neutralized when tested with different antisera including the homologous antiserum. STIB 228A and STIB 68M were grown in immunocompetent hosts in which antigenic variation could have occurred before cryopreservation.

One of the main difficulties in our experiments was the limitation of antigen material obtained from mice. Therefore, complete test series combining all the stabilates with all antisera could not be done.

We have no final evidence that a basic antigen does not exist in our *T. congolense* strains. But some observations we made, indicate a possible antigenic heterogeneity of metacyclic forms. In preliminary investigations, only a proportion of metacyclic forms from saliva probes reacted positively with homologous antisera in the IFAT (unpublished results). As already stated by Wilson and Cunningham (1970), neutralization tests with metacyclic forms could lead to more accurate results and information about antigenic reversion of *T. congolense*.

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