Ann. Soc. belge Méd. trop.
1977, **57**, 4-5, 369-381

FIRST TSETSE FLY TRANSMISSION OF THE « AnTat » SERODEME OF *TRYPANOSOMA BRUCEI* (*)

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Summary — The « AnTat » serodeme derived from *Trypanosoma brucei brucei* EATRO 1125 was transmitted through *Glossina morsitans* and the variable antigen types (VATs) of resulting metacyclic and bloodstream populations were investigated by VAT-specific indirect immunofluorescence and trypanolysis.

Expression of the VAT repertoire characteristic of the syringe-transmitted « AnTat » serodeme was resumed by trypanosomes after cyclical transmission. Fly transmission resulted in an increased antigenic instability and lowered virulence of the VATs as compared with their long-term syringe-passaged counterparts. The first patent bloodstream population was shown to be markedly heretogeneous and composed chiefly of 19 distinct VATs. VATs present in the first patent parasitaemia could not be detected in the initiating metacyclic trypanosome population.

The bearing of these observations on epidemiological investigations and on the prospect of vaccinating against African trypanosomiasis is discussed.

KEYWORDS : *Trypanosoma brucei*; « AnTat » serodeme; cyclical transmission; metacyclic trypanosomes; first patent bloodstream population; antigenic variation; variable antigen type (VAT).

Introduction

Eighty years after its discovery, African trypanosomiasis is still a major problem for human health and cattle breeding and its control is urgently required. Vaccination against the causative agents, the salivarian trypanosomes transmitted by tsetse flies, would represent one of the most satisfactory ways of achieving such an aim. Prospects for active immunization are in theory quite favourable as *Trypanosoma brucei* is one of the few parasites for which an immunogenic (protective) antigen has been characterized (Le Ray, 1975; Cross, 1975; Fruit *et al.*, 1977). This antigen is expressed only by flagellates infecting the vertebrate host and is the main or sole component of the cell surface coat of *T. brucei.*

(*) Supported by Grants from the U. K. Ministry of Overseas Development (Scheme R 2940) and the International Atomic Energy Agency (Contract no. 1353/RB).

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The achievement of immune protection against African trypanosomiasis, however, has so far been hindered by the trypanosome's ability to vary the specificity of its surface antigen throughout the infection of the mammal and consequently to escape the host's immune response.

From comparative studies (Gray, 1965a and b; Cunningham, 1966) on bloodstream populations using agglutination and neutralization tests, salivarian trypanosomes are believed to revert to a basic antigen type at the metacyclic stage in the tsetse fly and to undergo a semi-predictable sequence of antigenic changes in the ensuing bloodstream populations (Vickerman, 1969). The exact nature of these antigenic changes is as yet unknown, and this situation precludes further progress towards effective vaccination.

In order to obtain precise information on the antigenic changes accompanying tsetse fly transmission to the mammalian host, the « AnTat » serodeme of *Trypanosoma brucei* was cyclically transmitted and the resulting metacyclic and bloodstream populations were cloned and analyzed by the methods of Van Meirvenne *et al.* (1975a) for determining the antigenic identity of individual trypanosomes. The first results presented in this paper describe the antigenic and biological characteristics of trypanosomes of the first patent parasitaemia following fly transmission and their relations with the initiating metacyclic organisms.

Materials and Methods

Glossina morsitans morsitans were infected with the variable antigen type (VAT) AnTat 1 belonging to the set (Van Meirvenne *et al.*, 1975b) of thirteen VATs isolated from a syringe-maintained clone line of *Trypanosoma brucei brucei* EATRO 1125. The VATs of the trypanosomes derived from an infective fly were investigated by cloning and by VAT-specific trypanolysis (TL), neutralization (N) and indirect immuno-fluorescence (IF) tests carried out as described by Van Meirvenne *et al.* (1975a) unless otherwise stated.

Tsetse flies

Glossina morsitans morsitans received as pupae from the Langford Tsetse Laboratory, Bristol, were kept as batches of ten in Kilner jars at 25 °C and 60 per cent relative humidity. After hatching the flies were fed within 36 h on six-week-old female CFLP mice infected with the VAT AnTat 1. Subsequently the flies were allowed to feed daily either through an agar-Parafilm membrane (Langley, 1972) on defibrinated sterile horse blood (Burroughs-Welcome) or on lop-eared rabbits. Eighteen days after the infective meal, the flies were transferred into individual Sterilin tubes fitted with gauze at both ends and maintained thereafter on clean CFLP mice. Salivary gland infection of single flies was checked daily by phase contrast microscope examination of saliva probed onto glass slides warmed up to 37 °C and of tail blood wet smears from the bitten mice.

As depicted in the adjoining flow diagram (figure 1), a pleomorphic clone of *T. b. brucei* EATRO 1125 was isolated at the 20th passage after the original field primary isolation and typed as VAT AnTat 1 using reference antisera.

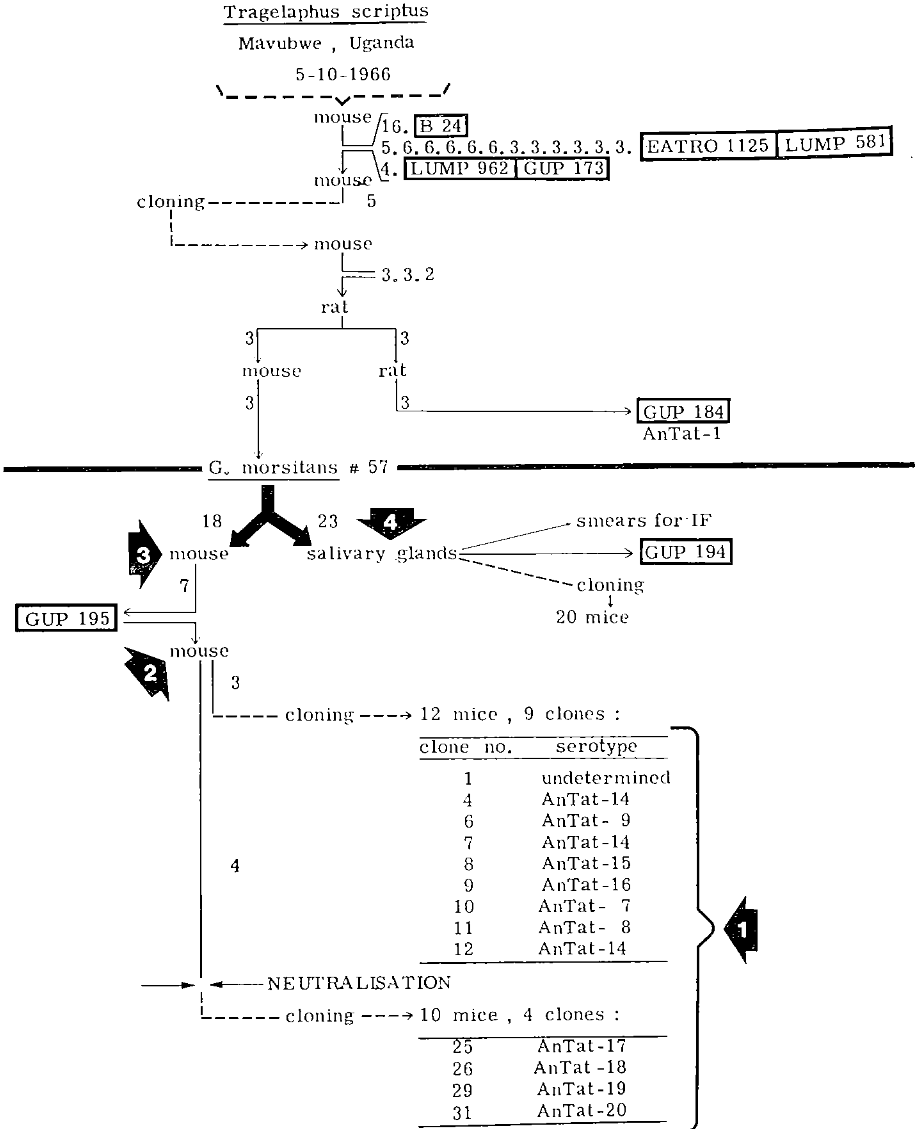

Figure 1

History of the stock of *Trypanosoma b. brucei* EATRO 1125 and of the line and clones described in the present study. The successive VAT analyses subsequent to fly transmission are indicated by the large arrows.

The clone population was checked with VAT-specific antisera and tsetse flies were fed on mice infected for 1 to 3 days. One fly out of 14 developed a salivary gland infection as shown by probe examination on day 18 after the infective feed; this fly was offered a mouse on the same day and dissected; the metacyclic trypanosomes were smeared and cloned and a stabilate (GUP 194) was prepared. The first patent parasitaemia developing in the mouse was smeared on day 5 after the infective fly bite and was stabilated on day 6 (GUP 195). A series of clones were derived from GUP 195.

Results

1. *Isolation of novel AnTat variable antigen types*

Our initial aim was to clone metacyclic and first patent parasitaemia trypanosomes and to prepare specific antisera in order to determine the actual composition of these two populations.

Infective salivary glands were dissected in a 9 : 1 mixture of Grace's insect saline : guinea pig serum and cloning of metacyclic trypanosomes was attempted; twenty such attempts were unsuccessful.

The stabilated first parasitaemia (GUP 195) was injected into mice and cloned 3 days later. Nine clones out of 12 were isolated (75 per cent cloning efficiency). The VAT of the clones was determined by VAT-specific trypanolysis and indirect immunofluorescence tests using reference antisera. Seven different VATs were identified — 3 previously described types (AnTat 7, 8 and 9) and 4 new ones which were allotted numbers in the AnTat series, i.e. AnTat 14 (isolated three times), 15, 16 and 21. Monospecific antisera to the new AnTat VATs were prepared as described below (and see figure 3).

Further isolation of new VATs was carried out by neutralization of the already identified VATs. Trypanosomes collected 4 days after sub-inoculation of the stabilate GUP 195 were suspended in a pool of trypanolytic antisera specific to AnTat 1 to 16. After incubation for 2 h, flagellates still showing motility were cloned. Four clones out of 10 (40 per cent cloning efficiency) developed and were shown to be new distinct VATs, designated AnTat 17 to 20 respectively.

The variety of VATs isolated from the first patent parasitaemia suggested a marked antigenic heterogeneity in this trypanosome population.

2. *Maintenance of fly-transmitted clones and production of corresponding monospecific antisera*

The handling of clones derived from a fly-induced first patent parasitaemia, and consequent production of monospecific antisera against them, proved difficult owing to the low virulence of the clones in mice and the marked antigenic variability of such clones.

A low virulence subsequent to fly transmission (already suggested by the failure of metacyclic clones to develop in mice) characterized the first

patent parasitaemia clones as demonstrated by the length of the pre-patent period (ranging from 4 to 7 days), by the scanty parasitaemia developed (from 5×10^3 to 4×10^6 organisms per ml of blood on day 7 in the original mice) and by the fact that 4 of these clones did not grow up on serial 3-day passaging in mice with 0.5 ml of retro-orbital sinus blood.

Such a reduction in virulence was aggravated, during the maintenance of the clones, by their pleomorphism. After four to five 3-day subinoculations, most of the trypanosomes were short stumpy forms (figure 2) which can no longer divide and the clones were easily lost during serial passage.

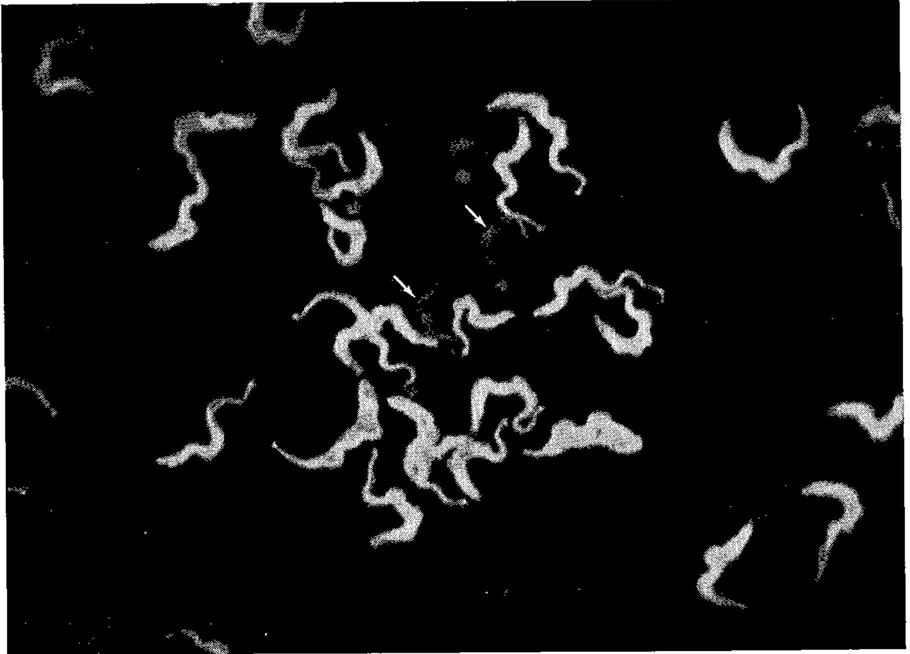

Figure 2

VAT-specific immunofluorescent staining of pleomorphic AnTat 15 with homologous antiserum at passage 4 from the fly (second 3-day passage from cloning). The short stumpy forms are expressing the homotype AnTat 15; the heterotypes (arrows) are long slender forms.

A third striking feature of the clones derived from a fly-transmitted population was the fast development of heterologous VATs (heterotypes), suggesting a marked antigenic instability. Data illustrating this are presented in table 1 and were obtained by trypanolysis using a pool of monospecific antisera to the known AnTat VATs. Three conclusions can be drawn from table 1. First, a 3-day passage regime, as was practised for AnTat 21 (see table 1), does not preclude development of, and overgrowth by, heretotypes — an observation which argues against antigenic variation being an antibody-induced event. Second, the degree of instability differs markedly among VATs maintained in a similar way. Third, such instability may be subject to drastic reduction during prolonged syringe maintenance, as shown by the comparison of the present results with those obtained by Van Meirvenne *et al.* (1975a) for similar VATs.

TABLE 1

Antigenic instability of clone populations isolated after fly transmission : frequency of heterotypes

Clone no.	VAT	Passages (days) from cloning	Heterotypes %
1	AnTat 21	3.3.3.3.3.	94
4	AnTat 14	7.3.3.	14
8	AnTat 15	7.3.3.	< 1
9	AnTat 16	7.3.3.	6
11	AnTat 8	7.3.3.	5

An obvious way of keeping the overgrowth by heterotypes under control was to neutralize them repeatedly with appropriate antisera until the antigenic instability of the original VAT was reduced. The data presented in table 1 suggest that stability may be achieved only after a considerable number of passages and might not succeed for certain VATs.

As a consequence of the low virulence and of the antigenic instability described above, production of monospecific antisera to the fly-transmitted VATs proved difficult. Several precautions had to be observed during the adaptation of the clones to mice — as exemplified for AnTat 14 (figure 3)

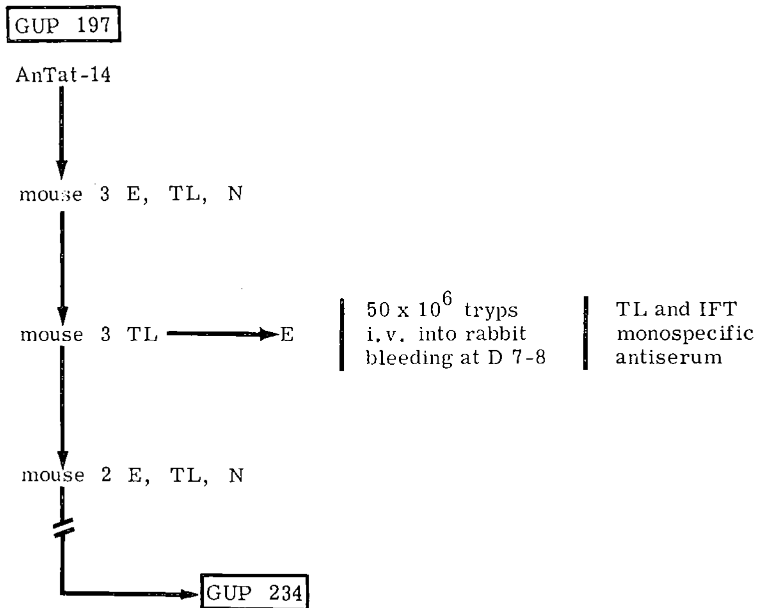

Figure 3

To maintain the antigenic homogeneity of a fly-induced clone (e.g. AnTat 14) and to prepare a monospecific antiserum, the trypanosomes at every 2- or 3-day passage were concentrated after elution (E) on an anion exchange column and checked by VAT-specific trypanolysis (TL); heterotypes were neutralized (N) prior to subinoculation.

TABLE 2

Persistence of the « AnTat » serodeme following cyclical transmission : immune lysis reactions performed with polyvalent antisera against VATs of two serodemes

Fly-induced clone VATs		End-infection antisera from rabbits infected with											
		AnTat							ETat				
Clone no.	VAT	A-1	A-2	A-4	A-5	A-6	A-10	A-13	E-8	E-9	E-10	E-11	E-12
10	AnTat 7	+	+	+	+	+	+	+	0	0	0	0	0
7	AnTat 14	±	+	+	+	+	+	+	0	0	0	0	0
8	AnTat 15	0	+	±	0	0	+	+	0	0	0	0	0
9	AnTat 16	+	+	+	+	+	+	+	0	0	0	0	0
25	AnTat 17	+	+	+	+	+	+	+	0	0	0	0	0
26	AnTat 18	+	+	+	+	+	+	+	0	0	0	0	0
29	AnTat 19	+	+	+	+	+	+	+	0	0	0	0	0
31	AnTat 20	+	+	+	+	+	+	+	0	0	0	0	0

+ = 100 percent lysis. ± = partial lysis. 0 = no lysis.

— namely. (i) repeated checking for the presence of, and neutralization of, heterotypes with a comprehensive set of lytic antisera; (ii) increasing the size of inoculum for both passaging and immunization by concentrating the trypanosomes (by elution on an anion-exchange column followed by centrifugation); (iii) frequent stabilation.

3. *Persistence of the AnTat serodeme after fly transmission*

Fly transmission of AnTat 1 might have resulted in the production of new antigenic types not present in the syringe-passaged serodeme. To test this possibility, end-infection antisera from rabbits infected by syringe with one of the AnTat 1 to 13 clones and bled after 4 to 6 weeks, when they had experienced a broad spectrum of VATs, were obtained through courtesy of N. Van Meirvenne and E. Magnus. Their lytic activity on the first patent parasitaemia-derived VATs was tested. Similar antisera from rabbits infected with VATs belonging to another serodeme (ETat) were used as control.

The results presented in table 2 show that (i) the fly-induced AnTat VATs has been expressed also in clone populations maintained by syringe passage for more than two years; (ii) the new VATs isolated after cyclical transmission were still specific to the AnTat serodeme initially described.

4. *Antigenic composition of fly-induced first patent parasitaemia*

The composition of the first patent parasitaemia which developed after an infective fly bite was examined by VAT-specific indirect IF of acetone-fixed smears using a set of monospecific antisera to the VATs AnTat 1 to 20.

Two different populations were analyzed. One was smeared 3 days after injection into mice of a stabilate (GUP 195) of the first patent parasitaemia frozen on day 6 following fly bite. The presence of nineteen different VATs was observed (table 3) with relative frequencies ranging from 0.7 to 18.4 per cent and 100 per cent of the trypanosomes present in this population were typed (figure 4).

When the same set of antisera was applied to the population smeared on day 5 following fly bite, however, only 1.3 per cent of the organisms could be typed (figure 4) and were shown to belong to 12 different VATs — the relative incidence of which ranged from 0.07 to 0.4 per cent of the whole population (table 3).

The striking differences in VAT composition observed between these two populations may be due to a variety of reasons : (1) the initial mammalian host had mounted an effective immune response against the homotype (majority type) between day 5 and stabilation; (2) more virulent heterotypes had overgrown the original population between day 5 in the first host and day 3 in the second; (3) stabilisation and passage had selected out for survival the more infective VATs.

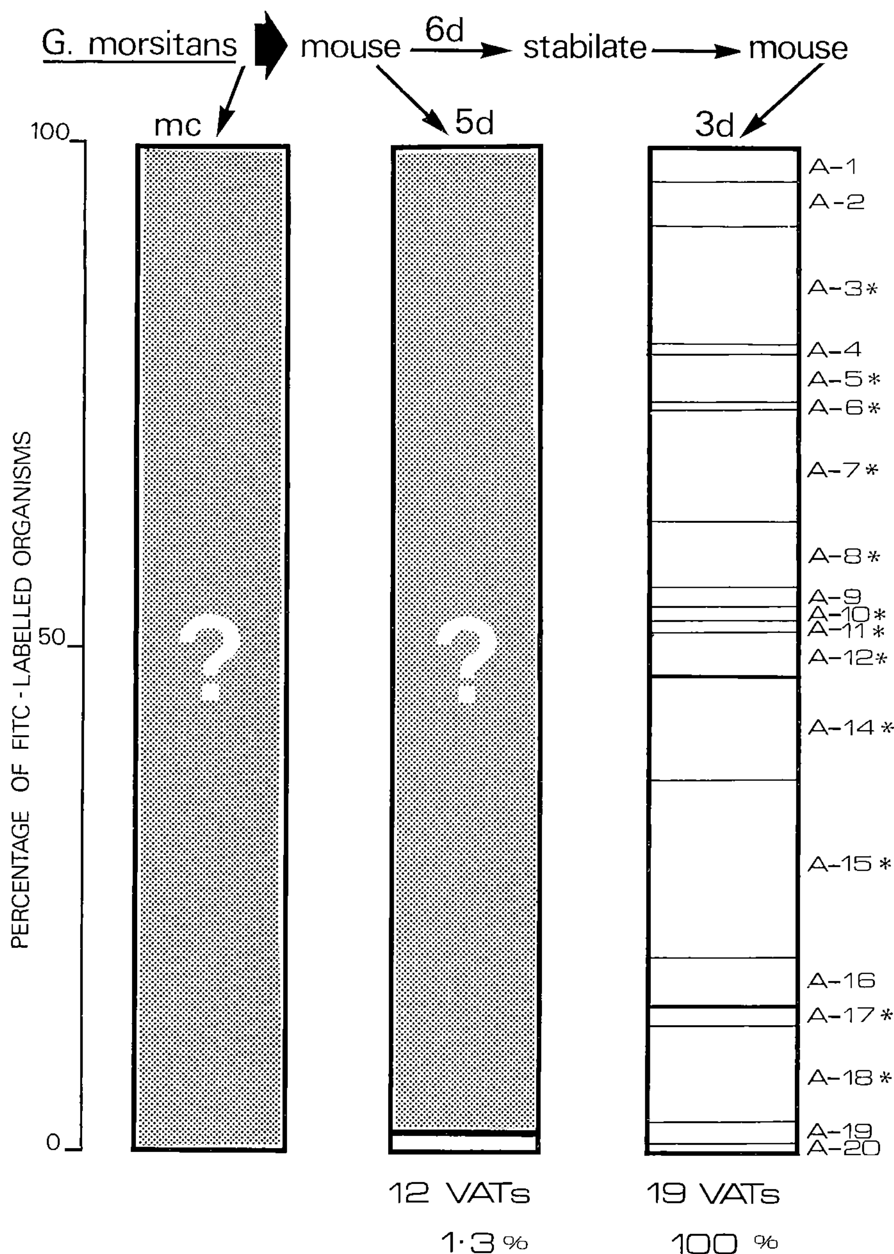

Figure 4

VAT composition of metacyclic (MC) and ensuing bloodstream populations as assessed by IF with antisera specific to the known VATs.
* denotes 12 VATs identified in 5d trypanosome population in original mouse.

TABLE 3

VAT-specific immunofluorescence analysis of fly-transmitted populations (see figure 4)

Antiserum to	No. metacyclics (absolute figures)	First patent parasitaemia (%)	
		Population 1 *	Population 2 **
AnTat 1	0/53	0	3.6
AnTat 2	0/16	0	4.6
AnTat 3	0/14	0.05	12.4
AnTat 4	0/15	0	0.7
AnTat 5	0/8	0.07	4.9
AnTat 6	0/7	0.15	0.8
AnTat 7	0/2	0.12	11.5
AnTat 8	0/7	0.02	7.4
AnTat 9	0/8	0	2.4
AnTat 10	0/9	0.02	1.6
AnTat 11	0/9	0.01	1.5
AnTat 12	0/19	0.20	4.7
AnTat 13	0/118	0	0
AnTat 14	0/3	0.07	11.7
AnTat 15	0/19	0.07	18.4
AnTat 16	0/22	0	5.5
AnTat 17	0/1	0.09	2.0
AnTat 18	0/1	0.46	11.4
AnTat 19	0/35	0	2.1
AnTat 20	0/13	0	1.5

* Original mouse, 5 days after fly bite.
** 2nd mouse, 3 days after injection with stabilate (GUP 195) of 6-day blood from original mouse.

5. *Attempt to characterize the metacyclic population*

A suspension of metacyclic trypanosomes was obtained by dissection of the infective fly's salivary glands and was dispensed onto Teflon-coated Multispot slides (Burroughs-Wellcome). The droplets were allowed to dry out at room temperature. The slides were processed for IF as described above, but 5 per cent pyronin was added to the Evans Blue counterstain in order to stain the kinetoplast and nucleus and hence to distinguish morphologically metacyclic trypanosomes from any other stages in the life cycle.

About 400 metacyclic trypanosomes were tested with the twenty different VAT-specific antisera prepared so far. None of the metacyclics reacted positively (table 3 and figure 4). This result suggests that the bulk of the metacyclics were antigenically different from the first patent parasi-

taemia on day 6 after the fly bite. It should be emphasized that the number of metacyclic trypanosomes tested with a single antiserum in this experiment was too small to exclude the presence of such VATs as minor metacyclic types (metatypes).

Discussion

Metacyclic trypanosomes and first patent parasitaemia

In the metacyclic population we were unable to detect by VAT-specific IF either the VAT ingested by the fly or any of the 20 AnTat VATs so far characterized. The antigenic identity of the metacyclic trypanosomes still remains, therefore, an open question which is currently being investigated. Monospecific antisera to clone lines from metacyclic trypanosomes would be of major help in investigating the antigenic identity of metacyclic populations. Metacyclic trypanosomes appeared to be of low infectivity as indicated by our failure to set up clone lines from them.

The fly-induced first patent populations expressed a marked antigenic heterogeneity; at least nineteen different VATs were demonstrated by immunofluorescence and by cloning. It has not yet proved possible to demonstrate the existence of VATs specific to these populations. The existence of a semi-predictable sequence of antigenic types which was suggested (Gray, 1965a, b) to take place during the early development of a trypanosome population in a cyclically infected mammal appears, therefore, to be questionable in the light of results presented here. With respect to vaccination, the prevention of trypanosomiasis by immunizing against early fly-induced bloodstream VATs is most probably a remote possibility.

Comparison of the effects of cyclical transmission and syringe passage on the serodeme

Cyclical transmission of the AnTat serodeme did not modify its characteristic VAT repertoire. Most of the 13 VATs previously isolated (Van Meirvenne *et al.*, 1975a and b) from a syringe-transmitted clone population were also found here after fly transmission. The novel VATs isolated in the course of the present study were shown to be produced during syringe-initiated infections with several VATs of the serodeme, as corresponding lytic antibodies were elicited by rabbits injected with AnTat clones which had been transmitted by syringe over two years. Since the completion of the present work, direct evidence for presence of the novel AnTat VATs in old syringe-passaged lines has been obtained by IF and cloning assays (Van Meirvenne and Magnus, personal communication).

The persistence of the antigenic repertoire of a serodeme following cyclical development in the vector demonstrates its constancy. Accordingly, information gained from previous studies on antigenic variation in laboratory-maintained populations of trypanosomes is relevant to the field

situation. With respect to epidemiology and diagnosis, our results emphasize the feasibility and the usefulness of mapping the actual serodemes of African trypanosomes circulating in the field.

Another prominent feature of cyclically-transmitted trypanosomes is reduction of infectivity and/or virulence to the mammal accompanied by an increased VAT instability. Such a situation is complex and appears to be due to several factors. Virulence might be a VAT-linked character as already suggested by McNeillage and Herbert (1968). VAT instability could also result from the early bloodstream flagellates undergoing transformation to short stumpy forms while the heretotypes appearing by that time are almost exclusively long slender trypanosomes, as illustrated in figure 2. A preliminary comparative study in this laboratory of two distinct, monomorphic lines of the same VAT which are respectively transmissible and no longer transmissible through the tsetse fly, has indicated an increase in virulence as well as in antigenic stability in the latter.

What about vaccination ?

In the light of our present results, the key questions to be answered before planning an immunization project with variable antigens are : (i) are there several variable antigen types (« metatypes ») expressed by the metacyclic trypanosomes or just one such type (the « basic antigen ») ? (ii) is there any relationship between the metatype(s) and the ensuing bloodstream VATs ? (iii) is there any specificity of the metatype(s) to different serodemes ?

If there is only one metatype expressed by the metacyclic trypanosomes, the antigenic heterogeneity of the first patent parasitaemia and the failure to tag the metacyclic trypanosomes with antibodies specific to the ensuing VATs suggest that a complete antigenic change takes place between the metacyclic stage and the bloodstream stage. Should this be the case, immunization would have to be carried out with the metacyclic trypanosomes themselves or with their purified variable antigen; protection against homologous challenge should then be complete.

If there are several metatypes, they could vary in infectivity and virulence, the most infective ones giving rise to the first patent parasitaemia. Then, antibodies against some of the first patent parasitaemia VATs could neutralize only some of the metacyclic trypanosomes and could afford only a partial protection against an infected fly-challenge.

If every serodeme has its own specific metatype, or metatypes, any attempt at vaccination would require previous field surveys in order to define the serodemes circulating in the wild. Transmitting uncloned populations of trypanosomes through the fly could, of course, induce a false heterogeneity of the metatypes if the fly had fed on a mixture of serodemes.

Résumé — Première transmission cyclique du sérodème « AnTat » de *Trypanosoma brucei.*

Le sérodème « AnTat » dérivé du stock EATRO 1125 de *Trypanosoma brucei brucei* a été transmis cycliquement par *Glossina morsitans.* Les populations de trypanosomes métacycliques et sanguicoles qui en résultèrent furent analysées par immunofluorescence indirecte et par trypanolyse spécifiques de type antigénique variable (TAV).

Le répertoire TAV caractéristique du sérodème « AnTat » fut réexprimé après transmission cyclique. Celle-ci eut pour effet d'intensifier la variabilité antigénique et de réduire la virulence des TAVs. La première population sanguicole patente exhiba une hétérogénéité marquée due à la présence de 19 TAVs distincts au minimum. Aucun de ces TAVs ne fut retrouvé chez les trypanosomes métacycliques initiaux.

La portée épidémiologique et immunoprophylactique de ces observations est développée.

Samenvatting — Eerste cyklische transmissie van het serodeme « AnTat » van *Trypanosoma brucei.*

Het « AnTat » serodeem, afgeleid van *Trypanosoma brucei brucei* EATRO 1125, werd cyklisch overgebracht door *Glossina morsitans.* De daaruit voortkomende metacyklische- en bloedvormpopulaties werden op hun variantsamenstelling onderzocht door middel van variantspecifieke indirekte immunofluorescentie en trypanolyse.

De antigeenvarianten, karakteristiek voor het variantrepertorium van het « AnTat » serodeem werden na de cyklische transmissie teruggevonden.

In vergelijking met gedurende lange tijd mechanisch overgeënte bloedvormpopulaties, gaf de cyklische transmissie aanleiding tot een verhoogde antigenische variabiliteit en tot een verlaging van de groeisnelheid der antigeenvarianten.

De eerste waargenomen bloedvormpopulatie, ontstaan na cyklische transmissie, was zeer heterogeen van samenstelling en bestond hoofdzakelijk uit 19 verschillende antigeentypes. Geen verband werd waargenomen tussen de varianten, aanwezig in deze populatie en de aanvankelijke metacyklische trypanosomen.

De draagwijdte van deze waarnemingen wordt besproken in funktie van vaccinatie en epidemiologie.

Acknowledgements — *We thank Patricia McKail for her skilful technical assistance and Dr A. M. Jordan for supplying the tsetse fly pupae.*

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(Discussion see page 386.)