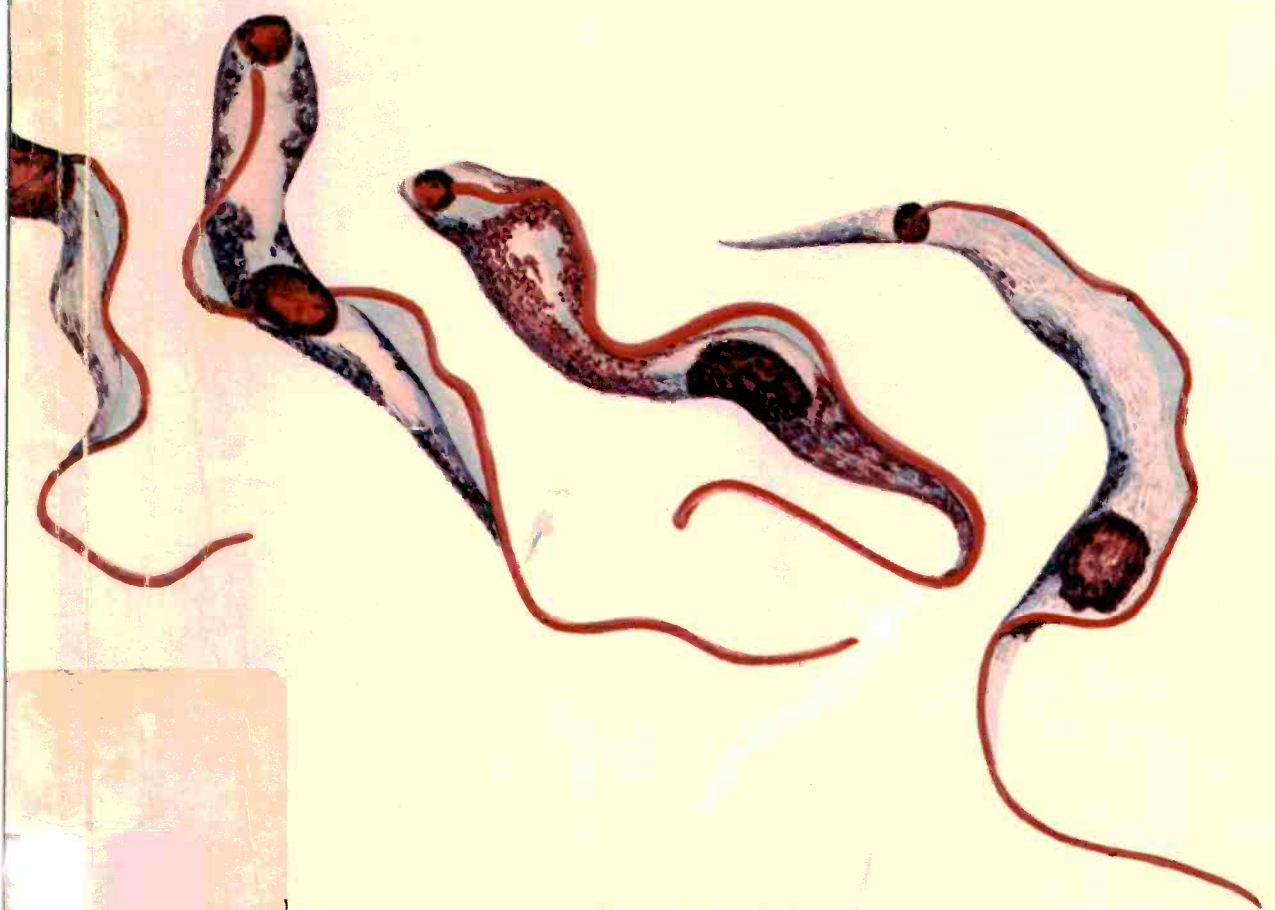
IDRC-132e

Pathogenicity of Trypanosomes

Proceedings of a workshop held at Nairobi, Kenya, 20-23 November 1978



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Editors: **George Losos[1] and Amy Chouinard[2]**

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Contents

Antigenic heterogeneity of bloodstream and metacyclic forms of *T. brucei*

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Abstract. Heterogeneity of variable antigen type (VAT) in bloodstream populations of *T. brucei* has been widely reported. The mechanism allows the parasite to persist in an immunized host. To determine whether heterogeneity extends back to the metacyclic population, which *Glossina* introduces into mammals, we extended previous studies (Le Ray, Barry, and Vickerman 1978) that demonstrated metacyclic VATs expressed by bloodstream forms. Using a syringe-passaged line of trypanosomes, we isolated clones from a rabbit at the onset of lytic activity against metacyclics. Monospecific antiserum to one of the clones (with VAT AnTat 1.30) showed lytic and immunofluorescent activity against 11–20% of metacyclics. Another VAT, AnTat 1.6 accounted for 8–10% of metacyclics. The significance of the results to vaccination and the genetic control of antigenic variation is discussed.

When the tsetse fly introduces metacyclic forms of trypanosomes into the mammalian host, it initiates the pathogenesis of trypanosomiasis. The trypanosomes not only manage to keep ahead of the host's immune response but also cause, directly or indirectly, the gross pathological effects characteristic of the disease. The most obvious and most studied means by which they evade the host's response is antigenic variation.

To date, most studies of antigenic variation have been done on the subgenus *Trypanozoon*. Because of its high infectivity and virulence in mice and its ease of handling in vitro, it is more suitable for laboratory study than are the economically important *Duttonella* and *Nannomonas*. For similar reasons, most studies have focused on the trypanosome in the mammalian bloodstream.

Syringe-Passaged Populations

In the past decade, trypanosome clones have been extensively isolated and used to analyze antigenic variation during single bloodstream infections (McNeillage, Herbert, and Lumsden 1969; Van Meirvenne, Janssens, and Magnus 1975; Van Meirvenne et al. 1975; Capbern et al. 1977). With syringe-passaged trypanosomes, investigators found that each trypanosome possesses a variable antigen type (VAT)[1] conferred by the glycoprotein antigen in its surface coat. Their results indicate that a parasitemic peak is usually a mixture of VATs in different proportions (McNeillage, Herbert, and Lumsden 1969; Van Meirvenne, Janssens, and Magnus 1975). The host eliminates the major type or types at each remission, leaving others (heterotypes) to multiply and form the next peak. During multiplication, the parasites may compete, some growing faster than others, to establish the major VATs of the next peak

[1] The terminology used here (e.g., VAT) conforms to that agreed by an international discussion group (WHO 1978). A subsequent meeting on VAT nomenclature has now extended the system proposed by Lumsden, Herbert, and McNeillage (1967) to include the VAT repertoire of each trypanosome. Thus the series of VATs originally designated AnTat 1–13 (Antwerp *Trypanozoon* antigen type 1–13) (Van Meirvenne, Janssens, and Magnus 1975) are now designated AnTat 1.1–1.13, meaning that these are VATs 1–13 in the VAT repertoire 1 studied in Antwerp. This repertoire also has a new code: AnTAR 1 (Antwerp *Trypanozoon* Antigen Repertoire 1).

(McNeillage and Herbert 1968; Herbert 1975; Van Meirvenne, Janssens, and Magnus 1975). The existence of minor variants in a peak is clearly important for continuation of the infection and, in keeping with this, antigenic variation would appear to occur spontaneously, without being induced by specific antibody (Van Meirvenne, Janssens, and Magnus 1975; LeRay et al. 1977; Barry in press).

Certain VATs tend to predominate early in infections (Gray 1965; Van Meirvenne, Janssens, and Magnus 1975; Capbern et al. 1977), emerging in a loosely defined sequence regardless of the infecting VAT (Capbern et al. 1977). The number of VATs that a single trypanosome can express is known as its VAT repertoire, and although the extent of this is as yet unknown, Capbern et al. (1977) have shown that one clone of *T. equiperdum* produced at least 101 VATs. Within the subgenus *Trypanozoon*, different repertoires have common or cross-reacting VATs (Van Meirvenne et al. 1975; Van Meirvenne, Magnus, and Vervoort, 1977).

The overall picture is of the trypanosome expressing a large number of VATs in a loosely defined sequence. Presumably, the greater the number of VATs at any one time, the better the chance of the infection's persisting. Thus, a high degree of heterogeneity in the trypanosome population seems to be more important than the individual parasite responding to changing host conditions.

T. vivax can now be cloned, and similar studies on antigenic variation can be applied. We have intravenously injected into fresh mice single organisms of a naturally mouse-infective line isolated by Leeflang, Buys, and Blotkamp (1976). Ten clones, including 6 VATs, have been isolated. Interestingly, none of these shows antigenic cross-reaction with any of 9 *Trypanozoon* VAT repertoires investigated by Van Meirvenne, Magnus, and Vervoort (1977).

Preadaptation of Metacyclics

Some findings on the biology of metacyclics are relevant to this study of their antigenic variation: for instance, initiating infections with single metacyclics is relatively easy (Le Ray, Barry, and Vickerman 1978). In one experiment five mice were injected intravenously and five intraperitoneally with single metacyclics of *T. brucei* obtained by allowing a single tsetse fly to probe into guinea pig serum. In the intravenously injected group, all five developed infections, whereas only three in the other group were infected. The course of parasitemia of the intravenous clones was 1 day in advance of the intraperitoneal clones (Fig. 1), demonstrating clearly the metacyclics' high infectivity to mice. As previously hypothesized by Vickerman (1969b), the metacyclics appear to be preadapted for life in the bloodstream without needing to adapt extravascularly in the chancre before invading the vascular system.

Cyclically Transmitted Populations

To examine antigen variation in populations arising in mice following cyclical transmission, Le Ray et al. (1977) passaged AnTAR 1 trypanosomes (Van Meirvenne, Janssens, and Magnus 1975) through *Glossina morsitans* and used cloning, immunofluorescence, and trypanolysis to identify the ensuing VATs. Cloning of trypanosomes (derived from mice bitten by single infective flies) yielded 13 clones of which 3 were of types previously identified within the series AnTat 1.1–1.13; the remainder (10) included 8 new types.

Two features were immediately evident. Firstly, the cyclically passaged material provided clones that were less virulent and antigenically much less stable than did the syringe-passaged material

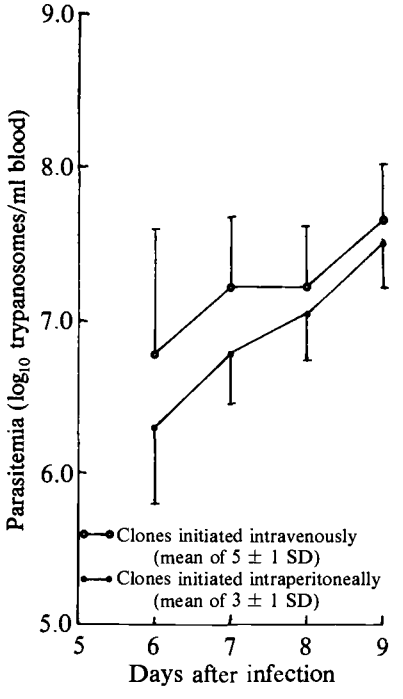

Fig. 1. *Parasitemias (estimated by the method of Herbert and Lumsden 1976) for* T. brucei *metacyclic clones.*

Table 1. Antigenic instability of clones from fly-transmitted populations.[a]

Clone	Passages from cloning (d)	Variants (%)
1	3.3.3.3.3	94
4	7.3.3	14
8	7.3.3	<1
9	7.3.3	6
11	7.3.3	5

[a]Variants were detected in trypanolysis using pooled antisera against AnTAR 1 trypanosomes (based on Le Ray et al. 1977).

studied by Van Meirvenne, Janssens, and Magnus (1975) (Table 1). The differences were detected in populations rather than individual trypanosomes and may reflect differences in degree of pleomorphism (Le Ray et al. 1977; Barry, Le Ray, and Herbert in press). To obtain VAT-specific antisera against the new clones, Le Ray et al. (1977) adapted the trypanosomes to mice by repeated syringe passage, neutralizing heterotypes at every 3-day passage. By this method, specific antiserum was raised successfully for most of the VATs.

Secondly, the populations arising in mice after fly bite proved to be antigenically very heterogeneous, possessing in one case at least 13 VATs after 5 days and 19 of 22 sought after a further 3-day syringe passage in a mouse (Le Ray et al. 1977). Whether the heterogeneity resulted from the antigenic instability of cyclically transmitted trypanosomes or from heterogeneity among metacyclics was not clear, but the general results support the idea that *T. brucei* presents an antigenic mixture to its host: the population is of greater importance than the individual trypanosome.

Metacyclic Populations

To look at the VATs of metacyclics, Le Ray, Barry, and Vickerman (1978) used a variety of sera in trypanolysis and immunofluorescence on freshly probed metacyclics of VAT repertoire AnTAR 1. They allowed flies to feed on rabbits and after 7 days obtained antisera that were capable of lysing all or most metacyclics in one sample (Table 2). Immunofluorescence gave comparable results. This method of obtaining antiserum is similar to that used by Jenni (1977a) and gives similar results. However, it is completely unsuitable for use in identifying individual antigen types: if the immunizing metacyclics are antigenically heterogeneous, the antiserum will be correspondingly heterospecific, with activity against most or all metacyclics.

Use of more characterized antisera produces a completely different picture (Le Ray, Barry, and Vickerman 1978). Pooled monospecific antisera against AnTat 1.1−1.22 lyse less than 10% of metacyclics in a preparation where up to 100% are lysed by other antisera. Quite clearly then, the metacyclics within one probe are antigenically heterogeneous. Le Ray, Barry, and Vickerman found that all the metacyclics in single probes are lysed with long-term sera from rabbits infected by syringe with bloodstream forms. The significance of these findings is that the metacyclic VATs are not specific to the fly stage of the life cycle; they are expressed also by bloodstream forms.

To demonstrate unequivocally that metacyclic VATs are heterogeneous, we needed to identify at least two types within one probe. The cyclically transmitted trypanosomes, being antigenically unstable, are unsuitable for the production of VAT-specific antisera against clones of either

Table 2. Homology of metacyclic VATs with bloodstream form VATs.

	Trypanolysis (no. lysed / total)			
		Antisera against:		
Fly	Control	Metacyclic[a]	Bloodstream VATs[b]	Bloodstream VAT repertoire[c]
A	0/14	14/16	3/10	7/10
B	0/10	65/76	1/12	10/11
C	0/6	7/8	3/6	–
D	2/100	–	14/169	70/70
D	0/25	–	0/25	50/50
Total	2/155	86/100	21/222	137/141
%	1.29	86.00	9.45	97.16

[a]Rabbit serum 7 days after bite by infective flies.

[b]Pooled monospecific antisera against AnTat 1.1−1.22.

[c]Pooled long-term infection sera (32−43 days) from five rabbits infected with bloodstream form AnTAR 1 trypanosomes.

Source: Le Ray, Barry, and Vickerman (1978).

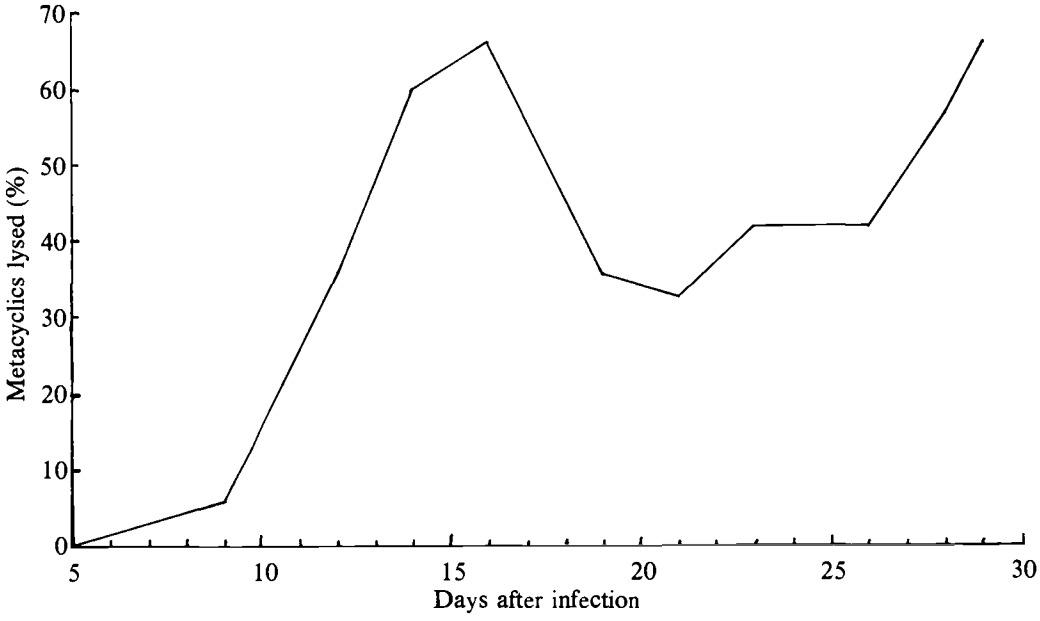

Fig. 2. Development of antimetacyclic activity in a rabbit infected by syringe.

bloodstream forms (Le Ray et al. 1977) or metacyclics (Le Ray, Barry, and Vickerman 1978). Trypanosome lines adapted to mice by at least 20 syringe passages are more stable so we used them to identify and clone single trypanosomes antigenically homologous with metacyclics and subsequently to produce monospecific antisera. The following protocol was devised (Barry et al. in press). We infected a rabbit with a clone from the syringe-passaged line investigated by Van Meirvenne, Janssens, and Magnus (1975). Every 2 or 3 days we took samples of serum and passaged the blood into mice. When parasitemia was patent in the mice, we prepared stabilates in liquid nitrogen. We then tested the whole range of sera in trypanolysis against metacyclics from a single probe and recorded the percentages of metacyclics lysed (Fig. 2). Two or possibly three peaks of activity, none of which reached 100%, were apparent. The finding provides further evidence for antigenic heterogeneity and suggests that at least two or three VATs homologous to metacyclics arose at different times. We initiated cloning, using the stabilate material that was prepared from blood drawn at the onset of the first peak of lytic activity against metacyclics. We tested the VAT of each clone in trypanolysis with antimetacyclic sera taken from rabbits 7 days after fly bite (Table 2).

The 10th clone isolated, whose VAT has now been designated AnTat 1.30 within the AnTAR 1 series, was completely lysed by the antimetacyclic sera. We used it to prepare monospecific antiserum in a rabbit by the method of Van Meirvenne, Janssens, and Magnus (1975) and conducted serological testing on metacyclics. Using immunofluorescence, we found that 11–20% of trypanosomes stained within single probes, and we obtained corresponding figures using trypanolysis. Monospecific mouse antiserum gave the same results — strong evidence for the heterogeneity of metacyclic VATs (Barry et al. in press).

To identify a second metacyclic VAT, we systematically applied to probes 22 monospecific antisera, each against one of AnTat 1.1–1.22, which had previously been shown to account for ca. 10% of metacyclics (Table 1). We expected at least one to exhibit activity. The results indicated that AnTat 1.6 probably accounted for the entire 10%. The conclusion: metacyclics, at least within the AnTAR 1 trypanosomes, are antigenically heterogeneous within a single probe.

The specific antisera against the VATs have also been applied, in a preliminary study, to different probes. Regardless of what VAT was originally ingested by the fly, the age of infection, or the period between probes (at least 2 days), the percentage of the two VATs always fell within certain ranges: 8–10% for AnTat 1.6 and 11–20% for AnTat 1.30 (tables 3 and 4). The behaviour of AnTat 1.30 in a cyclically infected mouse has also been studied: by day 3 post infection 50% of the bloodstream forms were AnTat 1.30. The percen-

Table 3. Presence of AnTat 1.30 and AnTat 1.6 in metacyclic populations.

	Immunofluorescence			
Fly	Anti-AnTat 1.30 (no. labeled/ no. counted)	%	Anti-AnTat 1.6 (no. labeled/ no. counted)	%
C[a]	38/200	19	–	–
1	41/220	18.6	17/200	8.5
1	–	–	28/290	9.7
2	81/466	17.4	8/100	8
2	14/100	14	–	–
3	25/140	17.8	20/200	10

[a]Same as fly C in Table 2.

tage decreased on day 4, although the absolute number of AnTat 1.30 rose to its maximum on day 4 and then declined sharply. There was a great increase in numbers of AnTat 1.30 trypanosomes; however, by day 4 they were being outgrown by trypanosomes of a different VAT and were destroyed the following day. The transformation of metacyclics to long slender bloodstream forms would appear in this case not to be linked to antigenic variation. Some of the predominant VATs previously demonstrated by Van Meirvenne, Janssens, and Magnus (1975) were found to grow up as a mixture even from the 4th day of infection.

These data lead to the conclusion, reached before, that *T. brucei* presents an antigenically heterogeneous population as a means of coping with the host's immune system. Thus, individual metacyclics are less important than the whole metacyclic population, within which certain VATs, such as AnTat 1.30, are capable of amplification in the mammalian host and consequent immune destruction. Others may remain at low levels and give rise to the full range of predominant bloodstream VATs. Whether metacyclic VATs can interfere with the expression of each other in the host bloodstream, as postulated for bloodstream populations (McNeillage and Herbert 1968; Herbert 1975; Van Meirvenne, Janssens, and Magnus 1975) remains an open question.

Vaccination

The age-old problem of vaccination merits some discussion in the light of our results. The existence of a single "basic" metacyclic antigen type, and its potential in immunization, must now be viewed as unlikely. A VAT mixture in the tsetse salivary gland appears to be advantageous to the trypanosome, affording greater resistance to the host's immune response. There may be a constant appearance of certain VATs, and at constant percentages, in the metacyclic population; therefore, a "basic VAT repertoire" is possible.

Table 4. Results of immunofluorescence of AnTat 1.30 in sequential probes of one fly.

Age of infection (d)	AnTat 1.30 (%)
26	16
37	20
46	11

If this proves to be the case, vaccination can be reevaluated. Our protocol permits the preparation of large numbers of antigenically stable bloodstream forms of trypanosomes, from which variable antigen can be purified readily (Cross 1975; 1977b) and used effectively for VAT-specific immunization at low concentrations (Baltz et al. 1977). It should also permit the isolation of pure mRNA for potential use in gene cloning in microbial systems and large-scale production of variable antigen (R.O. Williams p. 46).

Of obvious necessity, then, are both the further application of this protocol to characterize fully the VAT complement of probed populations and the determination of the number of serodemes circulating in the field. This approach may be more applicable to *T. vivax* and *T. congolense* than to *T. brucei*, because in these cases substantially fewer trypanosomes, and thus possibly fewer VATs, are probed.

Control of Antigenic Variation

To date, nothing is known about the control of antigenic variation or about the process that initiates the expression of the antigen. Even the demonstration of predominant antigen types does not reveal much, because they are detected as populations and may not reflect what occurs at the level of individual parasites. Nevertheless, our findings on expression of metacyclic VATs may open the way for basic studies on control mechanism(s).

It appears that certain VATs are always expressed in the salivary gland population, and furthermore they are expressed at constant percentages. Although further investigation is required, current evidence suggests that the preinfective epimastigote stage of *T. brucei* is found attached to the secretory epithelium of the salivary gland and gives rise, via immature forms, to the unattached mature infective metacyclic (reviewed by Vickerman 1978). The qualitative and quantitative constancy of these

metacyclic VATs implies that one (or more) of the following conditions exists:

- Up to 20% of epimastigotes are programed to give rise to metacyclics coding for AnTat 1.30 and up to 10% for AnTat 1.6;
- Equal numbers of epimastigotes are programed for eventual expression of different VATs but give rise to metacyclics at different rates;
- Each epimastigote produces 20% metacyclics expressing AnTat 1.30 and 10% expressing AnTat 1.6;
- Metacyclics of different VATs interfere with each other and control their relative numbers in the population, as postulated for bloodstream form VATs.
- The signal for expression is not present in epimastigote forms but is initiated in the immature metacyclic and at different frequency for different VATs.

These possibilities are easy to examine initially just by using monospecific antisera in situ with appropriate markers. Now, there are the means to look at the crucial stage between nonexpression and expression of variable antigen.

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