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SEROLOGICAL CROSS-REACTIONS AMONG TRYPANOSOME VARIABLE ANTIGEN ISOTYPES OF THE SUBGENUS *TRYPANOZOON*

by

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Summary — Clones of 4 serologically similar Variable Antigen Types (Iso-VATs) were derived from mouse-passaged lines of *T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense* and *T. evansi*. Cross reactions were observed in immunolysis, agglutination and immunofluorescence tests. However, comparative titration and absorption experiments yielded distinct patterns of reactivity. The serotypical identity of trypanosome VATs is probably determined by a complex combination of several antigenic determinants in the surface coat glycoprotein.

KEYWORDS : Antigenic variation; Variable Antigen Type; Variable Antigen Isotype (Iso-VAT); Variable Antigen Repertoire; Antigenic determinant; Immunofluorescence; Immunolysis; Agglutination; Immuno-absorption; *Trypanozoon; T. b. brucei*; *T. b. rhodesiense*; *T. b. gambiense*; *T. evansi*.

Introduction

In the mammalian host, a trypanosome clone of the subgenus *Trypanozoon* can produce more than one hundred distinct variable antigen types (VATs) differing in their surface coat protein (Capbern *et al.*, 1977; Cross, 1980; Vickerman, 1978). Comparative studies of variable antigen repertoires (VARs) have shown that distinct VARs tend to produce their own characteristic series of predominant VATs during the first few weeks of infection (reviewed by Gray and Luckins, 1976). However, differentiation of VARs on this basis is complicated by some overlap due to the occurrence of serologically similar VATs, so-called Iso-VATs, which cross-react in immunolysis, agglutination and immunofluorescence tests (Gray and Luckins, 1976; Van Meirvenne *et al.*, 1975b, 1977).

We have prepared several sets of clones of such Iso-VATs. The serological relationships of one set of 4 Iso-VATs were studied during the present experiments.

Materials and methods

Trypanosomes

A clone of each VAT was derived from mouse-passaged clone lines of *T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense* and *T. evansi*. Pedigrees are given in figures 1 to 4.

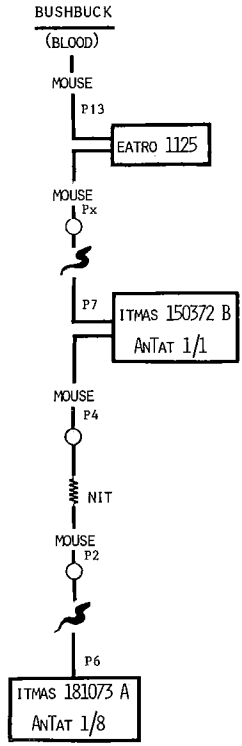

Figure 1.

Pedigree of clone AnTat 1/8.

P13, P4, a.s.o. = number of syringe-passages at 3-4 days interval.

Px = undefined large number of syringe passages.

NIT = Selective Neutralization-Infection Test using lytic antisera.

ITMAS = Institute of Tropical Medicine Antwerp — Serology (stabilate code).

= cloning.

= population sample.

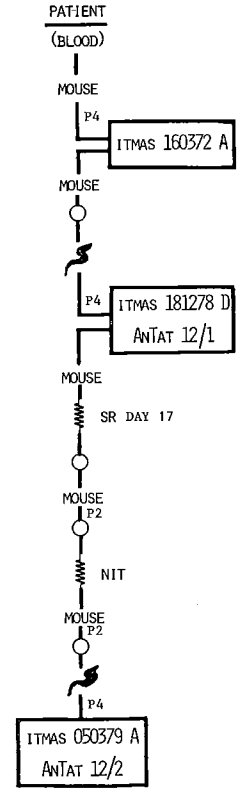

Figure 2.

Pedigree of clone AnTat 12/2.

SR = Spontaneous Relapse *in vivo*.

— *AnTat 1/8* (Antwerp *Trypanozoon* antigen type 8 from antigen repertoire AnTAR 1) is one of the VATs expressed by a clone line of *T. b. brucei* derived from stabilate EATRO 1125/LUMP 581 (Van Meirvenne *et al.*, 1975a, 1977). The original stock was isolated from a bushbuck at Mavubwe, Uganda in 1966.

— *AnTat 12/2* was derived from a stock of *T. b. rhodesiense* isolated in 1971 from a patient, infected in the Kagera Park, Rwanda.

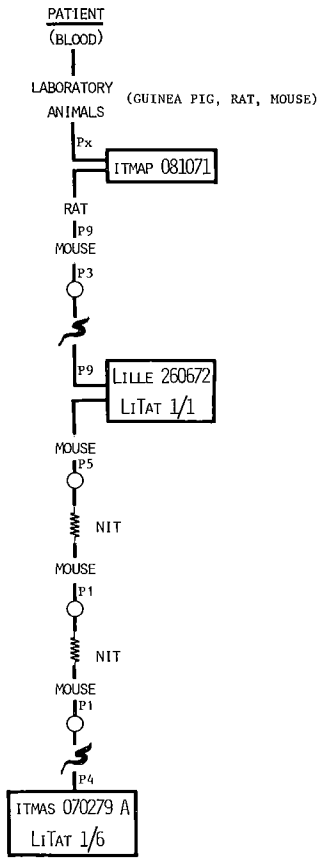

Figure 3.
Pedigree of clone LiTat 1/6.

ITMAP = Institute of Tropical Medicine Antwerp — Protozoology (stabilate code).

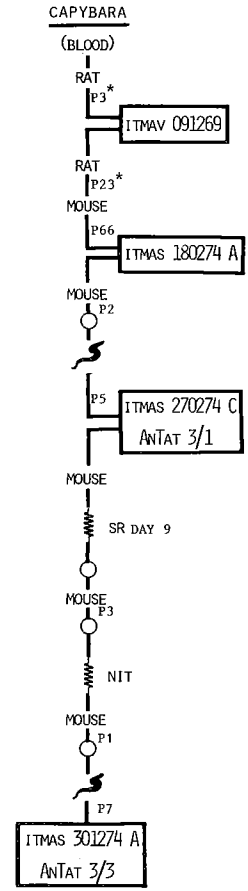

Figure 4.
Pedigree of AnTat 3/3.

* interval : several days to several weeks.

ITMAV = Institute of Tropical Medicine Antwerp — Veterinary department (stabilate code).

— *LiTat 1/6* (Lille *Trypanozoon* antigen type 6 from antigen repertoire LiTAR 1) was derived from a clone line of *T. b. gambiense* originally isolated from a patient in Ivory Coast in 1952 (Afchain, 1976).
— *AnTat 3/3* was derived from a stock of *T. evansi*, originally isolated from a South-American Capybara (Kageruka and Mortelmans, 1971).

The first findings of AnTat 12/2, LiTat 1/6 and AnTat 3/3 were made by immunofluorescence tests using antiserum to AnTat 1/8. For cloning purposes, populations containing sufficiently high proportions of these Iso-VATs were obtained by relapsing infections or by immune selection *in vitro* (Neutralization Infection Test).

Antisera

Sixth-day infection antisera against the VATs were raised in 4 groups of 2 New Zealand White rabbits (animals A to H) by intravenous inoculation of 2×10^7 clone trypanosomes in 1 ml of Phosphate-Saline-Glucose buffer (PSG, pH 8.0).

Antisera of increasing antibody-complexity were collected from 4 other groups of 2 rabbits (animals I to P) on days 4, 5, 6, 7, 8, 11 and 14 (15).

Immunolysis

Tests were carried out as described by Van Meirvenne *et al.* (1975a).

Immunofluorescence on smears

Parasitized blood smears were dried, fixed with acetone for 15 minutes and further processed as described earlier (Van Meirvenne *et al.*, 1975a). Antisera were titrated by testing serial twofold dilutions of 1 : 20 to 1 : 160 in Phosphate-Buffered-Saline (pH 7.3). Readings were made using a Leitz-Orthoplan microscope (10 × 40), Ploem-illumination, a mercury vapour lamp and a KP 490 excitation filter. The intensity of fluorescence was scored as +++ (strongly positive), ++ (positive), + (weakly positive) or − (negative). The highest serum dilution still giving a + result was considered as the end-titre.

Immunofluorescence on live trypanosomes

Tests were carried out in tubes at 4 °C. 0.25 ml-volumes of serial twofold antiserum dilutions (1 : 2.5 to 1 : 320) were prepared in PSG (pH 8.0) supplemented with 10 per cent fetal calf serum (FCS). A suspension containing 5×10^7 column-eluated (Lanham, 1968) trypanosomes per ml was prepared in PSG-FCS and 0.25 ml aliquots added to the antiserum dilutions for 45 minutes. Trypanosomes were washed with PSG-buffer (4 ml) by centrifugation (4 °C; 10 min; 1,400 g). Supernating fluid was discarded and the washing procedure repeated twice. The pelleted trypanosomes were resuspended in 0.25 ml PSG-FCS and 0.25 ml aliquots of FITC-conjugated sheep-anti rabbit Ig serum (Institut Pasteur, Paris), diluted 1 :50 in PSG containing Evans Blue (1/10,000, w/v) added to each tube. After 30 minutes, the trypanosomes were washed again by 3 centrifugations and fixed by adding 0.25 ml of a 2 per cent solution of formaldehyde in PSG. Wet preparations were examined and end-titres determined as for blood smears. Dotted patterns of surface coat fluorescence, often obtained with high serum dilutions were recorded as weakly positive (+).

Direct agglutination

Trypanosomes were separated from parasitized mouse or rat blood (Lanham, 1968; Lanham and Godfrey, 1970), washed twice by centrifugation

(4 °C; 15 min; 1,400 g) with cold PSG-buffer and a suspension containing about 10^8 organisms per ml of PSG was prepared.

Fifty microliters of twofold serum dilutions in PSG and equal aliquots of the trypanosome-suspension were mixed in Microtiter U-plates and incubated at 4 °C for 60 minutes. Results were read under an illuminated magnifying glass. The highest serum dilution yielding a sediment of aggregated trypanosomes was considered as the end-titre.

Absorption

Column-separated trypanosomes from the blood of infected mice or rats were spun down and washed twice with cold PSG-buffer. Aliquots (± 0.35 ml) of packed live trypanosomes were kept in tubes in an ice bath at 0 °C.

To a first tube, 1.5 ml of heat-inactivated antiserum (56 °C, 30 min) was added, mixed gently with the trypanosomes and left at 4 °C for 90 minutes. The serum was separated by centrifugation, re-incubated for 90 minutes in a second tube, recovered by centrifugation and stored at − 80 °C.

Though strong agglutination of the trypanosomes always occurred during the first and often during the second incubation, morphology and motility were not affected.

Results

Cross-titrations of 6th-day infection antisera

The 6th-day infection antisera of rabbits A to H were cross-titrated by immunofluorescence on acetone-fixed blood smears and by immunolysis, agglutination and immunofluorescence on live trypanosomes. The results are given in table 1.

When considering the results obtained with live trypanosomes, it may be seen that :

1. In general, the highest end-titres were obtained in homologous combination.
2. AnTat 1/8 and LiTat 1/6 showed the highest degree of cross-reactivity.
3. Clear differences in reactivity between AnTat 3/3 and AnTat 1/8-LiTat 1/6 were seen with some antisera and some test systems.
4. Clear-cut differentiation between AnTat 12/2 and AnTat 3/3 was made by both antisera to AnTat 12/2, both antisera to AnTat 3/3 and, in some tests, by one antiserum to AnTat 1/8 (rabbit B) and one antiserum to LiTat 1/6 (rabbit E).
5. Using antisera to AnTat 1/8, AnTat 12/2 and LiTat 1/6, the reactivity of AnTat 12/2 was clearly distinct from that of AnTat 1/8 and LiTat 1/6. This difference was less pronounced with antisera to AnTat 3/3.

TABLE 1

End-titres (reciprocals) of 6th-day infection antisera of rabbits A to H obtained by cross-testing against the four VATs in four test systems

6th-day ANTISERUM		VAT	END-TITRES			
VAT INOCULATED	RABBIT	TESTED	IMMUNOLYSIS	DIRECT AGGLUTINATION	IMMUNOFLUORESCENCE on LIVE TRYPs	IMMUNOFLUORESCENCE on BLOODSMEARS
AnTat 1/8	A	AnTat 1/8	256-512	256	NT	160-320
		AnTat 12/2	64	64	NT	80
		LiTat 1/6	256-512	128-256	NT	160-320
		AnTat 3/3	128	64	NT	160
	B	AnTat 1/8	256	256	320	160
		AnTat 12/2	8	8-16	10	80
		LiTat 1/6	256	128-256	160-320	80
		AnTat 3/3	128	32	40	80
AnTat 12/2	C	AnTat 1/8	8	16	5	40
		AnTat 12/2	64	128	80	80
		LiTat 1/6	16	16-32	20	40
		AnTat 3/3	16	32	20	40
	D	AnTat 1/8	16	16	10	80
		AnTat 12/2	64	128	160-320	160
		LiTat 1/6	32	16-32	20	80
		AnTat 3/3	32	16	20-40	80
LiTat 1/6	E	AnTat 1/8	256	256	160-320	320
		AnTat 12/2	32	32	80	320
		LiTat 1/6	256-512	128-256	320	320
		AnTat 3/3	128	64	160	320
	F	AnTat 1/8	256-512	256	160	320
		AnTat 12/2	128	64	80	320
		LiTat 1/6	256-512	128-256	160	320
		AnTat 3/3	128-256	128	160	320
AnTat 3/3	G	AnTat 1/8	64	64	80-160	80
		AnTat 12/2	32	32	80	40
		LiTat 1/6	64-128	32-64	160	80
		AnTat 3/3	256	128	320	160-320
	H	AnTat 1/8	64-128	NT	160	160
		AnTat 12/2	32	NT	80	80
		LiTat 1/6	64	NT	160	160-320
		AnTat 3/3	256	NT	320	320

NT = NOT TESTED

As compared with tests on live trypanosomes, poor differentiation was obtained by immunofluorescence on acetone-fixed blood smears. Five out of 8 antisera failed to detect differences in reactivity between the 4 VATs (rabbits B, C, D, E and F).

Kinetics of cross-reacting lytic antibodies

The serum samples, sequentially collected from rabbits I to P after various periods of infection were titrated for lytic antibody against each VAT. The results are shown in figures 5 to 8.

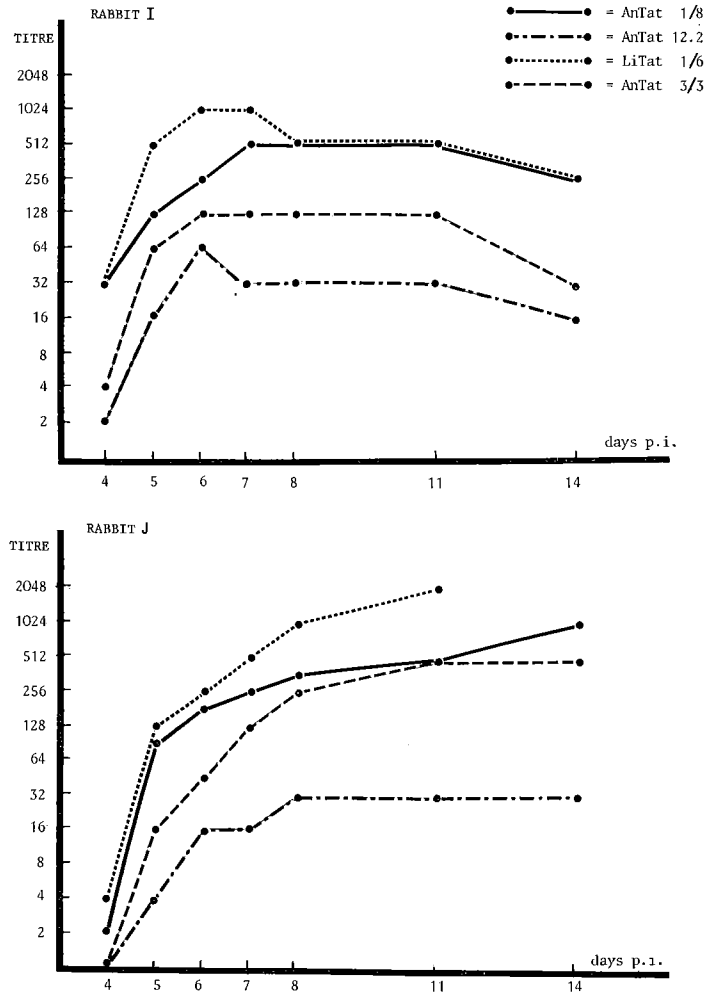

Figure 5.

Evolution of lytic antibody titres against the four VATs in two rabbits (I and J) inoculated with AnTat 1/8.

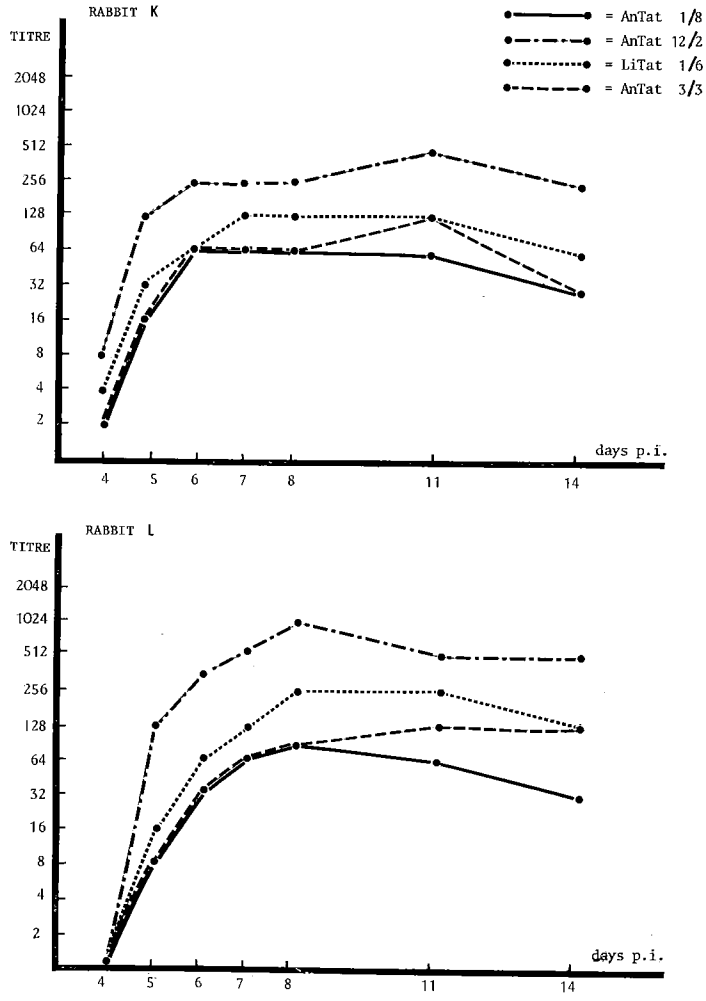

Figure 6.

Evolution of lytic antibody titres against the four VATs in two rabbits (K and L) inoculated with AnTat 12/2.

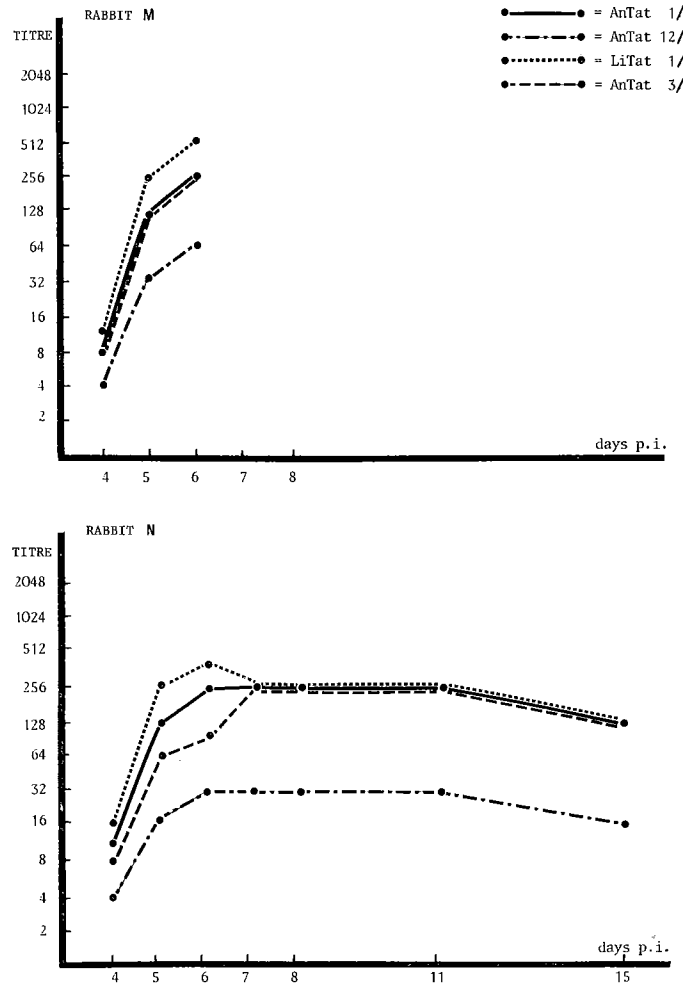

Figure 7.

Evolution of lytic antibody titres against the four VATs in two rabbits (M and N) inoculated with LiTat 1/6.

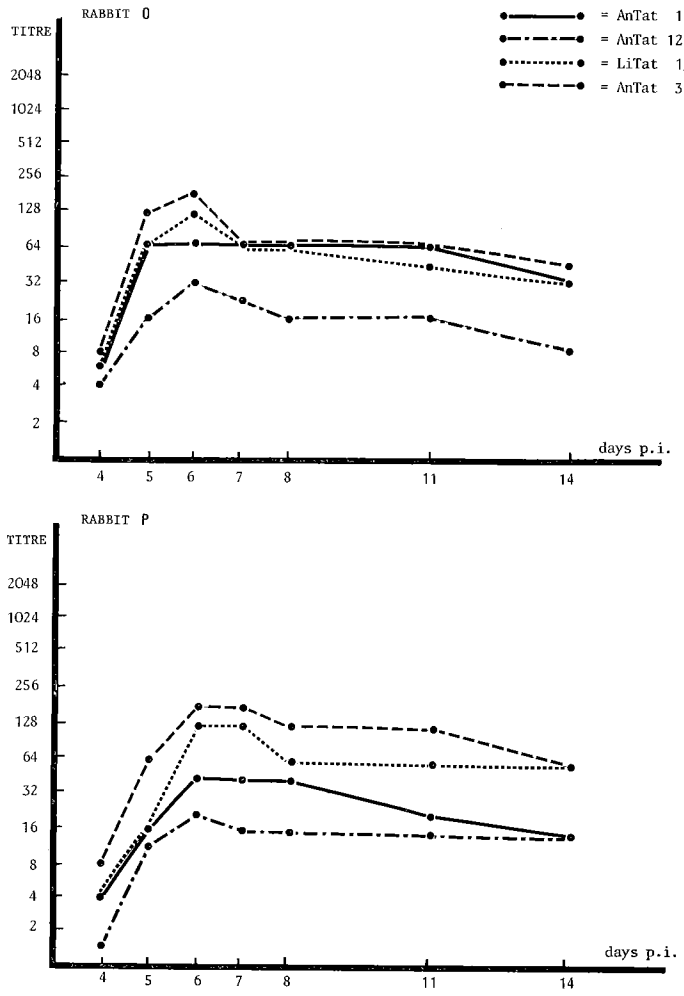

Figure 8.

Evolution of lytic antibody titres against the four VATs in two rabbits (O and P) inoculated with AnTat 3/3.

From day 4 or 5 on, all sera contained lytic antibodies against each of the 4 VATs. Maximal titres of 1 : 16 to 1 : 1024 were reached within 6-8 days of infection. During the second week, plateau levels or a two- to fourfold decrease in titre were observed. On day 14-15, the end-titres varied from 1 : 8 to 1 : 1024.

Considering individual animals, the differentiating capacity of the antisera largely depended upon the length of infection. Detailed comparison of the results of different infection groups was complicated by the occurrence of variations within each group. However, in all instances the curves of AnTat 12/2 were quite distinct from those of the 3 other VATs.

Comparing the relative position of the curves within each infection group, the degree of reactivity of the 4 VATs in decreasing order was as follows :

— group AnTat 1/8 : LiTat 1/6, AnTat 1/8, AnTat 3/3, AnTat 12/2
— group AnTat 12/2 : AnTat 12/2, LiTat 1/6, AnTat 3/3, AnTat 1/8
— group LiTat 1/6 : LiTat 1/6, AnTat 1/8, AnTat 3/3, AnTat 12/2
— group AnTat 3/3 : AnTat 3/3, LiTat 1/6, AnTat 1/8, AnTat 12/2

Lytic activity of cross-absorbed 6th-day infection antisera

The 6th-day antisera of rabbits J (inoculated with AnTat 1/8), L (AnTat 12/2), N (LiTat 1/6) and P (AnTat 3/3) were absorbed with live trypanosomes of each VAT in 16 different combinations. Untreated and absorbed serum samples were titrated for lytic activity against each VAT. Results are shown in tables 2 to 5.

TABLE 2

Trypanolytic end-titres of 6th-day antiserum to AnTat 1/8 (rabbit J) before and after absorption

Sorbent	VAT tested			
	AnTat 1/8	AnTat 12/2	LiTat 1/6	AnTat 3/3
None	128/256	16	256	32/64
AnTat 1/8	Neg.	Neg.	Neg.	Neg.
AnTat 12/2	128	Neg.	128	32
LiTat 1/6	8	Neg.	Neg.	Neg.
AnTat 3/3	32	4	32	Neg.

TABLE 3

Trypanolytic end-titres of 6th-day antiserum to AnTat 12/2 (rabbit L) before and after absorption

Sorbent	VAT tested			
	AnTat 1/8	AnTat 12/2	LiTat 1/6	AnTat 3/3
None	32	256/512	64	32
AnTat 1/8	Neg.	128	8	16
AnTat 12/2	Neg.	Neg.	Neg.	Neg.
LiTat 1/6	Neg.	128	Neg.	4
AnTat 3/3	4	128	4	Neg.

TABLE 4

Trypanolytic end-titres of 6th-day antiserum to LiTat 1/6 (rabbit N) before and after absorption

Sorbent	VAT tested			
	AnTat 1/8	AnTat 12/2	LiTat 1/6	AnTat 3/3
None	256	32	256/512	64/128
AnTat 1/8	Neg.	4/8	4/8	4
AnTat 12/2	128	Neg.	128/256	64
LiTat 1/6	Neg.	Neg.	Neg.	Neg.
AnTat 3/3	32	8	32/64	Neg.

TABLE 5

Trypanolytic end-titres of 6th-day antiserum to AnTat 3/3 (rabbit P) before and after absorption

Sorbent	VAT tested			
	AnTat 1/8	AnTat 12/2	LiTat 1/6	AnTat 3/3
None	32/64	16/32	128	128/256
AnTat 1/8	Neg.	4	4	16/32
AnTat 12/2	32	Neg.	64	64/128
LiTat 1/6	Neg.	2	Neg.	16/32
AnTat 3/3	Neg.	Neg.	Neg.	Neg.

All absorptions rendered antiserum non-reactive with the absorbing VAT. When absorbed with the VAT inoculated into its donor animal the antiserum became inactive against any VAT. Absorption of heterologous antisera with AnTat 12/2 had little effect on their end-titres to AnTat 1/8, LiTat 1/6 and AnTat 3/3.

Per antiserum, details are as follows :

— *antiserum to AnTat 1/8* (table 2) :

LiTat 1/6 absorbed out the activity against AnTat 12/2 and AnTat 3/3 but some activity with AnTat 1/8 was left. Sorbent AnTat 3/3 considerably decreased all end-titres.

— *antiserum to AnTat 12/2* (table 3) :

Absorption with AnTat 1/8, LiTat 1/6 or AnTat 3/3 had little effect on the reactivity with AnTat 12/2. Sorbent AnTat 1/8 substantially decreased reactivity with LiTat 1/6 and, to a less extent, that with AnTat 3/3. LiTat 1/6 completely absorbed out the activity against AnTat 1/8 and partially that against AnTat 3/3. Absorption with AnTat 3/3 resulted in low residual activity against AnTat 1/8 and LiTat 1/6.

— *antiserum to LiTat 1/6* (table 4) :

Sorbent AnTat 1/8 strongly reduced the activity against LiTat 1/6 and AnTat 3/3 and, less drastically, against AnTat 12/2. Sorbent AnTat 3/3 had a similar effect on reactivity with AnTat 1/8, LiTat 1/6 and AnTat 12/2.

— *antiserum to AnTat 3/3* (table 5) :

Sorbent AnTat 1/8 strongly decreased the end-titres for LiTat 1/6 and less for AnTat 12/2 and AnTat 3/3. Absorption with LiTat 1/6 had a similar effect on the reactivity with the latter two VATs and made the serum non-reactive with AnTat 1/8.

Discussion

The present results confirm the earlier findings (Van Meirvenne *et al.*, 1975b, 1977) of serologically cross-reacting VATs in different species of trypanosomes. Moreover they indicate that such Iso-VATs display several degrees of similarity.

The present 4 Iso-VATs cross-react with each other's infection antisera. As compared with immunofluorescence tests on acetone-fixed blood-smears, a more clear-cut serotype differentiation was achieved by tests on live trypanosomes (immunolysis, immunofluorescence, direct agglutination) and by absorbing out cross-reacting lytic antibodies. The patterns of serological overlap suggest that different combinations of several antigenic determinants are exposed at the surface of live trypanosomes of each individual VAT. Indirect immunofluorescence on acetone-fixed smears probably results in exposure and immune-amplification of extra common antigenic sites present in deeper regions of the surface glycoproteins. If correct, the antigen specificity, then follows a descending path from the exterior to the interior parts of the molecule. However, because of their complex antibody spectrum, infection antisera are not optimal tools for comparative dissection of variable trypanosome antigens. Using monoclonal antibodies in immunofluorescence tests, Pearson *et al.* (1980) demonstrated the existence of both exposed and non-exposed antigenic sites. Some antibodies did stain acetone-fixed trypanosomes but not the surface of live organisms while other antibodies stained both.

The practical implications of the Iso-VAT phenomenon remain difficult to assess. Our own long-term comparative studies on VAT-repertoires of cloned *Trypanozoon* stocks resulted in frequent findings of overlapping Iso-VATs and of antigen repertoires displaying various degrees of similarity and cross-protection. Unfortunately, no data are yet available about the occurrence of metacyclic Iso-VATs which, eventually, might provide interesting reagents for serodiagnosis and immune protection.

In the meantime, the variable surface glycoproteins (VSGs) of the present VATs have been subjected to comparative biochemical and immunochemical studies (Vervoort *et al.*, 1981).

Recent studies using recombinant DNA technology have provided clues to the mechanism of antigenic variation in salivarian trypanosomes. It has been demonstrated that VSGs are coded for by a large number of genes which are present in the nuclear DNA and which become alternately activated and expressed by genomic rearrangements (Boothroyd *et al.*, 1981; Borst *et al.*, 1980a, b; Englund *et al.*, in press; Hoeijmakers *et al.*, 1980; Marcu, 1981; Pays *et al.*, 1981b; Richards *et al.*, 1981; Williams *et al.*, 1981). The current hypothesis that different sets of VSG-genes arise and evolve by a process of duplication, mutation and deletion fits perfectly well with

the occurrence of Iso-VATs and overlapping serodemes. The expected cross-hybridization of the VSG-genes of the present Iso-VATs has recently been confirmed (Pays *et al.*, 1981a; Pays *et al.*, in press). On theoretical grounds one would expect as well the existence of « intra-repertoire » Iso-VATs within one and the same clone line. Monoclonal antibodies seem to be required for identification of such missing links.

An exciting perspective for the next future is the characterization of trypanosome isolates by hybridization of their nDNA restriction digests with cDNA probes of reference Iso-VATs.

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Serologische kruisreakties tussen variabele antigeen-isotypen (Iso-VATs) bij trypanosomen van het subgenus *Trypanozoon*.

Samenvatting — Uit muis-overgeënte lijnen van *T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense* en *T. evansi* werden kloons van 4 serologisch gelijkaardige variabele antigeentypes (Iso-VATs) geïsoleerd. Zowel in immunolyse, agglutinatie als immunofluorescentie werden kruisreacties waargenomen. Vergelijkende titraties en kruis-absorptieproeven brachten evenwel min of meer verschillende reaktiviteiten aan het licht. De serotypische identiteit van antigeenvarianten wordt waarschijnlijk bepaald door een komplexe kombinatie van meerdere antigeendeterminanten in het glycoproteine van de oppervlaktelaag der trypanosomen.

Réactions sérologiques croisées entre isotypes d'antigènes variables (Iso-VATs) de trypanosomes du sous-genre *Trypanozoon*.

Résumé — Quatre types antigéniques variables sérologiquement similaires (Iso-VATs) ont été clônés à partir de lignées de *T. b. brucei*, *T. b. rhodesiense*, *T. b. gambiense* et *T. evansi*. Leur similarité sérologique a été établie par des tests de trypanolyse, d'agglutination et d'immunofluorescence. Toutefois, une réactivité plus ou moins distincte a été démontrée par de titrations comparées et des absorptions croisées. L'identité sérotypique de variants antigéniques est probablement déterminée par plusieurs déterminants antigéniques différents dans la glycoprotéine de surface des trypanosomes.

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