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ANTIGENIC VARIATION IN SYRINGE PASSAGED POPULATIONS OF *TRYPANOSOMA (TRYPANOZOON) BRUCEI*.

I. RATIONALIZATION OF THE EXPERIMENTAL APPROACH

by

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Summary — Clone populations of thirteen different antigenic variants of *Trypanosoma (Trypanozoon) brucei*, designated AnTat 1 to AnTat 13, were obtained in mice and cryopreserved as stabilate material. The antigenic characteristics of these clones and of variant populations derived from them were studied, and their relationships to one another established by means of trypanolysis, neutralization and immunofluorescence tests. These methods greatly facilitated the systematic collection of clones of distinct antigenic type and yielded detailed information on the variant composition of populations. Clone populations were found to be heterogeneous mixtures of a major variant type and of several minor types. The results suggest that antigenic variation is a spontaneous random process capable of producing a large number of different antigenic types, some of which are produced more frequently than others and represent so-called predominant variants.

KEYWORDS : Trypanosoma (Trypanozoon) brucei; Antigenic variation; Predominant antigenic types; Trypanolysis; Neutralization; Immunofluorescence; Clones; Infectivity; Immunity; Serodeme.

Introduction

The degree of specifically acquired functional immunity against salivarian trypanosomiasis mainly depends on the variant specific immune state of the host. Ritz (1916) demonstrated that even a single trypanosome is capable of producing a large number of antigenic variant types. Lourie and O'Connor (1937), by setting up clones and typing them by trypanolysis tests, showed that heterogeneously behaving populations were mixture of individuals of distinct antigenic type. McNeillage, Herbert and Lumsden (1969) found that the first relapse populations arising in mice after infection with clone organisms were complex mixture of several antigenic types. Gray (1965a, b) demonstrated that antigenic variation is a reversible process and that any variant has a tendency to reverse to basic or predominant antigenic types when subpassaged into non immune hosts. In spite of intensive studies carried out by several other investigators the fundamental nature and the potential of the variation process remain unknown. The existing methods appear to be inadequate to provide a convincing solution to these problems. In the present paper, a simple but precisely accurate methodology is described for study of antigenic variation in syringe passaged *Trypanosoma (Trypanozoon) brucei* (Hoare, 1972).

Materials and methods

Microscopy

An optimally equipped Leitz Orthoplan microscope was used for observations under bright field, phase contrast and fluorescence microscopy. For general purposes, a pair of large field eye-pieces were used. Enumeration of trypanosomes was made with the help of grid-marked eyepieces. Supplementary details are described under relevant heads.

Trypanosomes

The frozen stabilate EATRO 1125/LUMP 581, consisting of trypomastigotes of *T. (T.) brucei* in infected mouse blood, was received as the initial material through the courtesy of Professor W. H. R. Lumsden. It represented mouse passage material 13 of a population originally isolated from a bushbuck at Mavubwe, Busoga District, Uganda, in 1966. Populations derived from it by further mouse passages were monomorphic and susceptible to the lytic action of normal human serum (Van Meirvenne *et al.*, 1973). Preceding strain material was earlier shown by the EATRO to be non-infective to man. Working quantities of variant populations were obtained by infection of random bred white mice. Unless otherwise stated the passages were done at 2 to 3 days intervals by intraperitoneal injection of heparinized blood, undiluted or diluted in phosphate-saline-glucose 6 : 4 buffer (Lanham and Godfrey, 1970). Concentration of trypanosomes in suspension was estimated by relating the number of organisms seen in 10 microscopic fields of a wet preparation to previously determined equivalents of haemacytometer counts. Rabbits were infected by subcutaneous or intravenous injection of diluted parasitized mouse blood. Isolation of trypanosomes from infected rabbits was done by subinoculation of 0.5 ml aliquots of their blood into mice and further mouse passages. Concentrates of living trypanosomes were obtained by Lanham's procedure (1968). Stabilates of variant populations were preserved in liquid nitrogen using 5 per cent dimethyl sulphoxide as cryoprotectant.

Clones

Isolation of single trypanosomes was done by diluting infected mouse blood in guinea-pig serum and then applying it in minute drops in the uncovered centre of teflon-coated cover slips. Hanging drop preparations were made by sealing the cover slips with silicone grease over a cavity slide containing a few drops of water along its slope. The microdrops were screened in bright field using a $\times$ 16 objective, large field eye-pieces, low condensor and moderate light intensity. The entire preparation had a diameter several times smaller than that of the microscopic field and the presence of a single organism could be confirmed with absolute certainty. The individual trypanosome was picked up by aspiration through a 1-ml syringe while flushing the teflon-free area with about 0.5 ml of guinea-pig serum and injected intraperitoneally into a mouse. The subsequent massive blood passages were continued until a population suitable for cryopreservation was obtained.

In order to avoid repeated cloning of identical antigenic types, the populations from which clones had to be prepared were previously examined for their variant composition by immunofluorescence. The unwanted variant types were removed by selective trypanolysis and the clone populations set up from the surviving organisms or from populations derived from them by infection of new mice.

Antisera

To obtain variant specific antisera, random bred rabbits, weighing about 3 kg, were inoculated intravenously with a 1-ml suspension of 10^6 clone trypanosomes in PSG buffer. On the 6th day post-inoculation about 15 ml of serum was collected by bleeding the marginal ear vein under vacuum, lyophilized in 0.3 ml aliquots and stored at room temperature. Multivalent antisera were obtained from rabbits after several weeks of infection with various cloned and uncloned populations.

Trypanolysis tests

Trypanolysis tests were carried out in Microtiter U-plates (*). Reactants were applied and diluted using pipette-droppers and microdiluters (*). To 0.05 ml volumes of twofold dilutions of antiserum in complement-rich guinea-pig serum, equal amounts of an appropriate suspension of parasitized mouse blood in the same diluent were added so as to obtain a final concentration of 10^6 trypanosomes per ml. Negative reference preparations of trypanosomes in diluent alone were also included. After incubation at room temperature for two hours, the reaction mixtures were examined under the $\times$ 25 or $\times$ 40 phase contrast objective of the microscope. The various stages of lysis were grouped in the following categories : fully or nearly completed lysis, strong immobilization, weak immobilization, and normal. Only the first category was taken into account for establishing the percentage of lysis. Although under standard conditions the lysed percentage in the negative control preparations was negligible, the test results of less than 5 per cent of lysis were recorded as negative. End-titres of clone specific antisera were determined as the highest twofold dilution still giving 95 per cent or more of lysis. The variant specificity of such antisera was controlled by using heterologous clone populations as the antigen.

Variant specific immunofluorescence

Phosphate buffered saline (PBS), pH 7.3, was used as diluent. Smears of parasitized mouse tail blood were dried at 37 °C for 1 hour and reaction zones marked out on the slides with nail varnish. Smears were rehydrated by dipping them a few times into two successive baths of PBS and allowing

(*) Cooke Microtiter.

them to stand in the third PBS bath for 15 minutes. The excess of PBS was removed and suitable dilutions of clone specific antisera allowed to react for 15 minutes. The unreacted serum was poured off and slides washed again in PBS by passing through two dipping baths and through a third bath of 5 minutes. After removal of the excess of PBS, a 1 : 200 dilution of goat anti-rabbit-globulin conjugate containing Evans blue 1 : 10,000 was then allowed to react for 15 minutes. Unreacted material was removed as before and slides mounted in 50 per cent glycerol. Readings were made under Ploem illumination using a mercury vapor lamp and a 5 mm thick BG-38 excitation filter. The × 16 objective and large field eye-pieces were used for screening the preparations for the presence of rare variant types. Working with heavily infected blood smears, many hundreds of trypanosomes were seen in each microscope field and several tens of thousands could be screened within a few minutes. Positive findings were confirmed under the × 40 objective. The proportion of rare variants was calculated by relating the number of organisms per microscope field and the number of fields examined to that of positive findings. The proportion of prevalent variant types was determined with the help of grid-marked eye-pieces.

At the specific dilution of the antisera the heterologous antigenic types appeared red coloured or mat green, whereas homologous ones showed brilliant green fluorescence. The mono-variant specific dilution of each antiserum was determined by extensive cross-combination of all available clone populations and antisera.

Neutralization and infection

Selective elimination of unwanted variants from a given heterogeneous population was accomplished by the trypanolytic action of one or more clone specific antisera. The concentration of each antiserum in the reaction mixture was adjusted so that it was two twofold dilutions below its lytic end-titre. Depending upon each individual experimental situation the trypanosome concentration was ranging from 10^6 to 10^7 per ml. The neutralized suspension was inoculated intraperitoneally into mice. The number of trypanosomes injected varied widely from one experiment to another and mainly depended on the proportion of surviving organisms in the neutralized suspension. The mice developing parasitaemia were selected for further experiments.

Preparation of first spontaneous relapse populations in mice

In order to derive spontaneous first relapse populations from samples of organisms of mono-antigenic type, mice were given an intraperitoneal inoculation of low numbers of clone organisms, ranging from 100 to 1,000. The variant composition of the successive trypanosome populations appearing in such mice was followed by immunofluorescence tests.

(*) Institut Pasteur, Paris.

Designation of antigenic types and populations

The antigenic types were designated according to the terminology proposed by Lumsden *et al.* (1967). Accordingly AnTat 1 represented Antwerp Trypanozoon antigenic type-1. Clone populations were designated by the code of their major antigenic type, followed by the total number of mouse passages removed from the inoculation of the single organism, e.g. AnTat 1.P12. The passage history of any other variant population was precised in the same way. In all instances where passage intervals exceeded 3 days the number of days elapsing between each passage were illustrated in a following parenthesis, e.g. P4 (4.3.2.5.).

Results

1. *Preparation of clones of AnTat 1 and AnTat 2*

Three clones of AnTat 1 were isolated from a trypanosome population obtained by numerous mouse passages of stabilate EATRO 1125. The antigenic similarity of these clone populations was shown by trypanolysis cross-tests with antisera prepared against each of them (table 1). It was found later on by immunofluorescence that the first passage material of EATRO 1125 contained about 40 per cent of AnTat 1, indicating that this antigenic type was a major representative of the stabilate itself. A trypanosome population derived from EATRO 1125 by numerous passages on the other hand at first sight contained AnTat 1 organisms only. However, following the neutralization of such a population with anti-AnTat 1 antiserum and injection of the lysate into mice antigenically new variant populations were obtained. From one such population two clones were isolated, both being of an identical serotype designated as AnTat 2 (table 1).

TABLE 1

Trypanolytic endpoints of the antisera prepared against 3 clones of AnTat 1 and 2 clones of AnTat 2, when combined with each of the populations

Clone population tested		Antiserum against				
		Serotype AnTat 1			Serotype AnTat 2	
Serotype	Clone N°	Clone 1	Clone 2	Clone 3	Clone 1	Clone 2
AnTat 1	1	1/64	1/256	1/256	neg	neg
	2	1/64	1/256	1/256	neg	neg
	3	1/64	1/256	1/256	neg	neg
AnTat 2	1	neg	neg	neg	1/128	1/1024
	2	neg	neg	neg	1/128	1/512

2. *General principles of methodology*

2.1. *Preparation of variant specific trypanolytic antisera.*

In order to develop a rapid and reliable method for the preparation of variant specific trypanolytic antisera, several rabbits were infected sub-

Photograph 1
Specific immunofluorescent staining of a given antigenic type and aspect of the surrounding heterologous variants

cutaneously or intravenously with 3 different doses of AnTat 2 clone trypanosomes, being respectively 20,000, 10^6, and 0.1 ml of column-concentrated organisms. One or more serum samples were collected within a few days following infection, and trypanolytic endpoints determined. Results are summarized in table 2. It is evident that the intensity of the early antibody response was greatly dependent on the number of organisms injected as also on their route of administration. In later stages the lytic titres had tendency to rise to comparable levels in all animals.

TABLE 2

Trypanolytic antibody-titer in rabbits following subcutaneous or intravenous infection with varying doses of AnTat 2 clone organisms

WAY OF INFECTION	DOSE	RABBIT NUMBER	DAYS AFTER INFECTION							
			3	4	5	6	7	8	10	14
SUBCUTANEOUS	20,000	238	-	-	-	-	1/8	-	-	1/2048
	10^6	208	neg	-	-	1/4	-	1/128	1/512	-
	10^6	209	neg	-	-	1/8	-	1/128	>1/1024	-
	col.conc. 0.1 ml	213	neg	-	-	1/256	-	1/256	1/512	-
INTRAVENOUS	10^6	210	neg	-	-	1/256	-	1/512	1/1024	-
	10^6	212	neg	-	-	1/128	-	1/128	1/1024	-
	10^6	237	neg	neg	1/16	1/128	-	-	-	-
	10^6	230	-	-	-	-	1/256	-	-	1/2048
	col.conc. 0.1 ml	211	neg	-	-	1/256	-	1/1024	1/1024	-
	col.conc. 0.1 ml	239	neg	neg	1/16	1/128	-	-	-	-

Preliminary information about the range of variant specific activities of anticlone antisera collected at varying intervals after infection, was obtained by trypanolysis tests in which trypanosome populations, isolated from the infected animals, were used as the antigen. Combination of the 3th-day isolates from rabbits 208, 209, 210, 211, 212 and 213, with the 6th, 8th and 10th-day serum samples from the same animals, yielded titration results which were indistinguishable from those obtained with the infecting clone organisms. The variants isolated on day 6 from rabbits 208 and 210 on the other hand were fully or partially resistant to the sera collected from these animals on days 6, 8 and 10. In a more detailed experiment of this kind both trypanosomes and serum samples were collected from rabbit 237 on days 3, 4, 5 and 6 post-infection. The qualitative results of the trypanolysis cross-tests undertaken with the materials obtained from this rabbit are summarized in table 3.

From the results shown in tables 2 and 3 it was concluded that suitable clone specific trypanolytic antisera may be obtained on the 6th day after intravenous inoculation of 10^6 organisms. However, as will be outlined afterwards, because of the antigenically heterogeneous character of clone populations, such antisera may in some instances possess a low grade of activity against variant types other than the putative one.

TABLE 3

Susceptibility of trypanosome isolates from rabbit 237 to lysis by serum samples of the same animal (Intravenous infection with 10^6 organisms of AnTat 2.P9)

TRYPANOSOME POPULATION TESTED	SERUM (DAYS AFTER INFECTION)			
	3	4	5	6
Infecting clone	NEG	NEG	LYSIS	LYSIS
3-day isolate.P3	NEG	NEG	LYSIS	LYSIS
4-day isolate.P2	NEG	NEG	LYSIS	LYSIS
5-day isolate.P5	NEG	NEG	NEG	NEG
6-day isolate.P5	NEG	NEG	NEG	NEG

2.2. *Variant specific immunofluorescence.*

The initial attempts for distinguishing the antigenic types by immunofluorescence tests were made on blood smears of mice infected with clone populations AnTat 1 or AnTat 2, and of mice with mixed infection. Heparination of the trypanosomized blood was found to give less consistent results. The addition of sodium citrate or EDTA had no deleterious effect on the antigenic reactivity. However, in order to exclude the possibilities of unknown variable factors, the blood smears used for routine tests were always prepared from the freshly collected mouse tail blood. The variant specificity of the reaction was obtained by adjusting the dilution of the reacting clone specific antisera. In the fluorescent preparation, the positively reacting organisms appeared much more larger than the surrounding heterologous ones. The variant specific fluorescent staining was located all over the surface of the trypanosomes (photograph 1). It thus appeared highly probable that the surface coat (Vickerman, 1969a, b) was involved in the reaction. When progressively higher dilutions of antiserum were used, the fluorescence became restricted to some granular material located along the undulating membrane. This dotted pattern of fluorescence suggested that the reacting antigens were not homogeneously distributed. Variant specific fluorescence of internal cell structures was never observed. No efforts were made to verify if this was due to the absence or too low concentration of internal variable antigens, or, eventually, to their inaccessibility for antibodies. With some antisera non variant specific immunofluorescence of the kinetoplasts and of the nuclear chromatin masses was observed, but this probably was due to non trypanosomal antibodies.

2.3. *Neutralization and infection.*

Lytic neutralization of heterogeneous variant populations with one or more variant specific antisera, resulted in lysate preparations containing more or less high proportions of surviving organisms, depending on the composition of the population and the specificity of the antisera included. In some instances the proportion of intact organisms in the lysate was so low that their presence appeared from the positive outcome of infectivity tests only. With a view to ascertain if the clone populations raised in mice were antigenically homogeneous, several of them were neutralized by the corresponding antiserum and the lysates injected into mice. If a sufficiently large number of clone organisms was included, infection could always be established. The trypanosomes of such infections were fully resistant to the lytic action of the original antiserum and remained unstained by it in immunofluorescence.

2.4. *Management of population studies.*

The feasibilities and possibilities offered by the combined use of all methods described heretofore are schematically represented in figure 1.

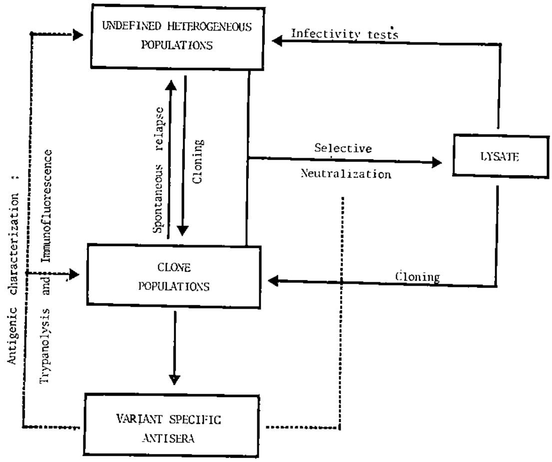

Figure 1
Management of variant population studies using trypanolysis, immunofluorescence, and neutralization and infection procedures

3. *Origin of clone populations of AnTat 3 to AnTat 9*

One or more clones of the antigenic types AnTat 3, 4, 7, 8 and 9, were isolated from different variant populations derived from AnTat 1 populations by the neutralization and infection procedure. Clones of the antigenic types AnTat 5 and 6 as well as two new clones of AnTat 2 were isolated from the variant population derived from AnTat 3. P20 by the same procedure. Data concerning the origin of the clones are summarized in table 4.

4. *Virulence of different antigenic types to mice*

McNeillage and Herbert (1968) have already described differences in infectivity and virulence which were associated with differences in antigenic type. Using immunofluorescence tests this fact could amply be confirmed by the follow-up of the variant composition of heterogeneous populations growing-up in individual mice or in subsequent mice at serial passage. A few examples of this kind are represented in table 5.

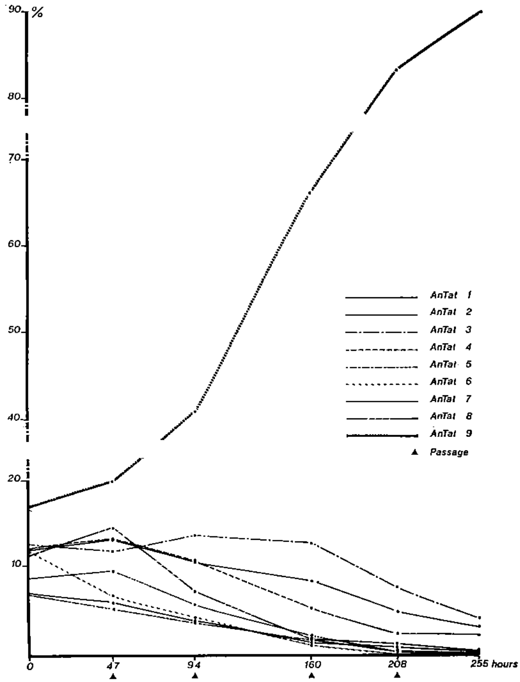

Figure 2a

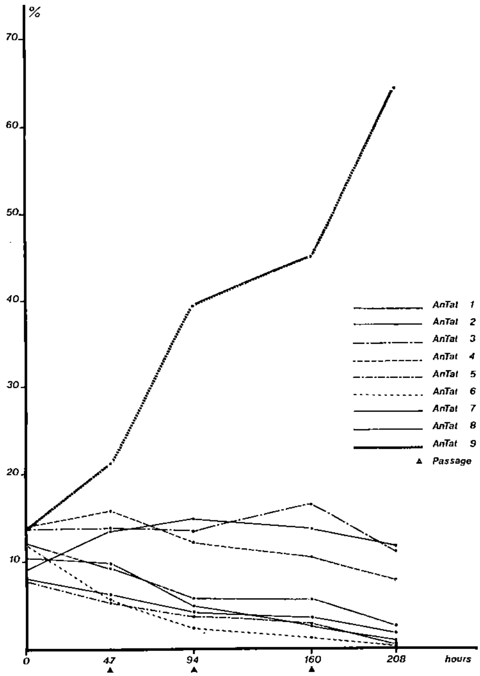

Figure 2b

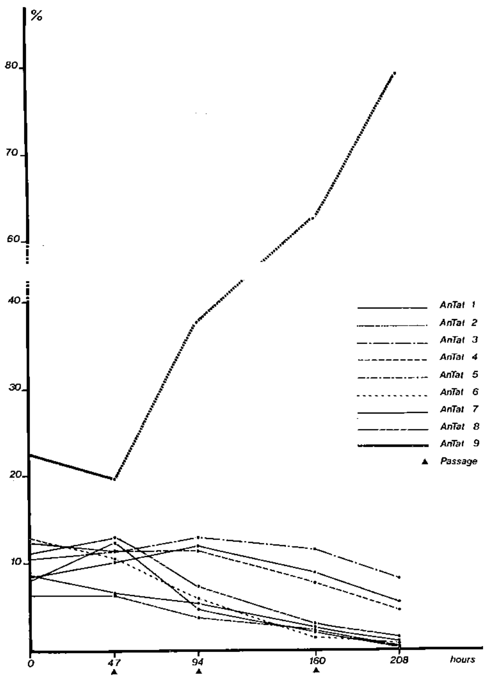

Figure 2c

Figure 2

Proportional representation of 9 different antigenic types in a mixed variant population passaged from mouse to mouse at short intervals.
Figures a, b and c represent 3 distinct passage series

TABLE 4

Isolation history of clones AnTat 2, 3, 4, 5, 6, 7, 8 and 9

STARTING CLONE POPULATION		NEUTRALIZATION	RELAPSE POPULATIONS USED FOR CLONING									CLONE ISOLATIONS	
MAJOR TYPE	Total number of passages since single organism	Specificity of antisera included	Passage history since injection of lysate	Immunofluorescence analysis : % of AnTat types 1	2	3	4	5	6	7	8	MAJOR TYPE	Times isolated
AnTat 1	P 20	AnTat 1	P 11	0	0							AnTat 3	1
AnTat 3	P 20	AnTat 3	P 2 (3-4)	7	40							AnTat 2 AnTat 5 AnTat 6	2 1 1
AnTat 1	P 12	AnTat 1,2,3	P 2 (4-3)	0	0	0						AnTat 4	2
AnTat 1	P 12	AnTat 1,2,3,4	P 2 (7-4)	0	0	0	0	0	0			AnTat 7 AnTat 8	2 2
AnTat 1	P 24	AnTat 1,2,3,4, 5,6	P 4 (5-4-3-2)	0	0	0	0	0	0	0	0	AnTat 9.	3

TABLE 5

Fluctuations in the proportional representation of two distinct variant types in heterogeneous populations, maintained in mice

1. SINGLE MOUSE.

DAYS AFTER INFECTION	AnTat 3	AnTat 6
3	79 %	14 %
4	>95 %	<1 %

2. SERIAL MOUSE PASSAGE (2 or 3 days intervals)

NUMBER OF PASSAGES	AnTat 1	AnTat 2
0	⩽20 %	⩽80 %
3	84 %	15 %
5	97 %	2 %
6	⩾99 %	1:1,000
9	⩾99 %	<1:10,000

In a more sophisticated procedure 3 mice were infected with a mixture of 9 distinct antigenic types containing about 500,000 organisms of each. From each of these mice passage series from mouse to mouse were started and continued by transfer of 2.10^6 organisms at intervals of no more than 3 days. The blood samples used for passage were simultaneously examined for the proportional representation of each antigenic type, this being based on the verification of 500 organisms. The antigenic type AnTat 9 rapidly overgrew all the other variants while AnTat 6 clearly behaved as the least virulent type (figures 2a, b and c).

5. *Antigenic heterogeneity of clone populations*

The new variant populations obtained by lytic neutralization of clones with homologous antiserum and injection of the lysate into mice, obviously resulted from infection by organisms resisting the lytic action of the antiserum. Upon painstaking microscopic examination of clone lysate preparations intact trypanosomes could occasionally be detected. Immunofluorescence analysis of blood smears of mice infected with clone populations confirmed that several antigenic types other than the putative one were present in low proportion. Detailed results concerning findings of minor antigenic types in clones are shown in tables 10 and 14.

The antigenically heterogeneous character of clone populations raised question regarding the mono-variant specificity of anti-clone antisera. Both clones and antisera were therefore assessed on their degree of heterogeneity by extensive cross-tests. Some results of trypanolytic cross-combinations are reviewed in tables 6 and 7. Concerning the results reported in table 6, it must be pointed out that with some antisera a prozone phenomenon occurred, in which the 1 : 2 dilution did not exhibit complete lysis of homologous organisms for few hours. This zone effect was probably due to anticomplementary factors rather than to excess of antibody because it was not correlated with the endtitres of the antisera.

TABLE 6

Trypanolytic activity of twofold dilutions of clone specific antisera in homologous combination : number of surviving organisms encountered upon examination of 500 trypanosomes

CLONE POPULATION TESTED	RECIPROCAL OF DILUTION OF HOMOLOGOUS ANTISERUM									
	2	4	8	16	32	64	128	256	512	1024
AnTat 1.P12	2*	0	0	0	0	0	1	3	---	---
AnTat 2.P10	6*	0	0	0	0	0	0	---	---	---
AnTat 3.P8	0	0	0	0	0	0	0	---	---	---
AnTat 4.P7	0	0	0	0	0	3	---	---	---	---
AnTat 5.P7	0	0	0	0	0	0	0	1	---	---
AnTat 6.P7	50*	0	0	0	0	1	1	---	---	---
AnTat 7.P6	0	0	1	0	0	0	0	1	---	---
AnTat 8.P7	0	0	0	0	0	1	---	---	---	---
AnTat 9.P5	0	0	0	0	0	0	0	0	---	---
AnTat 10.P9	0	0	0	0	0	0	0	0	0	---

* Due to prozone phenomenon.

--- Dilutions beyond trypanolytic endpoints.

The results of immunofluorescence cross-tests between clones and corresponding antisera are shown in table 8. The results only concern the observations made at the optimal dilution of each antiserum.

The results reported in tables 6, 7 and 8, illustrate that the mono-variant specific activity of anti-clone antisera could be controlled and guaranteed by its appropriate dilution.

TABLE 7

Trypanolytic cross-combination of various clone specific antisera with homologous and heterologous clone populations (Screening test with serum dilutions 1 : 2, 1 : 4 and 1 : 8; L = nearly 100 per cent of lysis at all dilutions; NEG = no significant lysis)

CLONE POPULATION TESTED	ANTISERA AGAINST CLONE POPULATIONS OF DIFFERENT VARIANT TYPES (dilutions 1:2, 1:4, 1:8)								
	AnTat 1	AnTat 2	AnTat 3	AnTat 4	AnTat 5	AnTat 6	AnTat 7	AnTat 8	AnTat 9
AnTat 1.P12	L	NEG	NEG	NEG	NEG	NEG	NEG	NEG	NEG
AnTat 2.P10	NEG	L	NEG	NEG	NEG	NEG	NEG	NEG	NEG
AnTat 3.P8	NEG	NEG	L	NEG	NEG	NEG	NEG	NEG	NEG
AnTat 4.P7	NEG	NEG	NEG	L	NEG	NEG	NEG	NEG	NEG
AnTat 5.P7	NEG	NEG	NEG	NEG	L	NEG	NEG	NEG	NEG
AnTat 6.P7	NEG	NEG	NEG	NEG	NEG	L*	NEG	NEG	NEG
AnTat 7.P6	NEG	NEG	NEG	NEG	NEG	NEG	L	NEG	NEG
AnTat 8.P7	NEG	NEG	NEG	NEG	NEG	NEG	NEG	L	NEG
AnTat 9.P5	NEG	NEG	NEG	NEG	1/2:L90% 1/4:L50% 1/8:NEG	NEG	NEG	NEG	L

*= Prozone phenomenon at 1:2

TABLE 8

Immunofluorescent activity of optimally diluted clone specific antisera when combined with homologous and heterologous clone populations. Global fluorescent aspect of the major type (— = red coloured, ± = mat green, aspecific fluorescence, ++ = typical variant specific fluorescence)

CLONE POPULATION TESTED	CLONE SPECIFICITY OF ANTISERA AT OPTIMAL DILUTION								
	AnTat 1	AnTat 2	AnTat 3	AnTat 4	AnTat 5	AnTat 6	AnTat 7	AnTat 8	AnTat 9
AnTat 1.P12	++	±	±	-	-	-	-	-	-
AnTat 2.P10	±	++	±	-	±	-	-	-	-
AnTat 3.P8	-	±	++	±	±	-	-	-	-
AnTat 4.P7	-	±	±	++	±	-	-	-	-
AnTat 5.P7	-	±	±	±	++	-	-	-	-
AnTat 6.P7	-	±	±	±	±	++	-	-	-
AnTat 7.P6	-	±	±	±	±	-	++	-	-
AnTat 8.P7	-	±	±	±	±	-	-	++	-
AnTat 9.P5	-	-	±	±	±	-	-	-	++

As far as the clone populations themselves were concerned, the major type which developed after a few passages in mice was likely to be the representative of the antigenic type of the initial single organism. The latter probably gave rise to a large number of antigenically identical descendants before the first heterologous variant organism appeared and, as the clone expanded, so several minor types accumulated in it. The clone population set up from the poorly virulent type AnTat 6 was found to be particularly susceptible to being super-imposed by the growth of more infective minor types. Table 9 thus shows the progressive replacement of clone organisms AnTat 6 by AnTat 3 with continued mouse passage.

From these results it clearly appeared that the earliest experimentally useful clone population of any newly isolated antigenic type should be used both for cryopreservation of reference material and for the preparation of antiserum.

TABLE 9

Progressive replacement of major type AnTat 6 by AnTat 3 in a mouse passaged clone

PASSAGE HISTORY OF CLONE AnTat 6	PROPORTION OF AnTat 3
P 8	1 : 5,500
P 11	1 : 60
P 13	1 : 20

6. *Relationship between the minor antigenic types of a clone population and the variant composition of populations derived from it by neutralization and infection tests*

The variant composition of a given clone population AnTat 1 . P12 was analyzed by means of immunofluorescence. The findings of minor antigenic types and their proportions are represented in table 10.

TABLE 10

Presence of minor antigenic types in clone population AnTat 1.P12 (per 10^6 organisms)

AnTat 2	AnTat 3	AnTat 4	AnTat 5	AnTat 6	AnTat 7	AnTat 8	AnTat 9
16	47	3	5	3	20	3	0

A part of the same population was used for the following neutralization and infection tests. A suspension of trypanosomes, containing 10^7 organisms per ml, was prepared in a 1 : 30 dilution of anti-AnTat 1 antiserum in guinea-pig complement and incubated at room temperature for two hours. Five mice were injected intraperitoneally with 1 ml of the undiluted suspension, corresponding to 10^7 of initial clone organisms. From the remaining part of the suspension dilutions of respectively 1 : 10, 1 : 100 and 1 : 1000, were prepared in PSG buffer. Groups of 5 mice were injected with a 1-ml volume of one out of these preparations, each individual of a group thus receiving respectively 10^6, 10^5 and 10^4 incubated trypanosomes. The mice were checked for parasitaemia on days 2 to 10 post-inoculation. All except 3 of the last group became infected. Blood smears were made from 15 out of the 17 animals developing parasitaemia and examined for the presence of the antigenic types AnTat 1 to 9. The results of these analyses (table 11) generally appeared agreeable with those given in table 10. The populations obtained by injection of high numbers of incubated trypanosomes obviously had a more pronounced heterogeneous composition than those obtained by injection of smaller numbers.

The unexpected occurrence of AnTat 1 in some populations must be explained by accidentally incomplete neutralization of the major antigenic type of the initial clone.

TABLE 11

Development of parasitaemia and variant-analysis of trypanosome populations appearing in mice, injected with varying numbers of organisms of clone population AnTat 1.P12 following its neutralization with anti-AnTat 1 antiserum

DOSE	MICE N°	DEVELOPMENT OF PARASITAEMIA (Days after injection)								VARIANT COMPOSITION OF POPULATIONS ⊕ (proportion per 5,000)									
		2	3	4	5	7	8	9	10	A1	A2	A3	A4	A5	A6	A7	A8	A9	Total
10^7	1	0	0	+	⊕	-	-	-	-	0	1700	2300	0	3	7	40	0	0	4050
	2	0	0	0	0	⊕	-	-	-	3080	900	170	0	20	50	350	10	0	4580
	3	0	0	0	0	⊕	-	-	-	0	140	50	0	110	80	50	200	0	630
	4	0	0	+	⊕	-	-	-	-	260	1130	310	0	560	55	240	260	0	2810
	5	0	0	0	0	⊕	-	-	-	0	850	550	250	270	250	550	80	0	2800
10^6	6	0	0	0	0	⊕	-	-	-	0	970	850	0	680	20	2010	0	0	4530
	7	0	0	0	0	⊕	-	-	-	0	3600	0	0	0	0	0	0	0	3600
	8	0	0	0	0	⊕	-	-	-	2950	590	1350	0	0	0	30	0	0	4920
	9	0	0	0	0	⊕	-	-	-	0	1750	30	0	0	0	1460	0	0	3240
	10	0	0	0	0	+	-	-	-	-	-	-	-	-	-	-	-	-	----
10^5	11	0	0	0	0	+	-	-	-	-	-	-	-	-	-	-	-	-	----
	12	0	0	0	0	⊕	-	-	-	0	0	0	0	3	0	0	0	0	3
	13	0	0	0	0	⊕	-	-	-	MT	0	0	0	0	0	0	0	0	----
	14	0	0	0	0	⊕	-	-	-	MT	0	0	30	0	0	0	0	0	----
	15	0	0	0	0	⊕	-	-	-	MT	0	20	0	0	0	0	0	0	----
10^4	16	0	0	0	0	+	⊕	-	-	1850	0	0	3000	0	0	0	0	0	4850
	17	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	----
	18	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	----
	19	0	0	0	0	0	0	0	0	-	-	-	-	-	-	-	-	-	----
	20	0	0	0	0	0	+	⊕	-	0	0	0	0	0	0	MT	0	0	----

0 = negatif result ; + = parasitaemia ; ⊕ = blood used for variant analysis ; - = not examined ; MT = major type

7. *Origin of clone populations of antigenic types AnTat 10 and 11*

Most of the populations resulting from injection of neutralized AnTat 1 . P12 contained high proportions of one or more unidentified variant types (table 11). Two such variants were isolated as clones from a population, obtained by including the 5-th day trypanosomes of mouse number 1 into a supplementary neutralization and infection procedure which ensured the removal of the unwanted variants AnTat 2, 3, 5, 6 and 7. Of the three clone populations successfully set up, one was designated AnTat 10 while the two others AnTat 11.

8. *Cinetics of the formation of relapse populations in mice*

Using immunofluorescence, detailed studies on the spontaneous relapse phenomenon in mice could easily be made. Figure 3 shows the evolution of the proportional representation of the major and of some of the minor variant types in a mouse infected with an heterogeneous variant population.

9. *Variant composition of first spontaneous relapse populations appearing in mice following infection with clone organisms : predominant antigenic types*

Considerable controversy still exists as to whether antigenic variation is an organized or a random process. According to Cantrell (1958), Watkins

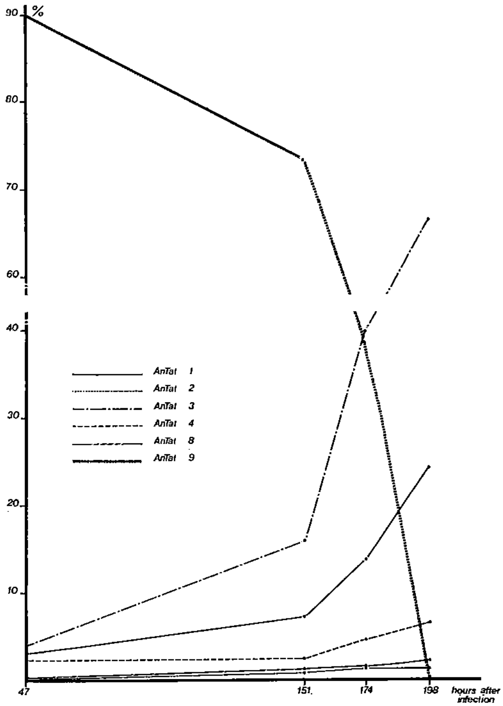

Figure 3

Cinetics of relapse formation in a mouse following infection with an heterogeneous population

(1964), and Seed and Gam (1966), the mechanism would be based entirely upon random mutation and subsequent selection by immune factors. Gray (1965a, b) and Vickerman (1969b), on the other hand, suggested that antigenic variation might be an adaptive process, induced by host antibodies. Gray (loc. cit.) observed that several identical antigenic types appeared in different rabbits during the first weeks following infection with various populations. In the later stage of infection, however, antigenic variation no longer occurred according to a predictable pattern. Gray postulated that any variant has a tendency to reverse to so-called « basic » or « predominant » antigenic types when cyclically or mechanically subpassaged into a non immune host.

This aspect was studied through immunofluorescence analysis of the spontaneous relapse populations appearing in mice following infection with trypanosomes of one and the same antigenic type. Infections of this kind were obtained by the injection of low numbers of clone trypanosomes.

Spontaneous first relapse populations, free from the infecting variant type, were obtained in all 5 mice infected with 10^3 organisms of AnTat 10. P9 (table 12). Complete relapse occurred in only 2 out of 5 mice infected with 10^3 organisms of AnTat 11.P8; the remaining 3 animals died before these could eliminate the infecting type (table 13).

TABLE 12

Variant composition of trypanosome populations appearing in mice after infection with 10^3 AnTat 10.P9 organisms

ORIGIN OF VARIANT POPULATION		PROPORTION OF AnTat VARIANT TYPES (per 500 organisms)											
MOUSE N°	DAYS AFTER INFECTION	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	Total
1	6	0*	0	0	4	0*	0*	0	0*	0	MT	0	---
	8	3	6	8	465	2	2	3	4	1	0	1	495
2	6	0	0	0	1	0	0	0	0	0	MT	0	---
	9	2	2	5	395	2	3	1	1	2	0	1	414
3	8	0	1	6	5	4	3	11	0*	1	0	0	31
4	6	0	0	0	0	1	0	0	0	0	MT	0	---
	9	0	15	3	11	180	13	26	3	0	0	2	253
5	8	1	10	3	455	3	0*	3	4	2	0	0*	481

MT = Major Type

* = present in a proportion between 1:500 and 1:5,000

TABLE 13

Variant composition of trypanosome populations appearing in mice after infection with 10^3 AnTat 11.P8 organisms

ORIGIN OF VARIANT POPULATION		PROPORTION OF AnTat VARIANT TYPES (per 500 organisms)													
MOUSE N°	DAYS AFTER INFECTION	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13	Total
1	6	0	0	0	0	0	0	0	0	0	0	MT	-	-	---
	8	0	0*	0*	0*	0	0*	0*	0*	0*	0	MT	0*	0	---
	10	0	7	60	220	27	18	47	68	7	4	0	7	0	465
2	6	0	0	0	0	0	0	0	0	0	0	MT	-	-	---
	8	0	0*	1	0*	2	2	1	0*	0*	0	MT	1	0	---
	10	0*	42	63	40	158	55	35	9	7	0	0	13	0	422
3	9	0	0*	0*	0*	0*	0	0*	0	0	0	MT	0*	0	---
4	8	0	0	*0*	*0*	0	0*	*0*	0	0	0	MT	0	0	---
5.	8	0	0	*0*	0	*0*	*0*	*0*	0	0	0	MT	0*	0	---

* = present in a proportion between 1:500 and 1:5,000

** = present in a proportion between 1:5,000 and 1:20,000

MT = Major Type

The results reported in tables 12 and 13 seem to be in full agreement with the observations and considerations made by Gray on the existence of predominant antigenic types. Some variants indeed appeared to have a greater chance to be produced than others. In spite of its low infectivity AnTat 6 in all likelihood would be a more predominant antigenic type than AnTat 9 and AnTat 1.

Regarding the antigenic variation system reported in the present paper, many of the predominant antigenic types obviously have been isolated as clone populations. It should not appear as surprising since the cloning procedures used were favourable for separation of such organisms.

10. *Origin of clone AnTat 12*

The spontaneous first relapse population arising from AnTat 10 in mouse number 3 (table 12) contained a high proportion of one or more unidentified antigenic types. A clone of one of such variants, designated AnTat 12, was derived from it. In view of the results reported in tables 13, 14 and 15, AnTat 12 must be one of the predominant types.

11. *Origin of AnTat 13* *and relationship between predominant and exceptional antigenic types*

With a view to verify if the predominant antigenic types had the greatest chance to arise from less predominant types as well, a clone of such an exceptional variant, designated AnTat 13, was set up from the 46-day isolate from a rabbit infected with mouse passaged EATRO 1125 organisms (see figure 4 and table 16). Trypanolytic tests with end-phase sera from infected rabbits confirmed that AnTat 13 was a rare variant type (table 17).

When tested by immunofluorescence the population AnTat 13.P5 was found to contain most of the predominant variants in low proportion (table 14). Using a part of the same population several mice were infected

TABLE 14

Presence (per 200,000 organisms) of minor antigenic types in a clone population AnTat 13.P5

AnTat 1	2	3	4	5	6	7	8	9	10	11	12	Total
0	3	9	4	4	4	5	1	3	2	0	6	41

TABLE 15

Variant composition of trypanosome populations appearing in mice after infection with respectively 100 and 500 organisms of clone population AnTat 13.P5

ORIGIN OF VARIANT POPULATION			NUMBER OF AnTat VARIANT TYPES SEEN PER 20,000 ORGANISMS												
INFECTION DOSE	MOUSE N°	DAYS AFTER INFECTION	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13
100	1	7	0	2	2	0	0	0	1	0	0	0	0	0	MT
	2	8	0	0	0	2	0	0	0	0	0	0	0	2	MT
		9	0	2	1	2	2	3	1	3	0	0	0	0	MT
	3	7	0	0	4	2	0	1	0	0	0	0	1	2	MT
500	4	7	0	8	4	8	2	4	2	5	1	2	3	8	MT
		8	0	29	21	28	7	16	11	8	12	14	9	24	MT
	5	7	0	0	1	0	2	0	0	0	0	0	0	0	MT
	6	8	0	0	0	0	0	0	0	0	0	0	0	0	MT
		9	0	0	0	0	1	0	2	0	0	0	0	0	MT
	7	7	0	6	2	2	8	1	4	6	2	8	3	8	MT
		8	0	13	24	15	3	3	7	18	3	22	11	7	MT

MT = Major Type

with as few as 100 or 500 trypanosomes. Although all the animals died before these could eliminate AnTat 13, all the identifiable predominant antigenic types, except AnTat 1, could once again be detected as minor representatives of the developing parasitaemias (table 15).

12. *Antigenic variation and variant specific immunity in rabbits*

Since the parasitaemia in infected rabbits remained very low, the study of the successive variant populations had to be made by indirect methods. The circulating trypanosomes were isolated and multiplied by subinoculation into mice. The variant composition of the populations thus obtained at weekly intervals from a rabbit infected with 10,000 organisms of mouse passaged EATRO 1125 is given in table 16. One might speculate that the variants found in the isolate of day 11 were the result of the presence of minor antigenic types in the inoculum. This, however, appeared unlikely since the population used for infection, when tested by trypanolysis, behaved as if it contained only AnTat 1 organisms. A more conceivable explanation would be that the predominant antigenic types encountered in mice were predominant in the rabbit as well. It should be mentioned that some variants found in extremely low proportion in the isolates of days 4, 18 and 46, could have been due to supplementary antigenic variation occurring during mouse passage of the isolated trypanosomes.

TABLE 16

Immunofluorescence analysis of variant populations isolated from rabbit C, infected with mouse passaged EATRO 1125/LUMP 581

ISOLATED VARIANT POPULATION		PRESENCE OF AnTat VARIANT TYPES (per 100,000 organisms)									
DAY	PASSAGE HISTORY	A1	A2	A3	A4	A5	A6	A7	A8	A9	Total
4	P 4 (4-3-3-3)	MT	4	10	0	0	0	0	0	0	---
11	P 2 (4-3)	0	4000*	28000*	20000*	6000*	350	360	1600*	0	60310
18	P 2 (4-3)	0	0	1	0	0	0	0	0	0	1
25	P 3 (3-4-3)	0	0	0	0	0	0	0	0	0	0
32	P 3 (3-4-3)	0	0	0	0	0	0	0	0	0	0
39	P 2 (4-3)	0	0	0	0	0	0	0	0	0	0
46.	P 3 (4-3-3)	0	0	0	0	3	1	0	0	0	4

MT = Major Type, present for more than 99%
* = calculated by extrapolation

Indirect information on the antigenic variation pattern of trypanosomes in rabbits was also obtained by testing the trypanolytic activity of end-phase sera from animals infected with a wide variety of cloned and uncloned trypanosome populations. The infection history of the latter group of rabbits is summarized in figure 4. When tested at the 1 : 5 dilution most sera were found to have lytic activity against the antigenic types AnTat 1 to 12, while only few of them against AnTat 13 (table 17).

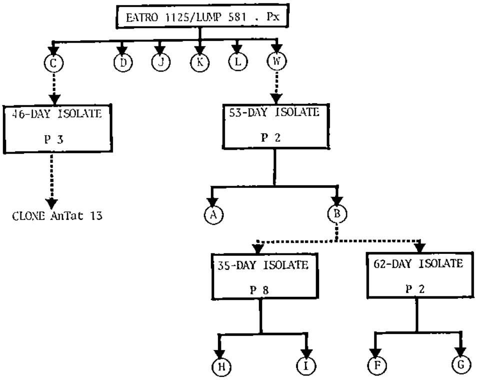

Figure 4

Infection history of rabbits infected with uncloned trypanosome populations and origin of clone population AnTat 13
(⟶ = infection; - - - ⟶ = isolation)

TABLE 17

Range of variant specific trypanolytic activity of end-phase sera from rabbits as observed at the 1 : 5 dilution (+ = nearly 100 per cent of lysis; ± = partial lysis; 0 = less than 5 per cent of lysis)

	ORIGIN OF THE ANTISERUM			TRYPANOLYTIC ACTIVITY AGAINST AnTat VARIANTS												
	INFECTING POPULATION	RABBIT	DAY	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12	A13
UNDEFINED	EATRO 1125/ LUMP 581 . Px	C	46	+	+	+	+	+	+	+	+	+	+	+	+	0
		D	53	+	+	+	+	+	±	+	±	+	+	+	+	+
		J	28	+	+	+	+	+	+	+	+	+	+	+	±	0
		K	21	+	+	+	+	+	+	+	+	+	+	+	+	0
		L	28	+	+	+	+	+	+	+	+	+	+	+	+	0
	53-DAY ISOLATE.P2 FROM RABBIT W	A	41	+	+	+	+	+	+	+	+	+	+	+	+	0
		B	62	±	+	+	+	+	±	+	±	+	+	+	+	+
	62-DAY ISOLATE.P2 FROM RABBIT B	F	59	+	+	+	+	+	+	+	+	±	+	+	±	0
		G	25	+	+	+	+	+	+	+	+	±	+	+	+	0
	35-DAY ISOLATE.P8 FROM RABBIT B	H	46	+	+	+	+	+	+	+	+	±	+	+	+	0
		I	52	+	+	+	+	+	+	+	+	+	+	+	+	0
CLONES	AnTat 1 . P12	221	30	+	+	+	+	+	+	+	+	+	+	+	+	0
	AnTat 1 . P6	229	30	+	+	+	+	+	+	+	+	+	+	+	+	0
	AnTat 1 . P9	228	30	+	+	+	+	+	+	+	+	+	+	+	+	0
	AnTat 2 . P7	226	30	+	+	+	+	+	+	+	+	+	+	+	+	0
	AnTat 2 . P15	230	35	+	+	+	+	+	+	+	+	+	+	+	+	0
	AnTat 4 . P6	219	33	+	+	+	+	+	+	+	+	+	+	+	+	0
	AnTat 5 . P7	232	29	+	+	+	+	+	+	+	±	0	+	+	+	0
	AnTat 6 . P7	236	29	+	+	+	+	+	±	+	+	0	+	+	+	0
	AnTat 7 . P6	241	32	+	+	+	+	+	+	+	+	+	+	+	+	0
	AnTat 9 . P5	242	30	+	+	+	+	+	+	+	+	+	+	+	+	0
	AnTat 10.P 7	266	30	+	+	+	+	+	+	+	+	+	+	+	+	0

In order to check if the trypanolytic activity against several antigenic types could be simultaneously induced by the injection of mixed populations, the following experiment was undertaken. Using clone populations of AnTat 1 to 9 a mixed suspension was prepared in which each of the variants was represented in a concentration of 10^6 organisms per ml. Two groups of 3 rabbits were inoculated intravenously with respectively 1 ml of the undiluted suspension and 1 ml of the 1 : 10 dilution, corresponding to 10^6 and 10^5 of each antigenic type. Serum samples taken from all these rabbits on day 7 and from the 4 surviving animals on day 30, were tested on their lytic activity against the infecting antigenic types. The results of these trypanolysis experiments (table 18) strongly suggest that immunization of animals against multivalent antigen mixtures is possible.

TABLE 18

Trypanolytic end-points of 7- and 30-day sera from rabbits infected with a mixture of 9 antigenic types

INFECTION DOSE	RABBIT N°	SERUM (DAY)	RECIPROCAL OF TRYPANOLYTIC END-POINTS (⩾95% LYSIS) AGAINST VARIANTS A1	A2	A3	A4	A5	A6	A7	A8	A9
10^6 OF EACH TYPE	257	7	64	64	64	32	64.	32	128	64	512
	258	7	128	64	32	32	32	32	128	64	256
	259	7	128	64	64	32	64	32	256	32	256
		30	16	128	128	32	64	32	32	64	16
10^5 OF EACH TYPE	260	7	32	32	16	32	32	32	128	32	256
		30	64	64	256	64	64	32	32	64	16
	261	7	32	32	16	32	16	16	32	32	128
		30	64	64	256	32	64	32	64	64	32
	262	7	64	128	64	32	64	32	128	128	256
		30	16	16	128	32	32	16	16	16	16

Discussion

The present report probably is the first to provide a detailed account of the possibilities of immunofluorescence as an aid to the study of antigenic variation in trypanosome populations. Weitz (1963 a, b), however, had obtained already some suggestive results with this technique.

Unequivocal evidence could now be obtained for the fact that clone populations grown-up in apparently non immune mice, contain several distinct antigenic types in low proportion. The antigenic variation process does not seem to produce variant types in a rigourously predetermined order. An hypothesis which could explain all the experimental results reported hitherto, and largely resolve a number of conflicting points of views, is that antigenic variation is a random process capable of producing a wide range of variants, each of which possesses its own specific chance to arise from any other type. Predominant antigenic types (Gray, 1965b) than represent a category of variants having the greatest chance of being produced. When defined in this way predominant variants need not at all to be the most infective ones. An efficient method for collecting the clone stabilates of such variants seems to be by milking out first spontaneous relapse populations.

In potential vaccination programmes the aim might be to immunize against a large number of predominant antigenic types. This however would require considerably much more work on the ranges of variants which may be produced by distinct trypanosome species and different strains. Of major importance would be a better knowledge of the number of serological types circulating as metacyclic forms in tsetse flies. Direct typing of such organisms by immunofluorescence may be envisaged. In this way the relationship between the antigenic type of the trypomastigotes ingested and of the metacyclic trypanosomes extruded might as well be studied.

For designating a collection of serological variants which can all be derived from one another by antigenic variation, such as those described in the present paper, the term « serodeme » (Hoare, 1972) might be used.

In practice, the characterization and comparison of serodemes may be done rapidly by means of immunofluorescence, using antisera against several predominant antigenic types representative for each serodeme defined. Experiments of this kind will be described in a following paper.

The present authors would welcome supply of their own stabilate materials and antisera on demand to any interested investigator.

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Samenvatting — **Antigenische variatie in populaties van** *Trypanosoma (Trypanozoon) brucei.*

I. Rationalisatie van de experimentele benadering.

Kloonpopulaties van 13 verschillende antigenische varianten van *T. (T.) brucei,* aangeduid als AnTat 1 tot AnTat 13, werden ingezameld bij muizen en bewaard als kryostabilaten. De antigenische eigenschappen van deze kloons en van variantpopulaties ervan afgeleid, werden bestudeerd en op hun onderlinge samenhang onderzocht bij middel van trypanolyse, neutralisatie en immunofluorescentie.

Deze methoden vergemakkelijken in aanzienlijke mate het inzamelen van kloonpopulaties van verschillende varianten, en geven gedetailleerde inlichtingen over de antigenische samenstelling van populaties.

Er werd aangetoond dat kloonpopulaties heterogene mengsels zijn, bestaande uit een hoofdtype en uit verschillende minderheidstypes.

De resultaten laten vermoeden dat antigenische variatie een spontaan, op toeval berustend proces is, waardoor een groot aantal verschillende antigenische types kunnen gevormd worden, waarvan sommige, de zogenaamde predominante varianten, meer frekwent ontstaan dan andere.

Résumé — **Variation antigénique dans des populations de** *Trypanosoma (Trypanozoon) brucei.*

I. Rationalisation de l'approche expérimentale.

Des populations clônées de 13 variants antigéniques différents de *T. (T.) brucei,* désignés AnTat 1 à AnTat 13, ont été obtenues sur souris et conservées sous forme de cryostabilats.

Les caractéristiques antigéniques de ces clônes, ainsi que celles de variants qui en dérivent, ont été étudiées et comparées à l'aide d'épreuves de trypanolyse, de neutralisation et d'immunofluorescence.

Ces méthodes ont considérablement facilité la collecte de populations de types antigéniques différents. Elles fournissent simultanément une information précise sur la composition antigénique d'une population quelconque.

On a pu démontrer qu'une population clônée est en fait un mélange hétérogène, composé d'un type majeur et de plusieurs types mineurs.

Les résultats suggèrent que la variation antigénique est un phénomène spontané et de hasard, au cours duquel un grand nombre de types antigéniques différents peuvent être formés, dont certains (types prédominants) apparaissent plus fréquemment que d'autres.

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