REDUCTION IN THE ABILITY OF *TRYPANOSOMA BRUCEI* TO REACT WITH ANTIBODIES AFTER LONG EXPOSURE TO IMMUNE MECHANISMS *IN VIVO*

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ACCORDING to the present view, it is believed that in infections of long duration *Trypanosoma brucei* produces antigenically distinct variants that appear in succession. The serum of an infected animal contains antibodies against all the variants that have previously appeared, but not against any of those yet to occur (Lumsden, 1967). The extent of variation possible within one species of trypanosome is not known; some workers believe that the possible range is infinite or at least very large (Watkins, 1964). There is still considerable controversy as to whether antigenic variation results from random mutation and the selection of viable mutants, or whether an intracellular control system is activated in response to contact with antibodies.

Many facts seem to support an adaptive process as the basis of antigenic variation. However, more work remains to be done on the fundamental nature of the phenomenon. The present studies suggest that in animals with chronic infection, *T. brucei* adapts to the immune response of the animal by losing or rendering inaccessible its surface antigens.

MATERIALS AND METHODS

Organisms. The trypanosomes used in this study consisted of two substrains of *T. brucei* Shinyanga III preserved at -79°C (Polge and Soltys, 1957). One substrain "M" was frequently passaged through mice for over 15 yr. The other substrain "R" was syringe-passaged 39 times through rabbits and preserved at -79°C .

Two rabbits were infected with each substrain. Sera and trypanosomes were collected from infected rabbits every week for a period of 6 wk. Trypanosomes were preserved at -79°C until ready for use. The sera from these rabbits were tested for agglutination and opsonic adherence against (a) trypanosomes before inoculation and (b) against trypanosomes isolated at intervals after infection.

Agglutination. Inactivated rabbit sera were mixed in appropriate dilutions with a suspension of living organisms containing approximately 2 million organisms, and incubated at 20°C for 2 hr. At the end of this time, the mixture was examined microscopically for agglutination.

Opsonic adherence. The peritoneal cavity of a normal rabbit was washed out with 100 ml Hanks' BSS, pH 6.5, containing 0.1 per cent. Bovine Serum Albumin (Nutritional Biochemicals Ltd) and 10 IU heparin (Connaught Labs) per ml. The cell suspension obtained was cultured on waxed microscope slides (Tizard, 1969) for 2 hr at 37°C in an atmosphere containing 10 per cent. CO_2 in air.

Suspensions of organisms in serum dilutions were added to the macrophage monolayers and examined for adherence as described in *Results*.

RESULTS

The agglutinin response to substrains "M" and "R"

Animals infected with organisms of substrain "M" produced very high titres of agglutinating antibody against this substrain with a maximum titre (1000) 2 wk after infection.

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In contrast, these same sera agglutinated substrain "R" to a maximum titre of only 32. Rabbits infected with substrain "R" organisms produced agglutinins that agglutinated substrain "R" to a titre of 16, and substrain "M" up to a titre of 256. The time of maximum titre was also 2 wk after infection (see table I).

The results derived from this group of rabbits indicated that substrain "R" carried antigens found also on substrain "M". These antigens were immunogenic, since the rabbits

TABLE I
The agglutinin response of infected rabbits to living Trypanosoma brucei of substrains "M" and "R"

Substrain used for infection	Rabbit no.	Antigen used for agglutination tests	Titre of agglutinating antibody				
			before infection	at weeks after infection			
				1	2	3	4
"M"	1	M R	0 0	2046 2	1024 32	1024 16	64 8
	2	M R	0 0	64 8	...* ...		
"R"	3	M R	0 2	64 2	256 16	64 2	32 0
	4	M R	0 2	32 8	64 16	64 16	64 8

* Rabbit died.

infected with live substrain "R" produced agglutinins against substrain "M". It was, however, apparent that substrain "R" organisms were relatively inagglutinable by antisera directed against them.

Agglutination of trypanosomes isolated from rabbits at intervals after infection

In the next experiment, sera and trypanosomes were collected from two rabbits, one infected with substrain "M" and the other with substrain "R", 2 and 5 wk after infection. The sera were tested for agglutinating activity against the original variants "M" and "R", and also against the variants isolated 2 and 5 wk after infection. The results are summarised in table II. Substrain "M" was agglutinated by antisera taken 2 and 5 wk after infection with either substrain "M" or substrain "R". In contrast, substrain "R" was agglutinated to a very much lesser extent by serum from the rabbit infected with substrain "R". Organisms isolated from infected rabbits 2 and 5 wk after infection were relatively inagglutinable, irrespective of their initial substrain.

Since the results of this preliminary experiment suggested that substrain "M" could, within a few weeks in rabbits, acquire the inagglutinability characteristic of substrain "R", the variant of substrain "M" isolated from rabbits 5 wk after infection was tested for its agglutinability and antigenicity relative to the original substrain "M".

Four rabbits were infected, two with substrain "M", and two with its variant isolated from rabbits 5 wk after infection and called M5. Sera and trypanosomes were isolated at weekly intervals and the organisms examined for their agglutinability with corresponding antisera. The results of this experiment are summarised in table III.

Variants of substrain "M" isolated at 1 and 2 wk were readily agglutinated, but those isolated 4 and 5 wk after infection were relatively less so. M5 could induce antibodies to substrain "M", and to the same degree as the original substrain.

TABLE II
Agglutination of isolates of T. brucei by sera obtained at intervals after infection of rabbits with two substrains of T. brucei

Substrain used for infection	Antigen used for agglutination tests	Agglutinin titre of sera obtained				
		before infection	at weeks after infection			
			1	2	3	4
"M"	M	0	0	128	512	...
	M 2 wk	0	0	0	8	...
	M 5 wk	0	0	2	8	...
	R	0	0	16	32	...
	R 2 wk	0	0	0	8	...
	R 5 wk	0	0	0	0	...
"R"	M	0	16	64	128	32
	M 2 wk	0	0	4	4	2
	M 5 wk	0	0	0	2	0
	R	0	4	2	8	4
	R 2 wk	0	2	8	4	0
	R 5 wk	0	0	0	0	0

The agglutination of M5, but not of substrain "M", was characterised by extensive prozone formation (table IV). This phenomenon implies a relative excess of antibody over antigen. If this explanation were true, then variant M5 organisms, when exposed to anti-serum in low dilutions, might be expected to have their surface antigens completely covered by antibody. Such organisms may be expected to adhere to and be avidly phagocytosed by mononuclear phagocytes.

The adherence of antibody-treated trypanosomes to macrophages

After exposure to dilutions of antiserum for 2 hr at 20°C trypanosomes were exposed to macrophages growing on glass microscope slides (see *Materials and methods*). After a further hour at 20°C the monolayers were gently washed by dipping them into saline, and examined for adherence between organisms and macrophages.

It was found that if the organism was of substrain "M" and if agglutinating antibodies were present even in low titre (2), then adherence occurred. Attachment of organisms to cells could take place at any point on the trypanosome surface. The adherence was relatively strong, since it still occurred when organisms were highly mobile and even though it appeared to stimulate membrane and flagellar movement. M5 could also adhere to macrophages

when agglutinins were present in quantities inadequate to cause prozoning. However, in the presence of prozoning quantities of antiserum, M5 did not show adherence.

TABLE III

Agglutination of isolates of T. brucei by sera obtained at intervals after infection of rabbits with substrain "M" and its variant M5

Substrain used for infection	Antigen used for agglutination tests	Agglutin titres of sera taken at weeks after infection				
		1	2	3	4	5
"M"	M 1 wk	16	128	256	512	512
	M 2 wk	2	32	32	64	128
	M 4 wk	0	8	8	64	64
	M5 2 wk	0	0	0	0	0
	M5 3 wk	8*	32	64	2	32
	M5 5 wk	0	0	32*	2	128*
"M5"	M 1 wk	4	32	64	256	512
	M 2 wk	32	128	256	512	512
	M 4 wk	0	0	8	32	128
	M5 2 wk	0	0	0	0	0
	M5 3 wk	0	32	2	0	0
	M5 5 wk	0	0	16	2	0

* Presence of prozone.

TABLE IV

Degree of agglutination of variant M5 when mixed with sera derived from a rabbit at stated times after infection with substrain M

Week after infection	Degree of agglutination of variant M5 when mixed with sera obtained from a rabbit at stated times after infection with strain M and diluted 1 in						
	2	4	8	16	32	64	128
1	+++*	++	+	—	—	—	—
2	—	—	—	+	++	+	—
3	—	—	—	+	++	+	—
4	—	—	+	++	++	+	+
5	+	+	++	+++	+++	++	+

* +++ = Massive clumping of all organisms; ++ = many clumps of organisms in the presence of significant numbers of free organisms; + = few clumps of organisms; the majority remain as free individuals.

This lack of opsonic adherence in the presence of prozone may indicate that the organisms are not extensively covered by fixed immunoglobulin, and consequently that prozone formation is due to the presence of relatively few accessible antigenic determinants on the surface of the variant M5.

It is possible, therefore, that the inagglutinability of substrain "R" and variant M5 are due, not only to antigenic change but also to loss of surface antigens.

Agglutination and opsonic adherence with sera obtained from rabbits hyperimmunised with dead trypanosomes

Four rabbits received subcutaneously 2 ml of a heavy suspension of trypanosomes containing 5 per cent. packed cells killed with 0.1 per cent. beta-propiolactone. Before inoculation the suspension of trypanosomes was mixed with an equal volume of complete Freund's adjuvant. Two of the rabbits received four injections of substrain "M" at weekly intervals, and two were similarly treated with the variant M5. After 5 wk the sera of these rabbits were tested for agglutinins and opsonic adherence against homologous and heterologous variants.

Sera of rabbits that received the dead substrain "M" agglutinated the substrain "M" to a titre of 16, but failed to agglutinate its variant M5.

Similarly the adherence to macrophages in the presence of these antibodies occurred only with the substrain "M", but not with its variant M5. The variant M5 failed also to adhere to macrophages in the presence of sera of rabbits immunised with dead M5, although agglutination occurred to a titre of 16.

DISCUSSION

It is generally accepted that trypanosomes isolated from animals in which they have been exposed to antibodies may be serologically different from the organisms used to infect the animals in the first instance. The work on this subject has been discussed by Soltys (1957*a*) and Gray (1962, 1967).

How extensive is the variation possible within one trypanosome is not known (Watkins, 1964).

Gray (1965) in his studies on antigenic variation of *Trypanosoma brucei* has shown that substrains of a clone isolated from rabbits at monthly intervals appeared to be less sensitive to agglutinating antibody than substrains of the same clone isolated from rabbits at weekly intervals. He concluded that since the antisera were shown to contain normal amounts of antibody to various antigens of the clone, there was apparently a tendency for trypanosomes of the clone to lose the ability to react with agglutinating antibody when they were passaged in rabbits at intervals of 3–4 wk. Soltys (1957*a* and *b*) has shown that trypanosomes may become insensitive to neutralising antibody and inagglutinable when passaged at 26–28-day intervals in rabbits.

Further studies presented in this paper not only confirmed early work of Soltys (1957*a* and *b*) and Gray (1965) but clearly demonstrated that trypanosomes isolated from rabbits 2 wk after infection become less reactive to antibodies than the variant before it had been inoculated into a rabbit, and that this may be due to loss of surface antigen. Loss of the response to agglutinins and in opsonic adherence to macrophages in trypanosomes isolated from rabbits was observed not only with a substrain that was previously passaged through rabbits, but also with a substrain that before inoculation into rabbits was passaged through mice.

It appears from these studies and from previous work reported by Soltys (1957*a* and *b*) and Gray (1965) that during antigenic transformation surface components may become gradually non-antigenic either by loss or by inaccessibility, and may allow trypanosomes to survive in spite of the host response to the parasite.

SUMMARY

Antigenic studies on variants of *Trypanosoma brucei* isolated from infected rabbits at different intervals were carried out by agglutination and by opsonic adherence tests with corresponding antisera isolated from the same rabbits. Organisms isolated from infected rabbits 2 and 5 wk after infection were relatively inagglutinable by sera collected at the same time and 3 wk later from the rabbit from which the trypanosomes were derived.

Experiments on adherence of trypanosomes to macrophages in the presence of antibodies indicated the lack of opsonic adherence particularly in the presence of a prozone. These results suggest possible antigenic loss in the presence of antibodies.

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