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Variation in the sensitivity of successive variable antigen types in a *Trypanosoma (Trypanozoon) brucei* subspecies clone to some African game animal sera

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Summary

A *Trypanosoma brucei* subspecies clone was passaged in rats at ten-day intervals and the sensitivity/resistance to a variety of mammalian sera, of the successive variable antigen types (VATs) produced, was examined sequentially in a modified version of the blood incubation infectivity test (BIIT). A proven homogeneous VAT was used to initiate this series of tests, in which seven successive and different VATs were each exposed in vitro at 38.5° C for 2 h to standard samples of pooled rat serum (PRS), normal human serum (NHS) and to sera from two different eland and three different hippopotami. Samples were then inoculated into susceptible rats to determine the effects of the individual sera on the subsequent infectivity of the trypanosomes. The seven VATs were found to vary widely in their sensitivity to the different game sera, though all remained strongly resistant to the pooled rat and the normal human serum samples. BII testing of isolates from positive test rats in the sequential study showed that their resistance to normal human serum was unaffected by their exposure in vitro to the different game sera.

Key words: Trypanosoma (T.) brucei; variable antigen types; variation in serum resistance; rat serum; normal human serum; game sera.

Introduction

In a recent series of experiments it was shown (Rickman, 1981) that, when clones of *T.b.brucei and T.b.rhodesiense* were incubated separately in vitro with serum samples from twelve different African game animal species, and their subsequent infectivity for rats examined in a modified version of the blood

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incubation infectivity test (BIIT) (Rickman and Robson, 1970), the sera of eland, waterbuck, hippopotamus and spotted hyaena showed significant trypanolytic activity. No clear correlation was found between this activity and trypanosomal antibody titres.

Differences of infectivity and virulence for mice, between VATs in a *T.b.rhodesiense* clone, have been reported by McNeillage and Herbert (1968), Herbert (1968), and Lumsden and Herbert (1975); more recently similar differences, in their resistance to normal human serum, have been found between VATs in the same *T.b.rhodesiense* clone (Van Meirvenne et al., 1973, 1975, 1976; Rickman, 1977) and also in a *T.b.brucei* clone (Van Meirvenne et al., 1975; Rickman, 1977).

Further evidence of such instability was provided by the finding of a change in the potential infectivity for man of a *T.b.brucei* clone during its passage through birds (Joshua et al., 1978).

More recently Rickman and Kolala (in press) have demonstrated changes from normal human serum sensitive (NHSS) to resistant (NHSR) in each of three *T.b.brucei* clones serially syringe-passaged in rats.

The possibility that the sojourn of the *T.brucei* subspecies trypanosomes in certain of the game animals may, in some way, modify their subsequent character, behaviour or transmissibility to other vertebrate hosts, has important and self-evident epidemiological implications.

This exploratory study was undertaken to determine, firstly, whether the trypanolytic factor, identified earlier in some game sera, is active against all, or only against some, of the VATs expressed in a trypanosome clone population; and, secondly, whether such effects could at times give rise to changes in the resistance of the trypanosomes to normal human serum.

Materials and methods

Materials

The trypanosome clone – *Trypanosoma (Trypanozoon) brucei* (supp.indet.) – TDRN[1] 13 = CVRS[1] 50 – isolated by Dr. D. Röttcher (1979) from a naturally infected duiker *(Sylvicapra grimmia)* in the Kakumbi area of the Luangwa Valley, Eastern Province, Zambia.

The original stock was NHSS but the clone used in this study was strongly NHSR in each of eight serial BII tests (i.e. typical of *T.b.rhodesiense*).

Sera

Control – Pooled rat serum (PRS) from clean, laboratory-bred white Wistar rats was dispensed in 2 ml vols. and stored at –20° C until required.

Human – One standard vene-puncture sample from one of us (FK) was dispensed in 2 ml vols. and stored at –20° C until required.

Game – All five game sera were collected in the Kakumbi area of the Luangwa Valley (Dillman and Townsend, 1979), the hippo samples in 1969 and those from the eland in 1973 (NB: all game sera have been maintained at –20° C since their collection).

[1] TDRN = WHO Tropical Disease Research Centre, Ndola, Zambia; CVRS = Central Veterinary Research Station, Mazabuka, Zambia

Experimental animals – Those used were in-bred 50–100 g white Wistar rats (Mazabuka strain). Animals were of even weight for each sequential test.

Methods

The VAT sample – To provide the initial VAT sample, equal volumes of positive rat blood were inoculated into each of three rats. When heavily parasitaemic Rat 1 cardiac blood was cryopreserved as antigen sample. Rats 2 and 3 were simultaneously treated intra-peritoneally (i. p.) with Berenil (at 25 mg/kg body weight) and bled twelve days later to provide the VAT-specific anti-serum.

Anti-sera to the VATs – Specific antisera to the seven different VATs were obtained in the same way as that for the donor VAT described above.

Donor VAT homogeneity test

Equal vols. of antigen were later incubated separately in vitro at 37° C/2 h with three times the volume of pooled rat serum (control) and the VAT specific anti-serum (test). After incubation equal volumes of the samples were inoculated into each of three rats, i.e. Rats 1–3 (control), Rats 4–6 (test). Parasitaemic cardiac blood from control Rat 1 was used to initiate the sequential study. Cardiac blood from Rats 2 and 3 was cryopreserved – test Rats 4, 5 and 6 remained aparasitaemic for thirty days and were then killed.

Serum samples – These were dispensed in 0.4 ml vols. into seven sets of seven clean, sterile 'Bijou' bottles and frozen at –20° C. Prior to each sequential test one set of samples was brought to ambient temperature, ready for use.

Sequential BII tests – 0.1 ml vols. of heparinised (50 iu/ml) donor cardiac rat blood were added aseptically to each of the seven serum samples and mixed by swirling. After incubation at 38.5° C (average game animal body temperature) for 2 h, samples were remixed and 0.45 ml vols. were taken up from each bottle in turn and inoculated, in 0.15 ml vols., into each of three rats.

Parasitaemia in the infected control rats was allowed to proceed for ten days when the following procedures were adopted – Rat 1 was exsanguinated under chloroform anaesthesia and 0.1 ml vols. of cardiac blood added to each of the bottles in the second set of serum samples (as above); the remainder of the blood was buffered with 7% glycerol and cryopreserved as the VAT No. 2 antigen sample.

Rats 2 and 3 were treated with Berenil and bled for specific anti-VAT 2-serum twelve days later. This process was repeated at ten-days intervals to give a series of seven sequential tests – during which the change in the VAT was the only varying factor.

Neutralisation tests

To confirm the successive VATs differed antigenically from one to the other, VAT antigen and specific anti-serum samples were later set up in a neutralisation test (Table 3).

Antigen samples (0.05 ml) of each of the seven VATs were incubated in vitro at 37° C/2 h with an excess of specific homologous anti-serum (0.125 ml) and 0.225 ml of phosphate-buffered glucose saline at pH 8.0 (PGS).

Similar antigen samples were simultaneously but separately incubated with anti-serum to the next VAT in the series (e.g. VAT 1 with anti-VAT 1, VAT 1 with anti-VAT 2, VAT 2 with anti-VAT 2, VAT 2 with anti-VAT 3, etc. – additionally VAT 7 with anti-VAT 1).

Incubated samples were then inoculated in equal volumes into each of two clean, numbered rats to test for infectivity.

BII tests – Finally, where individual VATs were resistant to the game sera in the sequential study, trypanosome isolates from these positive test rats were later examined in separate BII tests, samples being incubated in vitro at 37° C/1 h with pooled rat (control) and normal human serum (test). Equal vols. of each sample were then inoculated into three rats, i.e. Rats 1–3 (control) and Rats 4–6 (test).

Table 1. Showing results of the neutralization test to prove the homogeneity of the antigen type used to initiate the sequential study

Experiment	Control			Test		
Antigen	TDRN-13 (clone)			TDRN-13 (clone)		
(Anti)Sera	Pooled rat			Anti-TDRN-13 (clone)		
Rat number	1	2	3	4	5	6
Day 1	–	–	–	–	–	–
Day 2	–	–	–	–	–	–
Day 3	–	5.0	5.0	–	–	–
Day 4	–	5.0	5.3	–	–	–
Day 5	6.2	6.5	5.6	–	–	–
Day 6	8.0*	6.5	7.1	–	–	–
Days 7–30	D	D	D	–	–	–

* Control Rat 1 cardiac blood used to initiate the sequential study
Figures = log number of trypanosomes per ml
D = daily examination discontinued

Indirect fluorescent antibody test (IFAT) – Examination of the game sera for trypanosomal antibodies was carried out in the laboratories of the Bernard-Nocht-Insitut für Schiffs- und Tropenkrankheiten, Hamburg, in the manner described by Mehlitz (1975).

Microscopy – Daily wet tail-blood films from inoculated rats were examined at × 240 magnifications. Parasite levels were estimated by the 'matching' method of Herbert and Lumsden (1976) and expressed as a log number of trypanosomes per ml.

Results

VAT homogeneity test – All three control rats showed persistent and rising parasitaemia with pre-patent periods of 3–5 days (Table 1). By contrast, all three test rats remained aparasitaemic for the full thirty-day examination period (Table 1).

The sequential study – All seven VATs in the series proved fully resistant to the pooled rat and to the normal human sera (Table 2). However, they differed widely in their responses to the different game sera. Some (VATs 3, 4 and 5) were fully resistant to them all, while others (VATs 1 and 2) were fully sensitive to all but the first of the three hippo serum samples.

VAT 6 was sensitive to the second eland and the second hippo sera but was fully resistant to the rest.

The last VAT in the series (No. 7) was resistant to both eland sera but was fully sensitive to the three hippos samples (Table 2).

Retesting of game sera positives by the BIIT – Retesting of trypanosome isolates from parasitaemic test rats (i.e. those previously exposed to the game sera) showed that all had retained their resistance to normal human serum.

Table 2. Showing the results for rats inoculated with seven successive variable antigen types, of a *T. brucei* subspecies clone, following their in vitro incubation at 38.5° C for 2 h with seven different standard mammalian sera

Serial VAT/ test number	Date of test (1980)	Pooled rat serum (titre* nil)			Normal human serum (titre* nil)			Eland No. 1 serum (titre* 1:160)			Eland No. 2 serum (titre* 1:640)			Hippo No. 1 serum (titre* 1:160)			Hippo No. 2 serum (titre* 1:640)			Hippo No. 3 serum (titre* 1:160)		
		R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	R17	R18	R19	R20	R21
1	2. 3.	4	4	5	7	8	8	–	–	–	–	–	–	4	4	6	–	–	–	–	–	–
2	12. 3.	1	1	1	5	5	6	–	–	–	–	–	–	10	10	10	–	–	–	–	–	–
3	21. 3.	2	4	1	6	5	6	2	2	3	1	1	1	1	2	1	5	4	5	2	2	4
4	31. 3.	3	2	2	5	5	5	3	4	5	3	4	4	2	2	2	3	5	2	4	4	3
5	10. 4.	1	1	1	2	1	1	3	1	1	1	1	3	1	1	1	1	3	1	1	1	3
6	22. 4.	1	1	1	4	2	1	1	1	2	–	–	–	1	1	1	–	–	–	3	1	1
7	2. 5.	1	1	1	3	3	3	2	2	2	4	4	4	–	–	–	–	–	–	–	–	–

Figures in results column indicate pre-patent period in days
* Immuno-fluorescent antibody titre against *T. brucei* antigen
R1–R21 = rat numbers
– = rats aparasitaemic for 30 days

Table 3. Showing results of neutralization tests to confirm antigenic differences between seven successive variable antigen types produced in a *Trypanosoma (Trypanozoon) brucei* subspecies clone serially syringe-passaged in rats

Experiment No.	Antigen	Serum	Rat No.	Result
Control 1	VAT 1	Anti-VAT 1	R1	–
			R2	–
Test 1	VAT 1	Anti-VAT 2	R3	3
			R4	3
Control 2	VAT 2	Anti-VAT 2	R5	–
			R6	–
Test 2	VAT 2	Anti-VAT 3	R7	4
			R8	4
Control 3	VAT 3	Anti-VAT 3	R9	–
			R10	–
Test 3	VAT 3	Anti-VAT 4	R11	6
			R12	6
Control 4	VAT 4	Anti-VAT 4	R13	–
			R14	–
Test 4	VAT 4	Anti-VAT 5	R15	4
			R16	6
Control 5	VAT 5	Anti-VAT 5	R17	–
			R18	–
Test 5	VAT 5	Anti-VAT 6	R19	4
			R20	4
Control 6	VAT 6	Anti-VAT 6	R21	–
			R22	–
Test 6	VAT 6	Anti-VAT 7	R23	5
			R24	2
Test 7	VAT 7	Anti-VAT 1	R25	2
			R26	2

Figures in results column indicate pre-patent period in days
– = rat aparasitaemic for 30 days

Neutralisation tests – Where VAT antigen samples were incubated in vitro with their homologous anti-sera all inoculated rats remained aparasitaemic until they were killed thirty days later.

All rats, inoculated with antigen samples incubated with anti-sera to succeeding VATs, showed early persistent and rising parasitaemias (Table 3), confirming antigenic differences between the successive VATs.

Serology – IFAT antibody titres of the game sera to *T.brucei* antigen were as follows: Eland 1 (1:160), Eland 2 (1:640), Hippo 1 (1:160), Hippo 2 (1:640) and Hippo 3 (1:160).

Discussion

Over the years, attempts to isolate *T.b.rhodesiense* from its feral reservoir have generally met with little success and have served only to emphasise the comparative rarity of this parasite in nature (Willett et al., 1964; Ford, 1969). One reason for this has been advanced by Ashcroft (1959) who reasoned that the dilution of the *T.b.rhodesiense* in animals by the more prevalent *T.b.brucei* would result in mixed infections incapable of infecting man.

Another possible reason is suggested by the studies of Culwick et al. (1951); Vaucel and Jonchère (1954) and Vaucel and Fromentin (1958), who found that when *T.b.rhodesiense* and *T.b.brucei* were passaged together in rats the latter overgrew and finally extinguished the former, the mixture becoming non-infective for man.

When they sequentially BII tested mixed infections of *T.b.rhodesiense* and *T.b.brucei* clones grown in rats, Geigy et al. (1975) showed that such infections gave inconsistent results. By contrast a pure *T.b.rhodesiense* clone concurrently grown separately in rats gave consistently positive BIIT results.

While African game animals may not be entirely unaffected by trypanosome infection (Corson, 1934; Ford, 1971; Losos and Gwamaka, 1973) most of them show few, if any, clinical symptoms of the disease (Ashcroft et al., 1959; WHO, 1965; Desowitz, 1970).

At present, virtually nothing is known of this asymptomatic carrier state and, for obvious reasons, very few comparative studies on antibody levels in game, such as those of Dräger and Mehlitz (1978), have been carried out.

Corson (1939) attributed his own failure to infect tsetse flies from one particular dikdik *(Madoqua kirki)*, where he had succeeded with others, to the blood of that animal ... 'being unsuitable for the development of the trypanosomes in the flies'. Unfortunately, it is not stated whether these animals came from tsetse-free or tsetse-infected areas.

That the subsequent infectivity of the *T.brucei* subspecies trypanosomes may be differentially affected by their exposure to physical elements in the game hosts, is strongly suggested by the results of the wild life studies of Dräger and Mehlitz (1978) in northern Botswana. They found that seven isolates of pleomorphic trypanosomes from buffaloes *(Syncerus caffer)* were non-infective for laboratory mice, unlike eight similar isolates (out of nine tested) from lechwe *(Kobus leche)* in the same general area, which did infect mice.

Past studies of the interactions between the African pathogenic trypanosomes and game animals from tsetse-free areas, have established that such animals differ markedly in their susceptibility to trypanosome infection (Ashcroft et al., 1959).

In one experiment a common duiker *(Sylvicapra grimmia)* from a tsetse-infested area, was found to be totally refractory to a *T.b.rhodesiense* challenge infection (Desowitz, 1960). This is significant in the light of the earlier ex-

periments of Ashcroft et al. (1959), which showed that similar animals captured in tsetse-free areas (and which presumably lacked any specific acquired immunity) were readily infectible with the *T.brucei* subspecies.

It has been shown (Van Meirvenne et al., 1975, 1976; Rickman, 1977) that different VATs, produced in clones of *T.brucei* subspecies infections in mice, vary widely in their sensitivity/resistance to normal human serum. Thus it is, perhaps, not entirely surprising to find evidence of similar variation in their responses to other, non-human mammalian sera.

While it must be accepted that, with sera kept so long in the frozen state, results obtained from their use may be of questionable validity, the presence of very high IFAT antibody levels[2] and differences in the effects of individual serum samples on the different VATs in this study argue against any serious degradation of the serum components having taken place during storage.

However, recognition of this possibility and the limited data from this study clearly preclude any attempt to correlate the effects of the sera with their antibody levels. Despite this, it is interesting to note that, in those cases where a particular VAT proved sensitive to one, but not to all, of the eland or hippo sera, that sample had the higher IFAT titre.

Also worthy of note is the fact that both the trypanosome isolate and the game sera came from animals in the same comparatively limited area of the Luangwa Valley, thus increasing the likelihood of the game sera antibodies being specific to the test organism.

Although all seven VATs gave NHSR responses typical of *T.b.rhodesiense* four of them were fully sensitive to one or more of the game sera. If this evidence truly reflects the situation in the wild, the destruction of *T.b.rhodesiense* VATs in vivo, in certain of the game, could be another factor contributing to the apparent scarcity of this parasite in its non-human reservoir. If substantiated, the epidemiological implication of these findings is self-evident.

These preliminary results suggest that eland and hippopotamus might be particularly rewarding subjects for more intensive investigations into the natural immune status of the African game. Ideally, further studies of this kind should be made using fresh sera taken from game in tsetse-infested areas of Africa, as well as from similar species born and raised in captivity, in areas free from trypanosome challenge. Such studies can be expected to enhance the present, rather limited, understanding of the dynamic factors involved in the natural transmission of the *T.brucei* subspecies trypanosomes.

[2] During 1978–1979 fresh serum samples were collected from nine sleeping sickness cases in the Luangwa Valley, prior to treatment and at the time that diagnosis was confirmed. These samples were frozen at –20° C and subsequently examined by Dr. Mehlitz in Hamburg at the same time, and using the same antigen, as the game sera used in this study. Of these sera, eight gave IFAT titres of 1:640, the other had a titre of 1:160. Thus some of these game sera had IFA titres as high as most human trypanosomiasis cases.

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