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Identification of *T. brucei*-Subgroup Strains Isolated from Game

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Abstract

Several *T. brucei*-subgroup strains isolated from game were investigated with the blood incubation infectivity test (BIIT) and in human volunteers. Original isolates and their cloned derivatives were tested. In order to check the validity of the BIIT, volunteer tested clones were used under modified BIIT conditions. Inoculation of different trypanosome strains into volunteers yielded positive parasitaemia for original isolates from lion, hyaena and Coke's hartebeest. Changing antibody titers during the course of infection in the volunteers were checked with the indirect fluorescent antibody test (IFT). The BIIT was reliable (consistently positive) for cloned *T. b. rhodesiense*. When testing *T. b. brucei*, the BIIT results depended on the number of incubated parasites and on the parasitaemia peak number the tested trypanosomes derived from. It was further shown that the *in vivo* part of the BIIT is actually essential for the complete neutralization of *T. b. brucei* induced by the action of human plasma. Tests with trypanosomes originating from experimentally mixed *T. b. rhodesiense* and *brucei* infections gave inconsistent results. On the other hand, pure populations mixed *in vitro* – prior to incubation for BIIT – revealed results depending on the proportion of the 2 species: up to 20% *T. b. rhodesiense* gave negative, higher concentrations consistently positive results.

Introduction

Two surveys were carried out in and around the Serengeti National Park in 1970/71 (MWAMBU & MAYENDE, 1971; GEIGY, MWAMBU & KAUFFMANN, 1971; GEIGY & KAUFFMANN, 1973a), in order to shed more light on the complex epidemiological status of *T. b. rhodesiense* sleeping sickness as a zoonosis. Besides 10 *T. brucei*-subgroup strains isolated from cattle, 52 strains of the same pleomorphic trypanosome group were isolated from game. The latter were transferred as stabilates to Basle for further examinations. The 52 strains, 12 strains from 1970 and 40 strains isolated in 1971, were subsequently tested with the blood incubation infectivity test (BIIT) (RICKMANN & ROBSON, 1970a/b; GEIGY et al., 1973a/b). Some positive and "equivocal" strains, after extensive drug sensitivity tests, were checked in human volunteers (GEIGY et al., 1972; GEIGY et al., 1973c; ONYANGO et al., 1973); Coke's hartebeest, spotted hyaena and lion were thus identified as additional new reservoirs of *T. b. rhodesiense*. The present paper describes investigations in human volunteers and with the BIIT using several of the Serengeti isolates and clones derived from them.

Material and Methods

Trypanosomes: Original strains and clones

Out of all the 52 *T. brucei*-subgroup strains isolated in 1970/71 from game the following stabilates were selected for further analysis (drug sensitivity test,

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Table 1. Original strains and their respective clones

Original strains					Clones		
Year of isolation	Animal	Stabilate No.	BIIT		Stabilate No.	Prelim. BIIT	
			pos.	neg.		pos.	neg.
1970	Coke's Hartebeest	EATRO 1810	8	7	STIB 346	2	0
1971	Coke's Hartebeest	STIB 246	1	5	STIB 348	0	2
1971	Coke's Hartebeest	STIB 229	4	1	STIB 347	2	0
1971	Spotted Hyaena	STIB 235	13	1			
	Derivative of STIB 235 from pos. test rat	STIB 324			STIB 349	2	0
1971	Lion	STIB 241	13	4	STIB 350	2	0

volunteer test, BIIT with volunteer-tested clones) on the basis of their preliminary BIIT results and their capability of producing clones (Table I).

Besides the original strains, which showed a "*rhodesiense*-like" behaviour in the BIIT, STIB 246 and STIB 348 were regarded as presumptive *T. brucei* and used for studies of the selectivity of the BIIT under modified conditions.

Clones were prepared by picking up single slender bloodstream forms with a fine glass capillary followed by i.p. inoculation into a rat: trypanosome populations were isolated by heart puncture at the first peak parasitaemia (5–8 days p.i.) and split into 3 parts: The first was stabilated, the second one checked with the BIIT and the third one directly passed into mice for the drug sensitivity tests. It was impossible to obtain a clone from STIB 235. Nevertheless STIB 235 occurs in Table 1, since it was tested for drug sensitivity as were all the other stabilates included in this Table.

EATRO 1810 and 1873 are stabilates derived from the same hartebeest. The latter was already man tested and proved to be *T. b. rhodesiense* (GEIGY et al., 1973c).

Volunteers

9 young adult male Kenyans were selected after careful medical examination. They all came from near Kisii, which is an area free of tsetse flies. They were all hospitalized in a ward of the Homa Bay District Hospital, Kenya, and stayed under medical observation from the time of the first medical check until the end of the experiment.

Laboratory animals

In Basle, white mice NMRI (20–25 g) were used, obtained from IVANOVAS, Kisslegg, Germany; SIV rats (100–120 g) originated from several breeding stations. In Homa Bay all laboratory animals used came from the colonies of nearby EATRO, Tororo.

Table 2. Volunteer and BIIT experiments in Homa Bay 1973

Simultaneous BIIT						Volunteer		
Stabilate	Number of passages since isolation	ID when cells taken	Number of cells incubated per BIIT	Prepatent periods days		Volunteer No.	Inoculum in Volunteer	Volunteer prep. period days
				Plasma European	Blood Volunteer			
EATRO 1810	2	8	8.2×10^6	4	4	XII	4.1×10^7	11
STIB 324	9	6	1.5×10^7	3	3	XI	7.8×10^7	4
STIB 241	2	8	1.1×10^7	6	6	XIV	5.7×10^7	21
STIB 350	4	8	2.0×10^7	4	6	XV	9.8×10^7	7
STIB 346	4	6	5.4×10^6	6	4	X	2.7×10^7	∞
STIB 349	11	6	4.6×10^6	∞	∞	III	2.3×10^7	∞
STIB 348	4	11	4.2×10^6	∞	∞	XVI	2.1×10^7	∞
			4.2×10^6	∞	∞	XVII	2.1×10^7	∞
			4.2×10^6	∞	∞	XVIII	2.1×10^7	∞

∞ = No trypanosomes were detected during the period of the experiment (8 weeks).

Drug sensitivity tests

These tests were carried out as described by GEIGY et al. (1973c) with two modifications: instead of Melarsen Sodium we used Mel B at 30 mg/kg body weight dissolved in 0.1 ml Propylenglycol/water 1 : 2 (v/v). Antrypol was administered at 20 mg/kg body weight (single doses). The body weights of the test mice were 25 g.

Inoculation of volunteers

The stabilates chosen for inoculation into volunteers were transported to Homa Bay and kept in liquid nitrogen, as was the human European plasma, required for the BIIT.

One capillary of each strain was diluted with ESG and inoculated into 2 rats. Tail blood from the infected rats was checked daily until the initial rising parasitaemia reached a suitable level. Blood was then taken by sterile heart puncture, using heparin as anticoagulant.

Each volunteer received 1 ml of heparinized blood containing between 2×10^7 and 1×10^8 trypanosomes (Table 2). The injection was made subcutaneously on the medial aspect of the left forearm. Blood examination for trypanosomes with the hematocrit centrifuge technique (HCT) (Woo, 1970) and inoculation of volunteer blood into mice and/or rats was started one week later or as soon as any symptoms appeared. Body temperature was checked twice daily. All positive cases were first diagnosed by HCT. As soon as trypanosomes were found in a volunteer, blood was taken by venous puncture and inoculated into fresh rats, from which stabilates were made (e.g. STIB 360, STIB 362), and preserved in liquid nitrogen. Volunteers showing a trypanosome-positive HCT were

immediately treated with a full course of Antrypol: 25 mg on the first day and then 1 g each on day 3, 8, 13, 18 and 23. A final check with blood and cerebrospinal fluid was carried out three weeks after the end of the treatment.

BIITs were carried out simultaneously with each volunteer inoculation. Samples of the same trypanosome suspension injected into volunteers were tested with fresh whole blood (taken before the injection) from the respective volunteer and with plasma from a single human donor (European).

Serological investigations

Serum samples taken from the volunteers before and at different intervals after injection of trypanosomes (ID 8, ID 21 and in positive cases 3 weeks after treatment) were preserved in liquid nitrogen. They were tested later in Basle with the indirect immunofluorescent test (IFT) for the presence of possible specific antibodies against *T. b. brucei* and *T. b. rhodesiense*. The antigens used were the respective aceton-fixed bloodstream forms (SUTER & FRICKER, 1972).

BIIT experiments

Based on the results obtained with human volunteers the following clones were retested with the BIIT: STIB 348, STIB 349, STIB 350.

Blood from a single human donor (European) was prepared according to RICKMANN & ROBSON (1970a/b) and used throughout all the experiments (Homa Bay and Basle). From this blood, plasma (aliquots of 1 ml preserved in liquid nitrogen) was taken for each test. IFT with this plasma revealed no specific antibodies against *T. b. brucei* (STIB 348). Our preliminary BIIT experiments showed that 1 ml of human plasma had the same effect as 2 ml of whole blood, which is in accordance with earlier observations of other authors (AWAN, 1971).

Infected blood from donor rats was taken by heart puncture with potassium oxalate (2 % w/v) as anticoagulant. The number of bloodstream forms (in 0.2 ml) incubated in 1 ml of human plasma was counted and their pleomorphism determined. The whole content of each test bottle (1.2 ml) was inoculated after the usual 5-h incubation at 37 °C into one or two test rats. Each test rat received 0.17 ml human plasma/20 g body weight. Each rat was checked daily for trypanosomes by HCT for at least 21 days. Studies were carried out to investigate the effects of the following conditions on the results of the BIIT:

a) Variations in response of trypanosomes during serial subpassages.
b) Parasites from positive test rats (STIB 348) were retested in order to clarify "breakthrough phenomena".
c) Artificial mixtures of the clones STIB 348 *(T. b. brucei)* and STIB 350 *(T. b. rhodesiense)* were tested.
d) In one experiment, aliquots of 0.2 ml of STIB 348 bloodstream forms were simultaneously used as follows:
   A. Immediate injection of trypanosomes together with 1 ml of human plasma into the test rat.
   B. Normal BIIT; incubation for 5 hours at 37 °C.
   C. After normal BIIT, the plasma was removed by centrifugation and the trypanosomes were resuspended in 1.5 ml of ESG (WALKER, 1970) or phosphate buffered saline and subsequently injected.
   D. The "used" plasma from C was retaken and used for a second normal BIIT with a fresh trypanosome population.
   E. As in C but immediately followed by injection of 1 ml of "used" or fresh plasma.

Control incubations were carried out in parallel with each test: Instead of human plasma, 1.5 ml of sterile ESG were added to the 0.2 ml of infected rat blood. Test and control animals were kept in separate cages in order to prevent possible cross infection, which we previously observed and attributed to oral transmission (MOLOO et al., 1973). Tail blood smears were prepared from all positive test animals and checked microscopically for the presence of *T. congolense* which may survive the test incubation (RICKMANN & ROBSON, 1972). This was necessary because some of the orignal isolates from game were mixed populations of *T. brucei*-subgroup and *T. congolense*.

Results

1. Drug sensitivity tests

With the exception of STIB 229, STIB 347 and STIB 235, all other stabilates in Table 1 proved to be drug sensitive and were therefore considered suitable for testing in volunteers.

2. Volunteer tests

The results of the simultaneous BIIT and inoculation tests in human volunteers are given in Table 2. STIB 246 could not be tested for several reasons, e.g. shortage of time. EATRO 1810, STIB 324, STIB 241 and its respective clone STIB 350 caused parasitaemia in man and must therefore be regarded as *T. b. rhodesiense*. STIB 346, a clone of EATRO 1810 produced no parasitaemia in volunteer X, although the BIITs carried out simultaneously were positive. One explanation for this negative result could be that this volunteer showed a relatively high titer of antibodies at ID O (Table 3) against *T. b. brucei* and *T. b. rhodesiense*. STIB 348 was negative in simultaneous BIITs as well as in 3 volunteers and is therefore considered as *T. b. brucei*. Clone STIB 349 was negative in the BIITs and in volunteer III, although the original population STIB 324 from which this clone derived was positive when tested with the BIIT and in volunteer XI. Surprisingly, when the STIB 349 population from the same rat was retested later (ID 20) the BIIT with plasma (European) was positive.

3. Serological investigations

Serum samples from volunteers were tested with the IFT for the presence of antibodies against *T. b. brucei* (STIB 348) and *T. b. rhodesiense* (STIB 362 and STIB 360). STIB 362 is the stabilated trypanosome population from a rat infected with infected blood from volunteer XI (STIB 350), while STIB 360 was obtained in the same way from

Table 3. Reciprocal antibody titers in the serum of human volunteers as revealed with the IFT

Volunteer No.	STIB No. inoculated	Reciprocal Antibody titers * at ID			
		0	8	21	> 45
XII	1810***	20/20/20	40/20/20	40/40/40 **	40/20/20
XI	324	20/20/20	40/20/20 **	40/20/20	40/20/20
XIV	241	–/ –/ –	–/ –/ –	20/ –/ – **	20/20/40
XV	350	20/20/20	20/20/ – **	80/80/40	40/40/40
X	346	80/40/40	80/40/40	80/40/40	
III	349	20/20/ –	40/40/40	80/40/80	
XVI	348	20/ –/ –	80/80/20	80/80/40	
XVII	348	20/ –/ –	80/40/20	80/40/20	
XVIII	348	20/ –/ –	20/20/20	40/40/40	

* Serum tested against bloodstream forms of STIB 348/STIB 362/STIB 360. The lowest serum dilution tested was 1/20.

** First serum taken after starting drug treatment of volunteer.

*** EATRO.

volunteer XI (STIB 324). The antibody titers shown in Table 3 are rather low. Changes in titers during the course of infection ranged in some cases between 1 and 4 in relation to the starting titers. In 6 out of 9 volunteers a marked rise in antibody titer was detectable. The increase in antibody titer was rather early in the group of volunteers which showed no parasitaemia. In those volunteers in which the trypanosomes produced a parasitaemia an increase in antibody titer was detected in 2 out of 4 (i.e. volunteer XIV and XV) after the start of drug treatment. Serum samples taken from two sleeping sickness patients at Homa Bay Hospital showed the following corresponding antibody titer values to Table 3 when tested against the three different trypanosome antigens: 320/320/160 and 640/160/160. Serum samples from these two patients were taken 1 month after the start of drug treatment.

Volunteer XVI, XVII and XVIII were suffering from malaria on ID 16 and were immediately treated with the antimalarial drug.

4. Testing of man tested clones with the normal BIIT

In a first series of tests STIB 348, STIB 349 and STIB 350 were checked with the BIIT over several passages in rats in order to investigate the validity of the test. The successive passages were made by passing the trypanosomes forming the first parasitaemia peak, from the respective rat also used for the BIIT in each passage. When the two

Table 4. BIIT with man tested clone *T. b. brucei* STIB 348 in the course of successive passages in rats

Number of passages since isolation	ID	Total No. of parasites incubated	Number of slender forms	BIIT	Pre-patent period days	BIIT No.
4	11 *	not evaluat.	not evaluat.	0		452
	19	not evaluat.	not evaluat.	+ **	13	457
5	8 *	not evaluat.	not evaluat.	0		458
	16	not evaluat.	not evaluat.	0		461
6	9 *	not evaluat.	not evaluat.	0		465
	18	1.8×10^7	2.4×10^6	0		472A
	18	5.4×10^7	7.5×10^6	+ **	9	472B
	18	2.7×10^7	3.8×10^6	0		472C
	18	1.3×10^7	1.9×10^6	0		472D
	18	5.4×10^6	0.9×10^6	0		472E
7	8 *	7.7×10^6	1.0×10^6	0		470
	36	1.6×10^7	0.7×10^6	0		497
	36	5.9×10^7	1.5×10^7	0		498
	36	4.7×10^7	6.5×10^6	+	9	499
8	22	3.5×10^7	1.1×10^7	+ **	12	489
	36	5.4×10^7	1.1×10^7	0		501
9	9 *	6.1×10^6	1.3×10^6	0		485
	35	1.1×10^7	0.3×10^6	0		507
10	10 *	1.0×10^7	0.1×10^6	0		492
	27	1.8×10^7	3.5×10^6	0		502

* First rising parasitaemia.
** Retested and BIIT negative.

BIITs per passage were carried out the trypanosomes were taken twice from the same rat by sterile heart puncture. Whenever possible, trypanosomes from the first rising parasitaemia were chosen in each passage for the first (early) BIIT while for the second (late) BIIT parasites from a subsequent peak were incubated.

STIB 348 (Table 4) was tested over 7 passages. Out of 20 tests, 4 tests were positive (persistent and increasing parasitaemia) while 16 tests were negative. In all 4 positive BIITs the incubated trypanosomes derived from a second or subsequent parasitaemia peak population. Trypanosomes taken during the course of the first rising parasitaemia were always neutralized in the test, i.e. gave consistently BIIT negative results.

Parasites from initial rising parasitaemias from 3 positive test rats were retested and the BIITs were consistently negative. Test number 472A–472E were carried out with different trypanosome concentra-

tions (diluent: ESG) from the same rat. Whereas a total number of incubated slender forms up to 3.8×10^6 was inactivated by the human plasma in these test series, a higher number of slender forms (7.5×10^6) was not totally neutralized and therefore produced a parasitaemia in the test rat. The incubated trypanosomes in BIIT 497 (same rat as BIIT 470), BIIT 498 and BIIT 499 were derived from 3 different rats from the 7th passage animals.

STIB 349 and STIB 350 were also tested over 7 passages derived from one single stabilate capillary of each (Table 5).

For STIB 349 out of 14 tests, 13 were positive and 1 negative. The negative test was the first test carried out in these series. The trypanosomes incubated (10^7) belonged to the first peak forming population growing out from the thawed and subsequently inoculated forms. This phenomenon of human plasma sensitivity of the first peak trypanosomes occurred regularly when a capillary of STIB 349 was tested. Likewise tests of STIB 349 in Homa Bay were also negative (BIIT and volunteer) when trypanosomes from the initial parasitaemia were tested. As already mentioned it is interesting to note that in Homa Bay when trypanosomes were taken from the same rat with a second heart-puncture at ID 20 the BIIT with these forms was positive (prepatent period 3 days). It was not possible to test these forms from ID 20 in human volunteers. Looking at the rest of the successively positive BIIT results of STIB 349 in Table 5, one can assume that STIB 349 is of *T. b. rhodesiense* like nature.

In an additional test series, low numbers of trypanosomes were incubated (3×10^5/l/ml human plasma). In these tests STIB 349 continued to give a positive BIIT result (mean prepatent period of 3 days), which means that this clone is very resistant to the effect of human plasma.

STIB 350 showed consistently positive results in the BIIT (Table 5), which is in accordance with the results of Homa Bay.

Trypanosomes from several of the positive test rats in Table 5 were chosen for retests which were always positive. The prepatent period was consistently 1 day, which shows that the resistance to the action of human plasma is increased.

Subsequently, STIB 348 *(T. b. brucei)* and STIB 350 *(T. b. rhodesiense)* were used to investigate parameters affecting the results of the BIIT.

5. BIIT with mixed populations of known T. b. brucei *and* T. b. rhodesiense

BIITS were carried out with artificially mixed populations of STIB 348 and STIB 350. The two clones were either grown separately

Table 5. BIIT with man tested clone STIB 349 and STIB 350 *(T. b. rhodesiense)*

STIB 349				STIB 350			
Number of passages since isolation	ID	BIIT	Prepatent period days	Number of passages since isolation	ID	BIIT	Prepatent period days
11	10	0	∞	4	11	+	7
	17	+	4		17	+	5
12	9	+	6	5	8	+	8
	17	+	5		16	+	12
13	9	+	4	6	9	+	8
	20	+	4		17	+	3
14	5	+	7	7	5	+	7
	14	+	1		17	+	3
15	7	+	5	8	8	+	3
	16	+	4		16	+	5
16	5	+	9	9	5	+	5
	15	+	5		15	+	5
17	5	+	4	10	5	+	5
	15	+	4		15	+	4

The number of trypanosomes incubated per test ranged from 10^6 to 10^7.

in different rats and mixed together before the incubation in human plasma (*in vitro* mixtures) or grown together in one rat (*in vivo* mixtures).

a) *In vitro* mixtures

The two clones were tested separately (controls) and combined in various proportions. An amount of STIB 348 corresponding to the highest amount used in the mixtures was used in the controls which were consistently negative. Conversely, an amount of STIB 350 corresponding to the lowest proportion used in the mixture was taken for the controls which were consistently positive.

The results of the BIITs with mixture were always positive when the portion of STIB 350 in the mixture was higher than 20%. Lower portions of STIB 350 were neutralized, i.e. gave consistently negative results. In the parallel control tests the same low amounts, when incubated individually, were always positive.

These results indicate the possibility of obtaining "equivocal" BIIT results when freshly isolated and uncloned *T. brucei*-subgroups strains containing minimal portions of *T. b. rhodesiense* are tested. In order to investigate this possibility another series of tests was carried out with mixed populations grown *in vivo*.

Table 6. BIIT with mixtures of STIB 350 and STIB 348 obtained *in vivo* from a series of 4 rats

Tested at ID	Mixture of STIB 350/STIB 348			Control BIIT with STIB 350		
	Number of trypanosomes incubated	Rat No. taken	BIIT	Number of trypanosomes incubated	Rat No. taken	BIIT
3	2.4×10^6	1/2	0/+	0.1×10^6	2	+
4	8.8×10^6	3/4	0/0	2.1×10^6	1	+
5	1.8×10^6	1	0	2.0×10^6	2	+
6	8.8×10^6	3	0	5.2×10^6	3	+
7	9.0×10^6	4	+	8.5×10^6	2	+
8	9.0×10^6	1	+	9.0×10^6	1	+
9	10.0×10^6	3	0	10.0×10^6	3	+
10	10.0×10^6	4	0			
11	9.9×10^6	1	+	10.0×10^6	1	+
12	10.7×10^6	3	+			
14	5.6×10^6	4	+	7.7×10^6	1	+
15	9.3×10^6	1	+			

b) *In vivo* mixtures

In a series of rats both clones were injected together into the same rat in equal amounts and grown as mixed populations. These resulting mixtures were tested taking blood by means of sterile heart puncture using the same rats several times (Table 6). The growing populations were tested from ID 3 to ID 15. Concurrently, STIB 350 was grown separately and tested as a control. These controls were consistently positive.

Table 6 shows that he BIIT with the mixed populations gave inconsistent results. For example the parasites grown in rat No. 4 were neutralized by the BIIT at ID 4 and ID 9, whereas at ID 7 and ID 14 the tested populations gave positive BIIT results. In this experiment, proportions of *T. b. brucei* and *T. b. rhodesiense* were unknown, because the growth rates of STIB 348 and STIB 350 were not known when the two clones were grown together in the same animal. These results could reflect the natural characteristics of newly isolated uncloned strains from game and other reservoir animals, which may contain mixed populations of *T b. brucei* and *T. b. rhodesiense.*

6. Special series of tests with STIB 348

In a series of 37 tests, infected blood was tested simultaneously in several variations of the standard BIIT (see also Material and Methods). The tested number of trypanosomes ranged between 10^6 and

10^7. Parasites were taken from different peaks of parasitaemia (1–5) and from several successive passages.

A. The trypanosomes were mixed with the human plasma and immediately injected into the test rats (without *in vitro* incubation).

Out of 37 tests, 23 were positive and 14 were negative. There was a significant correlation between the test results and the number of tested parasites and the degree of pleomorphism (comparison of the number of incubated trypanosomes giving a positive test result with the number of parasites giving a negative result by means of the U-test by MANN & WHITNEY). The corresponding probabilities for the different pleomorphic forms were:

slender forms:	$2\alpha \leqq 0.001$
intermediate forms:	$2\alpha \leqq 0.01$
stumpy forms:	$2\alpha \leqq 0.05$

Parasites from first peak populations had a higher tendency to give negative results than parasites from successive peaks as revealed with the Fourfold Table Test ($2\alpha \leqq 0.002$).

B. Aliquots of trypanosomes were tested simultaneously with the normal BIIT in parallel with A. In this series, 13 tests were positive and 24 tests were negative. As in A there was a significant correlation between the corresponding number of tested trypanosomes and the corresponding results (U-Test):

slender forms:	$2\alpha \leqq 0.001$
intermediate forms:	$2\alpha \leqq 0.01$
stumpy forms:	$2\alpha \leqq 0.001$

15 tests were carried out with trypanosomes from first peak forming populations. They all revealed negative results, while tested parasites from successive peaks gave 9 negative results and 13 positive results. This tendency for negative results of tested parasites from first peaks in this particular test series was checked as in A with the Fourfold Table Test and was significant also ($2\alpha \leqq 0.002$).

C. When the plasma was removed after the 5 hours *in vitro* incubation and the trypanosomes were subsequently injected with ESG or saline into the test rats, all test animals were infected in these 37 tests. This means that the neutralizing action of the human plasma during the *in vitro* phase of 5 hours was not complete even with small numbers of trypanosomes (10^6).

D. 14 normal BIITs were carried out with the "used" and removed plasma from C. 4 tests were positive and 10 tests were negative. In-

cubated trypanosomes from first-peak-forming populations revealed 7 negative tests and no positive tests. The analysis of these test results showed no significant decrease of neutralizing activity of the human plasma due to successive use (absorption of neutralizing factor) or to the different numbers of incubated parasites.

E. In this test series the procedure was carried out as in C but immediately followed by an additional injection of 1 ml of either fresh or "used" plasma. All these tests were consistently negative, showing that it is the *in vivo* action of the human plasma in the test animals which is essential and not the action *in vitro*.

Discussion

The number of reservoir animals for *T. b. rhodesiense* was increased by three additional game animals, namely Coke's hartebeest, lion and hyaena. In how far carnivores like lions and hyaenas do play an active role for the transmission of human sleeping sickness is difficult to discuss at the moment. The relationship between tsetse flies, trypanosomes and carnivores needs further investigations including the identification of bloodmeals and infection rates of tsetse flies living close to these game animals. However, it is well known that carnivores may get infected when eating trypanosome infected prey (MOLOO et al., 1973). As for the question, whether the tsetse flies being often found close by the hyaena in culverts actually feed on this particular host, this will be investigated in a forthcoming survey.

The serum samples from the volunteers collected at different intervals during the course of investigations in Homa Bay presented an interesting material for studies of the antibody response to the inoculation of *T. brucei*-subgroup trypanosomes. Preliminary attempts to use the modified IFT according to LATIF & ADAM (1973) failed because of inconsistent results which revealed no differentiation on titer-bases between *T. b. brucei* and *T. b. rhodesiense*. Therefore aceton-fixed smears of trypanosome bloodstream forms were used for the IFT modified by SUTER & FRICKER (1972). Generally the reciprocal antibody titers were initially, after the inoculation, relatively low and followed by minor increases which reached in no volunteer the comparatively higher titers measured with the serum of the two sleeping sickness patients.

The characterization of the *T. brucei*-subgroup strains isolated during the two surveys was the major aim of the work presented in this paper, i.e. the differentiation of *T. b. rhodesiense* and *T. b. brucei*. Until recently the only means of making the distinction has been the inoculation of the parasites into human volunteers, for only *T. b. rho-*

desiense infects man. RICKMANN & ROBSON (1970 a/b) described a test (BIIT) which made it possible to differentiate the two subgroup species without using human volunteers. This test is based on the neutralizing action of human blood on *T. b. brucei,* which can be very efficient (RICKMANN & ROBSON, 1972) in giving consistent results. When our original isolated strains were tested with the BIIT, we mostly obtained inconsistent results. Other authors (MWAMBU & MAYENDE, 1971; TARGETT & WILSON, 1973) also reported inconsistent BIIT results. In order to investigate the validity of the test we combined the BIIT with the inoculation into volunteers using original strains and their cloned derivatives. The results with parallel BIIT and volunteers corresponded in 8 cases out of 9, i.e. in one case the volunteer showed no parasitaemia while the parallel BIIT was positive. It may be that this particular cloned strain (STIB 346) is of border-line infectivity to man, for the non-infectivity for man is not determined by tests on a single volunteer only. A similar result was obtained by BAKER & MCCONNELL (1973) when they tested the Gambela V strain.

Retests with 3 clones gave consistent positive results with STIB 350 and STIB 349. The latter was once negative in the first test of the series which corresponds to the results in Homa Bay. It well may be that cryopreservation and thawing affect the infectivity of strains to man. The third clone (STIB 348) was 16 times negative in a series of 20 tests. When the trypanosomes of 3 positive test rats were retested as soon as the parasitaemia reached the first peak, the BIITs were negative. It is interesting that in all cases, when trypanosomes (STIB 348) of this particular first peak-forming populations were tested the BIITs were negative.

The tests with modified BIITs with STIB 348 showed that whenever the trypanosomes after the *in vitro* incubation of 5 hours were freed of human serum by centrifugation, the resuspended parasites in ESG were always infective. VAN MEIRVENNE et al. (1973) reported a similar result with shortly incubated trypanosomes. In contrast to their results, small numbers (10^6) of STIB 348 also retained their infectivity after 5 hours of incubation. The absorption of the trypanocidal factor could not be demonstrated in our experiments. It might well be that the neutralizing effect of human serum on *T. b. brucei* is of enzymatic origin which is mainly active during the *in vivo* part of the test. AARONOVITCH & TERRY (1972) concluded that the trypanocidal factor is an immunomacroglobulin (IgM) while HAWKING et al. (1973) and HAWKING (1973) stated that two substances are involved which are α_2 macroglobulins.

As for the results we obtained with artificial mixtures of known *T. b. rhodesiense* and *T. b. brucei* clones it was interesting to see that the BIIT results were inconsistent. RICKMANN & ROBSON (1974) tested

a mixed infection in a calf and also got inconsistent results. This may reflect the natural state of this trypanosome sub-species in its reservoir host in the wild. It is well possible that not only these two sub-species show an "out-of-phase periodicity" (RICKMANN & ROBSON, 1974) but that also other trypanosome species are involved in this changing pattern of the parasitaemia in the blood of hosts with mixed infections. One hyaena when darted twice in 1971 showed at the first inspection a mixture of *T. brucei*-subgroup and *T. congolense* while at the following check only *T. congolense* was found (GEIGY et al., 1973a). It is essential to get as much clones as possible from the original isolated and non preserved strains in order to obtain possible different genotypes which then have to be investigated for their attributes.

When freshly isolated strains of *T. brucei*-subgroup are investigated with the BIIT the original populations as well as clones should be tested over several passages. Only trypanosomes from the initial growing population (until to the first parasitaemia peak) should be taken. The incubated number of trypanosomes should not be higher than 10^6 cells. Instead of one test animal one better takes three mice per test.

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Zusammenfassung

Verschiedene aus Wildtieren isolierte Stämme der *T. brucei*-Gruppe sind dem Blut-Inkubations-Infektivitätstest (BIIT) unterworfen sowie auf menschlichen Freiwilligen ausgetestet worden. Bei diesen Stämmen handelte es sich um Direkt-

Isolate bzw. deren geklonte Derivate. Auf Freiwilligen getestete Klone sind für modifizierte BIIT verwendet worden, um die Zuverlässigkeit des Blut-Inkubations-Infektivitätstestes zu prüfen. Inokulationen verschiedener Trypanosomenstämme erzeugten Parasitämie durch Isolate von Löwe, Hyäne und Kuhantilope *(Alcelaphus buselaphus)*. Bei den Freiwilligen wurde der Verlauf der Antikörper-Titer mit der Indirekten Immunfluoreszenzmethode (IFT) ermittelt. Der BIIT erwies sich als zuverlässig (stets positiv) für geklonte *T. b. rhodesiense*. Beim Testen von *T. b. brucei* ergab sich eine Abhängigkeit der BIIT-Resultate von der Anzahl der getesteten Trypanosomen und deren Herkunft aus verschiedenen Stadien der Parasitaemie. Es zeigte sich ferner, dass der *in vivo*-Anteil des BIIT ausschlaggebend für die vollständige Neutralisierung von *T. b. brucei* durch Menschenplasma ist. Experimentelle Mischinfektionen von *T. b. rhodesiense* und *T. b. brucei* in Ratten gaben unterschiedliche BIIT-Resultate. Demgegenüber hingen die Resultate von *in vitro*-Mischungen beider Arten direkt von der Inkubation vom Anteil an *T. b. rhodesiense* ab; bis zu 20% waren sie negativ, höhere Konzentrationen stets positiv.

Résumé

Diverses souches de *T. brucei*-subgroup isolées en brousse à partir d'animaux sauvages ont été soumises au "Blood Incubation Infectivity Test" (BIIT), ainsi qu'à des essais sur volontaires humains. Il s'agissait de souches isolées directement ou dérivées de clônes. Ces clônes testés sur volontaires ont été utilisés pour des BIIT modifiés, afin d'examiner la fiabilité de ce test. Les souches isolées d'un Lion, d'un Hyène et d'un Kongoni *(Alcelaphus buselaphus)* ont provoqué une parasitémie chez les volontaires. Les modifications des taux d'anticorps survenant au cours d'une infection de volontaire ont été mis en évidence par la méthode d'immunofluorescence indirecte. Le BIIT s'est révélé constamment positif avec les clônes de *T. b. rhodesiense*. En testant *T. b. brucei* on a observé que les résultats du BIIT dépendaient du nombre des trypanosomes, ainsi que du stade de la parasitémie au moment du prélèvement. On pense en outre que la réaction de neutralisation de *T. b. brucei* se complète *in vivo*. Les tests effectués à partir d'infections mixtes à *T. b. rhodesiense* et *T. b. brucei* ont donné des résultats variables. En mélangeant les souches *in vitro* avant l'incubation, le BIIT n'était positif que lorsque la proportion de *T. b. rhodesiense* dépassait 20%.