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Use of procyclic trypanosomes for detection of antibodies in sera from vervet monkeys infected with *Trypanosoma rhodesiense*: an immunodiagnostic test for African sleeping sickness

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Summary

Uncoated procyclic culture forms of African trypanosomes were used in immunofluorescence and simple agglutination assays to detect antibodies in the sera of vervet monkeys infected with *T. b. rhodesiense*. Antibodies to procyclic surface antigens were found in sera from animals with active, untreated infections or sera taken soon after treatment with trypanocidal drugs. The antibodies were detectable within 7 days of infection. No specific antibodies were detected in sera prior to infection or long after drug cure. The results indicate that antigens expressed on the surface of procyclic culture forms of *T. brucei* spp. are useful for the detection of antibodies produced in response to infection with *T. b. rhodesiense* and may allow the development of a simple immunodiagnostic test for African sleeping sickness. In addition, the use of a form of the trypanosome of a different differentiation state from the infecting organism illustrates the utility of this approach for detection of antibodies to common antigens.

Key words: protozoan parasites; procyclic culture forms; immunodiagnosis; *Trypanosoma brucei rhodesiense*.

Introduction

Diagnosis of African sleeping sickness in humans is usually performed by parasitological techniques involving microscopical detection of parasites (WHO, 1979). Such methods are often not definitive as the number of parasites may not be sufficiently high for detection. Detection of specific antibodies in

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human sera, although not necessarily indicative of active infection, has proven to be extremely useful for diagnosis of sleeping sickness caused by *T. b. gambiense* (WHO, 1981). The test most successfully used for antibody detection in this form of sleeping sickness is the Card Agglutination Trypanosomiasis Test (C.A.T.T.) which depends on agglutination of fixed, stained, bloodstream *T. b. gambiense* bearing a particular variant surface glycoprotein (VSG) (Magnus et al., 1978). Most humans parasitized with *T. b. gambiense* produce antibodies to this VSG, indicating that trypanosomes of this variable antigenic type appear often in an infected human population. The C.A.T.T. test cannot be used for diagnosis of infections caused by *T. b. rhodesiense* since antibodies to the particular VSG used are not produced in these infections, nor has a similarly frequently occurring variable antigenic type been found with *T. b. rhodesiense* (WHO, 1981).

In this paper we report the use of procyclic culture forms of *T. brucei* spp. for detection of antibodies produced in response to infections with *T. b. rhodesiense*. We used vervet monkeys and *T. b. rhodesiense* EATRO 1989, a model which, with respect to the course of the disease and to parasitological, haematological, immunological and histological parameters, closely resembles human infections with *T. b. rhodesiense* (Schmidt and Sayer, 1982a). Since it is known that procyclic culture forms and bloodstream forms of African trypanosomes share some common antigens (reviewed by Shapiro and Pearson, 1986), we used procyclic culture forms (which are devoid of surface VSG) to detect antibodies made to such antigens during infection.

Materials and Methods

Parasites. The organisms used for infection were *T. b. rhodesiense* KETRI 2537 or KETRI 2545, derived from EATRO 1989 (Fink and Schmidt, 1979), which in vervet monkeys causes chronic disease (Schmidt and Sayer, 1982b) and late phase encephalitis (Schmidt and Sayer, 1982b), both characteristic of human sleeping sickness caused by *T. b. rhodesiense*. Procyclic culture forms were established from cloned bloodstream populations of *T. b. rhodesiense* ViTat 1.1 (Richardson et al., 1986) using the methods of Brun and Schönenberger (1979) and were maintained in SDM-79 medium as described by the latter authors. Promastigotes of a clone of *L. major* NIH (Seidman strain) were grown in the same medium.

Monkeys, infections and treatment. Vervet monkeys (*Cercopithecus aethiops*) were used throughout. These monkeys were maintained at KETRI for studies under the UNDP/World Bank/WHO Special Programme on the Chemotherapy of human African trypanosomiasis.

Prior to infection, all monkeys had undergone quarantine of not less than three months duration. During this time the monkeys became adapted to cage life and the presence of humans. They were examined clinically and repeated checks made for evidence of disease, including tuberculosis, intestinal protozoa and helminths and various viral diseases, including Marburg disease, Rift Valley Fever, Ebola and Congo haemorrhagic fever.

The monkeys were infected by intravenous injection of 10^3 *T. b. rhodesiense* suspended in phosphate buffered saline containing 1% glucose (PSG). Capillary blood samples were taken daily from the ear tip and examined for trypanosomes. Every two weeks (and occasionally, weekly) after infection the monkeys were anaesthetised and examined clinically; blood was collected for serology and haematology; electrocardiography was performed and cerebrospinal fluid collected.

Between days 28–42 of infection, the monkeys were treated with trypanocidal drugs. The drugs used and the time of serum sampling since drug treatment are indicated in the results. A number of monkeys required drug treatment a second or third time following relapse. Other monkeys were not treated until signs of encephalitits became apparent four to six months after infection. Serum and CSP samples were collected at various intervals and stored at –20 °C.

Quantitation of IgM and IgG. Levels of IgM and IgG in vervet monkey sera were determined by radial immunodiffusion (Mancini, 1965) using commercially prepared plates for measurement of human IgM and IgG (Diffugen, TAGO, Inc., Burlingame, California). Human IgM and IgG were used as standards, thus the test may not be quantitative for monkey immunoglobulins. Nevertheless, relative immunoglobulin levels were accurately determined. Some sera were diluted 1/5 in saline in order to obtain accurate readings.

Immunofluorescence. Antibodies specific for the surface of *T. b. rhodesiense* ViTat 1.1 procyclic culture forms were measured by indirect immunofluorescence. Procyclic trypanosomes or *Leishmania* promastigotes were centrifuged (1000 × *g*, 15 min, 4 °C) from culture medium, washed once (1000 × *g*, 15 min, 4 °C) with PSG and 10% heat-inactivated foetal bovine serum (FBS) and adjusted to 1×10^8 cells/ml in PSG/10% FCS. To 15×75 mm glass test tubes were added 20 µl of parasite suspension and 50 µl of human or vervet sera diluted in PSG/10% FBS. The tubes were incubated on ice for 1 h, washed three times with 2.0 ml of PSG/10% FBS and 20 µl of fluoresceinated, affinity-purified goat anti-human IgM or IgG (TAGO, Inc., Burlingname, California) were added. After 1 h incubation on ice, the parasites were washed 3 times as above, resuspended in 50 µl of PSG/10% FBS and 10 µl amounts used to prepare slides for microscopy. Immunofluorescence was observed using a Zeiss Standard binocular microscope fitted with an epi-fluorescence attachment and a Zeiss Neofluor 63/1.25 oil immersion objective.

Agglutination test. Antibodies to surface antigens of procyclic culture forms of trypanosomes or promastigote culture forms of *Leishmania* parasites were measured in simple agglutination assays. The organisms were grown to log-phase, washed twice with PSG/10% FBS by centrifugation at 1000 × *g*, 10 min at room temperature and adjusted to 5×10^7/ml in PSG/10% FBS. Within 5 min, 25 µl amounts of the suspension were added to flat-bottom microtitration plate wells (Titertek, 96 well, Flow laboratories) containing 25 µl of sera dilutions (1/10–1/320 in doubling dilutions). Control wells contained PSG/10% FBS or sera from mice infected with *T. b. rhodesiense* ViTat 1.1 bloodstream forms, sera from humans with *T. b. gambiense* infections and sera from uninfected north Americans. The plates were shaken for 10 sec to mix their contents and incubated for 5 min at 26 °C. Agglutination was observed using an inverted mircoscope at 200× magnification and scored as 4+ (total agglutination – all organisms in one or two large aggregates) to 1+ (some agglutination – organisms in one or two large aggregates) to 1+ (some agglutination – organisms in small aggregates of 3–5 organisms). Since, within about 20 min, small amounts of aggregates were often seen in the PSG/10% FBS controls, it was important to perform the test quickly and to refer periodically to the negative controls.

Results

Sera from a total of twelve vervet monkeys were prepared prior to infection, during infection with *T. b. rhodesiense* or after treatment of the infected monkeys with trypanocidal drugs. Sera were numbered according to the number of the monkey and the number of bleeds, e.g. serum 128–1 is from monkey no. 128, bleed no. 1. For ease of discussion, sera (including controls), were also numbered consecutively, from 1 to 49. Total IgM and IgG levels and specific Ig titres in immunofluorescence and agglutination assays using living procyclic trypanosomes were determined for each serum sample. The results are shown in Table 1.

Agglutination of procyclic culture forms of *T. b. rhodesiense* ViTat 1.1 correlated with the status of infection. Thus, no agglutination was observed with

Table 1. Measurement[a] of anti-procyclic surface antibodies in vervet monkey sera before and during infection with *T. b. rhodesiense* and at various times after treatment with trypanocidal drugs

Serum No.	Monkey No.	No. of days since infection[b]	No. of days since treatment[c] (drug in brackets)	Remarks	Ig levels (mg/ml)		Immunofluorescence titre vs. *T. b. rhodesiense*		Agglutination titre vs. *T. b. rhodesiense*
					IgM	IgG	IgM	IgG	
1	34	940	859 (Mel. B.)		12.0	51.5	–	–	–
2	35	978	933 (Suramin/M. K. 436)		3.3	16.0	–	–	–
3	47-1	0	0	Preinfection	N.D.	N.D.	N.D.	N.D.	–
4	47-2	7	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	–
5	47-3	14	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/80
6	47-4	28	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/160
7	47-5	56	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	>1/320
8	49-1	0	0	Preinfection	N.D.	N.D.	N.D.	N.D.	1/320
9	49-2	7	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/20
10	49-3	14	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/160
11	49-4	28	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/80
12	49-5	56	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/80
13	61	875	793 (Berenil)	528 days since last pos. (CSP)	1.9	8.0	–	–	–
14	85–1	392	130 (Suramin/M. K. 436)	29 days since last pos. (blood, CSP) (2nd treatment)	N.D.	N.D.	N.D.	N.D.	–
15	85-2	605	343 (Suramin/M. K. 436)	213 days since last pos. (blood) (2nd treatment)	5.6	10.7	–	–	–
16	94-1	0	0	Preinfection	N.D.	N.D.	N.D.	N.D.	–
17	94-2	28	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	>1/320
18	94-3	352	158 (Suramin/TS. 88)	11 days since last pos. (3rd treatment)	77.5	30.0	1/100	1/5	1/320
19	96-1	0	0	Preinfection	2.3	15.3	1/10	–	1/120
20	96-2	14	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/40
21	96-3	28	0	Pretreatment	35.0	30.0	1/100	–	>1/320
22	96-4	58	14 (Suramin/TS. 88)	1st and only treatment	N.D.	N.D.	N.D.	N.D.	1/80
23	96-5	71	27 (Suramin/TS. 88)	1st and only treatment	11.5	26.5	1/10	–	1/40
24	96-6	202	158 (Suramin/TS. 88)	1st and only treatment	1.6	14.5	1/10	–	–
25	113	0	0	Preinfection	1.6	16.0	1/10	1/10	1/20
26	115-1	0	0	Preinfection	2.1	32.5	–	1/10	1/80
27	115-2	16	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/40
28	115-3	33	0	Pretreatment	21.0	31.0	1/10	–	1/160

Table 1 (continued)

Serum No.	Monkey No.	No. of days since infection[b]	No. of days since treatment[c] (drug in brackets)	Remarks	Ig levels (mg/ml)		Immunofluorescence titre vs. *T. b. rhodesiense*		Agglutination titre vs. *T. b. rhodesiense*
					IgM	IgG	IgM	IgG	
29	115-4	56	12 (TS. 88)	1st treatment (relapsed later)	N.D.	N.D.	N.D.	N.D.	1/320
30	115-5	70	26 (TS. 88)	1st treatment (relapsed later)	N.D.	N.D.	N.D.	N.D.	1/160
31	115-6	98	54 (TS. 88)	1st treatment (relapsed later)	24.0	51.5	1/10	1/10	1/320
32	124-1	0	0	Preinfection	2.6	30.0	1/10	1/10	–
33	124-2	16	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/40
34	124-3	33	0	Pretreatment	19.2	23.5	–	–	1/320
35	124-4	352	105 (TS. 88)	3rd treatment (Suramin/TS. 88, 1st + 2nd)	4.1	32.5	–	–	–
36	128-1	0	0	Preinfection	4.8	36.5	1/10	–	1/10
37	128-2	16	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/320
38	128-3	33	0	Pretreatment	24.0	31.0	1/100	1/5	1/80
39	128-4	41	0	Pretreatment	N.D.	N.D.	N.D.	N.D.	1/80
40	128-5	352	278 (Suramin/TS. 88)	132 days since last pos. blood (2nd treatment)	26.0	21.5	1/10	1/10	1/320
41	128-6	366	292 (Suramin/TS. 88)	146 days since last pos. blood	N.D.	N.D.	N.D.	N.D.	–
42	96-CSF	71	27 (Suramin/TS. 88)		N.D.	N.D.	N.D.	N.D.	–
43	115-CSF	33	0		N.D.	N.D.	N.D.	N.D.	–
44	neg.	0	0	Human serum (north American)	N.D.	N.D.	–	–	–
45	neg.	0	0	Human serum (north American)	N.D.	N.D.	–	–	–
46	pos.	unknown	7 (Arsobal)	Human serum (Ivory Coast)	N.D.	N.D.	1/100	1/100	1/320
47	pos.	unkown	7 (Arsobal)	Human serum (Ivory Coast)	N.D.	N.D.	1/100	1/100	1/320
48	neg.	0	0	Mouse serum (uninfected)	N.D.	N.D.	–	–	–
49	pos.	21	0	Mouse serum (infected with *T. b. rhodesiense*)	N.D.	N.D.	–	–	1/320

[a] each serum was tested twice in each assay each time by a different investigator

[b] animals were infected with *T. b. rhodesiense* KETRI 2537 except No. 61 which was infected with *T. b. rhodesiense* KETRI 2545

[c] drug treatment

ND = Not done

sera taken from monkeys prior to infection (sera nos. 3, 8, 16, 32) or sera taken long after drug treatment (days 105–933; sera nos. 1, 2, 13, 15, 24, 35, 41). In addition, no agglutination was seen with one serum (no. 4) taken 7 days after infection, with one serum taken 14 days after drug treatment (no. 14) or with two CSF samples, one taken during an active infection (33 days) and one taken 27 days after drug treatment of the animal (nos. 43 and 42, respectively).

Low agglutination titres (1/10–1/80) were found with sera from early untreated infections (days 7–56; sera nos. 5, 9, 11, 12, 20, 27, 28, 33, 38, 39) or with sera taken 14 and 27 days after drug treatment of infected monkeys (sera nos. 22 and 23, respectively). Preinfection sera (nos. 19, 25, 26, 36) showed titres of 1/20, 1/20, 1/80 and 1/10, respectively.

High agglutination titres (1/160–1/320) were associated with active untreated infections (sera nos. 6, 7, 10, 17, 21, 28, 34 and 37) and were observed as early as day 14 of infection (serum 10). High titres were also seen in two monkeys after drug treatment (sera nos. 18, 29, 30 and 31). Both of these animals later showed relapse infections. Only in one case (serum no. 40) was there a high agglutination titre long after drug treatment (132 days since parasites were last observed) and this animal became negative 24 days later.

Control sera from uninfected north American humans (sera 44 and 45) or from normal uninfected BALB/c mice (serum 48) did not cause agglutination of procyclic trypanosomes whereas sera from west African humans with *T. b. gambiense* infections (sera nos. 46 and 47), or from BALB/c mice infected for 14 days with *T. b. rhodesiense* ViTat 1.1 bloodstream form trypanosomes (serum no. 49), had agglutination titres of 1/320. All sera from humans (control sera) and from the vervet monkeys (test sera) were tested in agglutination assays with *Leishmania major* culture form promastigotes. No agglutination titres above 1/10 were found (data not shown).

Immunofluorescence reactions showed that specific IgM levels reflected the agglutination titres in some but not all sera. Specific IgG levels measured by immunofluorescence on living *T. b. rhodesiense* procyclics did not correlate with agglutination titres. None of the sera tested showed any reaction with the surface of *L. major* promastigotes when tested by immunofluorescence.

Table 2. Total IgM and IgG levels[a] in vervet monkey sera before and during infection with *T. b. rhodesiense* and after drug treatment

	Sera	IgM (Mean±S.D.)	IgG (Mean±S.D.)
Preinfection	n = 5	2.7±0.4[b]	26.1±9.1
Infected	n = 4	24.8±7.1	28.9±3.6
Drug treated	n = 10	16.8±23.1	26.3±15.5

[a] Determined by radial immunodiffusion
[b] mg/ml

Total IgM and IgG levels were determined by radial immunodiffusion using anti-human Ig immunodiffusion plates and human Ig as standards. The results are summarized in Table 2. IgM levels increased dramatically in infected animals and remained elevated after drug treatment. The total IgM levels thus correlated well with the agglutination titres. Mean IgG levels changed little during infection and after drug treatment although a few animals showed increased IgG levels. There was no obvious group correlation of IgG levels with agglutination titres.

Discussion

Procyclic forms of African trypanosomes share antigens with bloodstream forms (reviewed by Shapiro and Pearson, 1986). Thus it is not surprising that we detected antibodies to procyclic surface antigens in sera from mice, vervet monkeys and humans infected with bloodstream stages of the parasites. Our data showed that we were able to detect anti-procyclic antibodies in vervet monkey sera as early as 7 days after infection with *T. b. rhodesiense.*

Indeed, all sera from monkeys with active, untreated infections (except one 7 day infection serum – no. 4) gave good agglutination titres as did sera taken soon after treatment of infected monkeys with trypanocidal drugs. Thus 14 and 27 days after drug treatment, animal no. 96 (sera nos. 22 and 23) showed titres of 1/80 and 1/40, respectively. Animal 94 (serum 18) showed a titre of 1/320 158 days after final drug treatment but this animal was treated three times with suramin/TS 88 and the serum was taken only 11 days after parasites were last seen in the blood (147 days after the 3rd drug treatment). Sera from animal 115 (sera nos. 29, 30 and 31) taken 12, 26 and 54 days after drug treatment had high agglutination titres. This animal was treated only once with drug and subsequently relapsed.

In all cases but two (serum 18 – see above, and serum 40), sera taken long after successful drug treatment showed no agglutination. Thus, as early as 14 days after treatment with trypanocidal drugs and certainly by 146 days (serum no. 41 taken 24 days after serum 40) agglutination titres dropped to zero.

The only false positive agglutination reaction was with preinfection serum no. 26 which showed a titre of 1/80. All other preinfection or negative control sera showed titres of 0, 1/10 or 1/20.

It is clear therefore, that simple agglutination of procyclic culture forms of trypanosomes can give useful information regarding the infection status of monkeys infected with *T. b. rhodesiense* organisms. Indeed, positive agglutination reactions were found with 6/6 of animals with active trypanosome infections and in animal 96 (sera 22 and 23) taken early after successful drug cure. Negative agglutination reactions were seen with all sera taken long after drug cure except those discussed above which subsequently showed relapse infections.

That the agglutination reactions and immunofluorescence detected trypanosome-specific antibodies was shown by the negative reactions obtained using *Leishmania major* as antigen. Agglutination of procyclic culture forms of all three *T. brucei* subspecies (*T. b. brucei, T. b. rhodesiense,* and *T. b. gambiense*) occurred with all of several sera tested from infected vervet monkeys, humans and mice (data not shown). This indicated that antigens shared between both bloodstream and procyclic stages of the *T. brucei* subspecies may allow detection of antibodies produced in human infections with both *T. b. gambiense* and *T. b. rhodesiense* and in cattle infections with *T. b. brucei.* Diagnosis of *T. b. gambiense* infections in patients from the Ivory Coast has been achieved using the simple procyclic agglutination test described here (Gardiner et al., in preparation) and limited results obtained with patients from Western Kenya indicate that diagnosis of sleeping sickness caused by *T. b. rhodesiense* may also be possible.

The potential utility of procyclic trypanosome surface antigens in a simple immunodiagnostic test for African sleeping sickness will only be known after extensive testing of well documented sera from sleeping sickness patients and from control patients in endemic areas. Such testing is underway. In addition, attempts are being made to identify and biochemically characterize the relevant antigens to aid in the development of a stable, simple test for field use.

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