Failure of trypanosomal membrane antigens to induce protection against tsetse-transmitted "Trypanosoma vivax" or "T. brucei" in goats and rabbits

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Failure of trypanosomal membrane antigens to induce protection against tsetse-transmitted *Trypanosoma vivax* or *T. brucei* in goats and rabbits

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Summary

A purified protein, relative molecular weight 83 kilodalton (kD), and plasma membranes from *Trypanosoma brucei* were tested as potential vaccines against tsetse-transmitted *T. vivax* and *T. brucei* in goats and rabbits. The 83 kD protein was found in lysates of all clones of *T. brucei* examined, as well as in lysates of *T. vivax*, *T. congolense* and *T. rhodesiense*. Rabbits and goats were immunized with various amounts of antigen in Freund's complete adjuvant and boosted twice with antigen in Freund's incomplete adjuvant. Two weeks after the last inoculation, the goats were challenged with *T. vivax*-infected and the rabbits with *T. brucei*-infected *Glossina morsitans morsitans*. Although high antibody levels were detected in all the animals immunized with either antigen as measured by radioimmunoassay and immunodiffusion, they became infected and the course of disease was the same as that in unimmunized controls.

Key words: *T. vivax; T. brucei;* plasma membrane antigen; immunization; cyclical challenge; protection.

Introduction

Trypanosomiasis has been a major constraint to livestock development in vast areas of tropical Africa where the disease is largely transmitted by tsetse. Control of the vector and the use of curative or prophylactic drugs have been the conventional measures used against this disease. The former method has had little success in view of the enormous distribution of the tsetse (Ford, 1963), while the latter can be applied only to a limited scale for reasons of economy

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and logistics. In recent years, much greater interest has been directed towards the development of a vaccine against African trypanosomiasis as a third control measure (Murray et al., 1979, 1980). Studies into the feasibility of vaccinating livestock have shown some promise. However, so far the results have been of scientific interest but of no practical value. The characteristic antigenic variation expressed in the glycoprotein surface coat of the mammalian bloodstream trypanosomes (Vickerman, 1974a) and the heterogeneity of metacyclic variable antigen types (Barry et al., 1979; Nantulya et al., 1983) have hindered development of a vaccine against trypanosomiasis.

It is known that some other trypanosome components remain unchanged during the course of infection and are responsible for cross-reactions among strains in various serological tests (Gray, 1967; De Raadt, 1974). Antibodies against these non-variable antigens, however, do not confer protection against tsetse transmitted trypanosomes (Doyle, 1977). Recently, Tetley et al. (1981) reported that metacyclics of *T. vivax* may not have a surface coat; hence common membrane antigens might be exposed to the host immune system in the early stage of infection. This led to the speculation that the mechanism of antigenic variation in this species may be different from that of other salivarian trypanosomes and raised the hope that vaccination against *T. vivax* might be a simpler task than immunization against *T. brucei* or *T. congolense* which have coated metacyclics. In this paper we report studies in which a common membrane protein present in the bloodstream forms of *T. brucei*, *T. congolense* and *T. vivax*, and purified plasma membranes from *T. brucei* were tested as potential vaccine materials in susceptible goats and rabbits.

Materials and Methods

Animals

Adult East African Galla cross-bred goats used were from tsetse free areas in Kenya. Prior to use they were screened for antibodies against trypanosomes by indirect immunofluorescence (Wilson, 1969) and found negative. New Zealand white rabbits were obtained from the ILRAD colony.

Parasites

Trypanosoma brucei clones MITat 1.2 and MITat 1.52 were derived from Lump 427 (Cross, 1975); clones ILTat 1.2 and ILTat 1.3 from Lump 227 (Barbet and McGuire, 1978); and clone ILTat 2.1 was from STIB 247 (Nantulya et al., 1983). *T. rhodesiense* clone ANTat 12/X was obtained from Prof. Van Meirveinne of the Institute of Tropical Medicine, Antwerp, Belgium. *T. vivax* stock IL 417 was a derivative of Zaria Y486 (Leeflang et al., 1976).

Chemicals

L-^{35}S-methionine and ^{14}C-proteins used as standard were purchased from the Radiochemical Centre, Amersham, U.K. Films for autoradiography were no-screen NS-2T from Eastman Kodak, Rochester, N.Y., U.S.A. All other chemicals used were of analytical grade.

Polyacrylamide slab gel electrophoresis

Polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed as previously described (Rovis et al., 1978) using a gradient of acrylamide

concentration from 7.5 to 15%. For autoradiography, the gels were dried and exposed to X-ray films for different lengths of time. The relative molecular weights (Mr) of the radioactive protein bands were determined using both Coomassie Brilliant Blue stain and ^{14}C-labelled methylated standard proteins.

Immunological methods

Radioimmunoassays (RIA) were performed as previously described (Barbet and McGuire, 1978). The biosynthetic labelling of trypanosomes with ^{35}S-methionine, the lysis of labelled parasites, and immunoprecipitations with rabbit antisera in the presence of formalin fixed *Staphylococcus aureus* suspension were carried out as described by Rovis and Dube (1981). Immunodiffusion was done according to the method of Ouchterlony (1959). The indirect immunofluorescent staining on live procyclics of *T. brucei* and *T. vivax* was performed following the method described by Barbet and McGuire (1978).

Preparation of plasma membranes

Plasma membranes from *T. brucei* clone MITat 1.2 were prepared as previously described (Rovis and Baekkeskov, 1980). The preparation used for the immunization experiment was washed ten times with 10 mM Hepes-NaOH, pH 7.4. The amount of variable surface glycoprotein (Cross, 1975) present was determined by radioimmunoassay (McGuire et al., 1980); it was found to be less than 10 μg per mg of total membrane protein. The total protein content was measured according to the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Preparation and characterization of the 83 kilodalton protein

In previous work (Rovis and Baekkeskov, 1980) a major protein with apparent molecular weight of 83 kD was identified by SDS-PAGE of three subcellular fractions of *T. brucei*. Further experiments (Rovis, unpublished) showed that a protein with the same apparent molecular weight was also present in total lysates of five additional clones of *T. brucei* from Lump 427 and Lump 227; in *T. congolense* clone ILNat 2.1; in one stock of *T. vivax* (IL 417) and in one *T. rhodesiense* clone AnTat 12/X. The protein was purified from the soluble fraction obtained by fractionation of total trypanosome lysate (Fig. 1, lane 2) as previously described (Rovis and Baekkeskov, 1980). The homogenates from two unrelated clones of *T. brucei*, MITat 1.52 and ILTat 1.3 were used. The mixture of soluble trypanosomal proteins (50 mg) was separated according to size in six preparative sodium dodecyl sulphate polyacrylamide slab gels. After fixing the gels and staining in 0.2% Coomassie Brilliant Blue in methanol:acetic acid:water (4:1:6 by vol.) the 83 kD band was cut out, minced into small cubes and rinsed with water. Protein was eluted out of the gel by overnight incubation at 37 °C with shaking in a solution containing 0.05 M phosphate buffer pH 7.4, 0.1% sodium dodecyl sulphate, and 1 mM phenylmethylsulfonyl fluoride (Bray and Brownlee, 1973). The slurry was then centrifuged and cold 2.0 M KCl was added to the supernatant fluid to obtain a final concentration of 0.2 M. After 15 min on ice, the precipitate formed was collected by centrifugation at 10,000 $\times g$ for 15 min. The pellet was washed once with acetone/0.1 M HCl, twice with acetone and then dried under nitrogen. Sodium dodecyl sulphate was removed from the protein by the method described by Henderson et al. (1979) using acetone:triethylamide:acetic acid:water (85:5:5:5, by vol.) as extraction solvent. The protein was finally dissolved in 6 M guanidinium hydrochloride and dialyzed against several changes of 50 mM Tris-HCl pH 7.5, 0.2 M NaCl. The amount of recovered protein was determined by the method of Lowry et al. (1951) and the purity of the preparation was assessed by SDS-PAGE (Fig. 1, lane 3). Approximately 200 μg of purified protein were obtained from 50 mg of total starting material.

Rabbits were inoculated with the 83 kD protein purified from either clone ILTat 1.3 or MITat 1.52 according to the protocol shown in Table 1. After seven weeks from the first injection the rabbits were bled to death under anaesthesia and the sera collected.

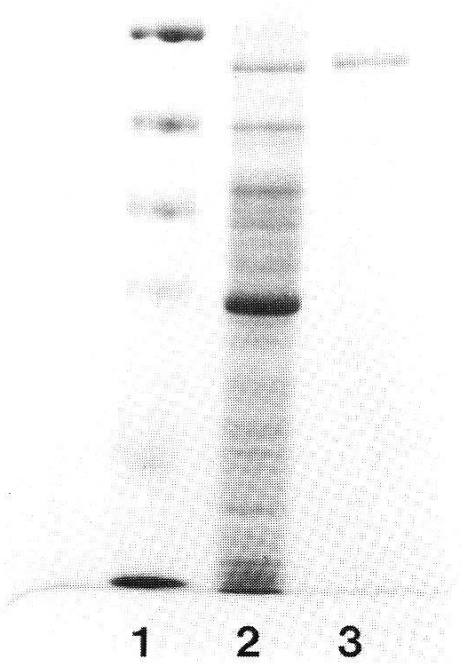

Fig. 1. Acrylamide gel electrophoresis of a lysate of *T. brucei* clone MITat 1.52 and the 83 kD protein purified from this material. Lane 1: Approximate molecular weights standard mixture. From top: Phosphorylase (92 kD), bovine serum albumin (64 kD), human IgG heavy chain (57 kD), ovalbumin (46 kD), human IgG light chain (23 kD), cytochrome b (12 kD). Lane 2: Profile of the soluble fractions of *T. brucei* clone MITat 1.52. Lane 3: The 83 kD protein purified from the lysate shown in lane 2.

Table 1. Preparation of rabbit antisera against the 83 kilodalton protein

Rabbit number	Source of 83 kD protein	Dose of inoculum (μg)		
		1st injection	2nd injection	3rd injection
11	ILTat 1.3	10	20	30
12	ILTat 1.3	20	40	60
13	MITat 1.52	25	50	100
14	MITat 1.52	50	100	200
15	MITat 1.52	100	200	300

The 83 kD protein was purified as described in Materials and Methods. First injection was in 1 ml phosphate buffered saline mixed thoroughly with 1 ml of Freund's complete adjuvant and divided among the footpads. Second and third injections were given at two-week intervals and consisted of the indicated amount of antigen mixed with 1 ml of Freund's incomplete adjuvant.

Immunization of the rabbits and goats

Three rabbits (16, 17, 18) were immunized with 10 μg of the 83 kD protein per animal in Freund's complete adjuvant and were boosted twice at two weekly intervals, each time using 20 μg, of the protein in Freund's incomplete adjuvant (Table 2). Two other rabbits (19, 20) were immu-

Table 2. Antibody levels in goats and rabbits immunized with 83 kD protein (Group I, IV, V) and plasma membranes (Group II) as determined by radioimmunoassay or double immunodiffusion in gel respectively

Group	Animal	Number	Antibody titre
I	Goat	261	10^{-3}
	Goat	262	10^{-3}
	Goat	263	10^{-3}
	Goat	264	10^{-4}
II	Goat	265	1:64
	Goat	266	1:128
	Goat	267	1:64
III (Control)	Goat	268	–
	Goat	269	–
	Goat	270	–
IV	Rabbit	16	10^{-4}
	Rabbit	17	10^{-3}
	Rabbit	18	10^{-4}
V	Rabbit	19	10^{-5}
	Rabbit	20	10^{-5}
VI (Control)	Rabbit	21	–
	Rabbit	22	–

nized similarly using higher antigen doses of 20 μg for the priming and 40 μg and 60 μg for first and second the boosting doses per rabbit respectively. Control rabbits (21, 22) received adjuvant alone following a similar time schedule. Sera collected from rabbits just before cyclical challenge were screened for antibody levels to the immunizing antigen by radioimmunoassay (Barbet and McGuire, 1978).

Four goats (261, 262, 263, 264) were immunized using a schedule similar to that for the rabbits. The priming dose was 10 μg antigen, followed by 20 μg and finally with 40 μg. Another group of 3 goats (265, 266, 267) was immunized with plasma membranes as follows: 20 μg protein as priming dose, 30 μg as first booster and 50 μg as second booster. The control group (268, 269, 270) received adjuvant alone. Antibody levels in goats 261, 262, 263 and 264 were detected by RIA, and in goats 265, 266, 267 by double immunodiffusion in gel.

Infection of tsetse

Three hundred teneral (young, unfed) *G. m. morsitans* from the ILRAD R[6] colony were fed on NMRI mice showing peak of parasitaemia with *T. brucei* ILTat 2.1. The tsetse were then fed on a rabbit every day except Sundays. On day 31 after the infected meal, the surviving tsetse were allowed to probe singly on warm slides at 38 °C. Those which showed metacyclics in saliva were used to challenge 5 immunized and 2 control rabbits: 6 infected tsetse feeds per animal. These rabbits were bled from the ear daily, and the packed red cell volume (PCV) of the samples determined; the buffy coat was also examined for parasites using the method of Woo (1971). Wet blood films were examined for parasites using a phase-contrast microscope at 400× magnification.

Four hundred teneral tsetse of the above sub-species were fed on a goat infected with *T. vivax* (IL-417) at peak of parasitaemia. These tsetse were then maintained as described previously, and on day 31 post-infected feed, the tsetse were used to challenge the 7 immunized and 3 control goats, 20 infected bites per animal. The parasitaemia and PCV were determined as described above.

Results

The antisera made against 83 kD reacted in immunodiffusion with the respective antigen as well as against total lysates or purified protein prepared from different clones (Fig. 2A and 2B). Precipitin lines were obtained also when the antisera were tested in immunodiffusion against NP-40 (0.5%) extract of *T. congolense* ILNat 2.1 and *T. vivax* IL 417 (Fig. 2C).

The immunodiffusion experiments suggested cross reactivity of the 83 kD protein with different clones of *T. brucei* and with different species of trypanosomes. In order to unequivocally prove that the protein is a common component of the three major pathogenic *Trypanosoma* species, additional experiments were carried out as follows: *T. brucei* clones MITat 1.2 and ILTat 1.1, *T. vivax* stock IL 417 and *T. congolense* clone ILNat 2.1 were metabolically labelled with ^{35}S-methionine and lysed. The radiolabelled proteins contained in the lysates were used in *Staphylococcus aureus* mediated immuno-precipitation experiments with the rabbit antisera described above. The specific precipitates and control precipitates (obtained with a pool of normal rabbit serum) were analysed by SDS-PAGE. Results of representative experiments are shown in Fig. 3. It is evident that the specific antisera raised against the purified 83 kD protein from either clone ILTat 1.3 or MITat 1.52 precipitated the same molecule from radio-labelled lysates of several unrelated *T. brucei* clones as well as from different trypanosomal species. The data indicate that the purified protein

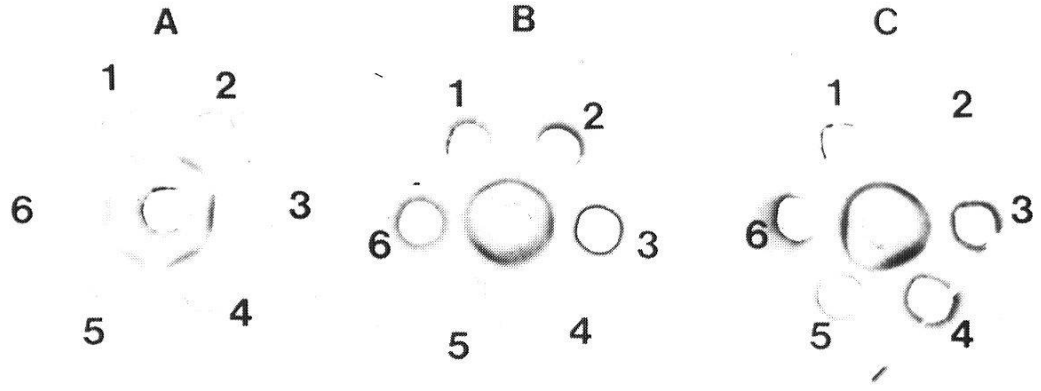

Fig. 2. Analysis by immunodiffusion, of the antisera listed in Table 1 and the different preparations of purified and crude 83 kD protein.

A: Central well: antiserum from rabbit No. 11; wells 1, 2, 3: three different preparations of purified 83 kD protein prepared from *T. brucei* clone ILTat 1.52; wells 4, 5, 6: the 83 kD protein prepared from *T. brucei* clone ILTat 1.3.

B: Central well: purified 83 kD protein from clone MITat 1.52; wells 1, 2, 3, 4: antisera from rabbits Nos. 13, 14, 15 and 16 diluted 1:20; wells 5, 6: antisera from rabbits Nos. 11 and 12.

C: Central well: NP-40 lysate of *T. brucei* clone MITat 1.2; wells 1, 2, 3: antisera from rabbits Nos. 13, 14, 15; wells 4, 5; antisera from rabbits Nos. 11 and 12; well 6: serum from rabbit No. 19 (Table 2). Unless indicated, all rabbit antisera were used at 1:10 dilution. Control rabbit sera (taken before immunization) gave no precipitin lines.

is a common antigen among these trypanosome species and a suitable candidate for the vaccination experiments undertaken in the present study. Five rabbits and 4 goats were then immunized with the 83 kD antigen as described under Material and Methods.

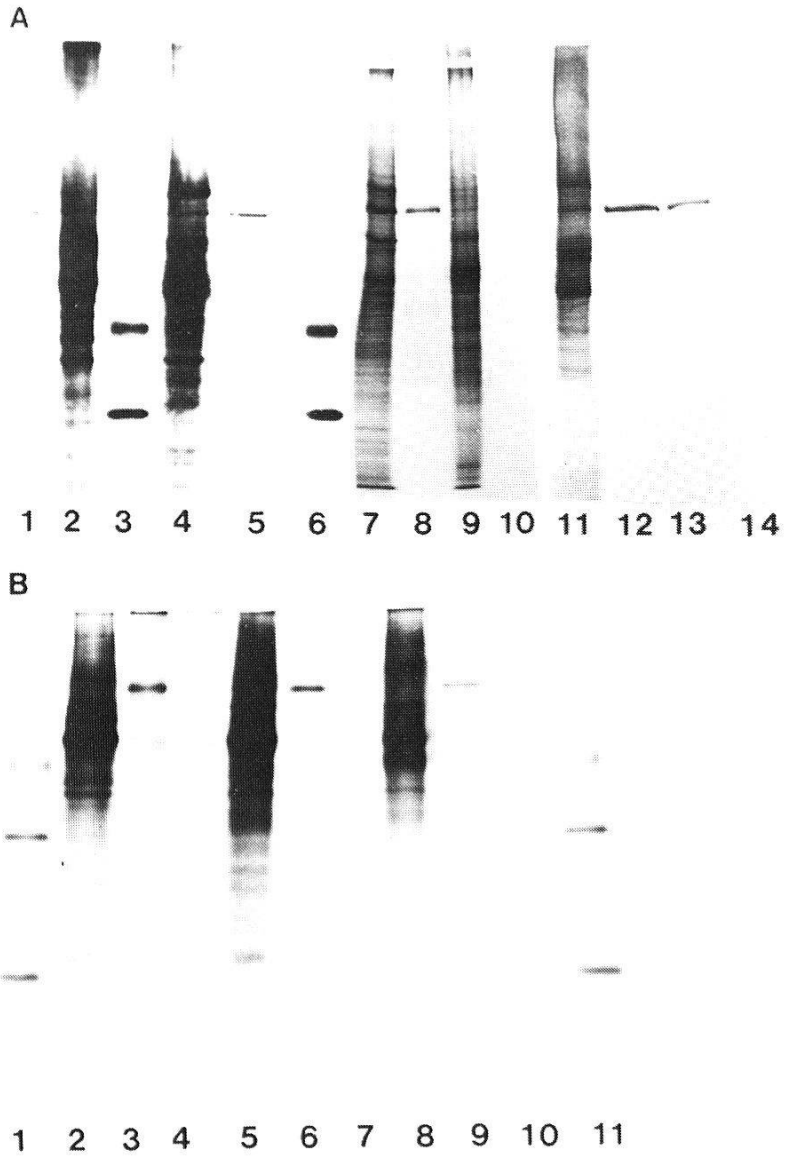

Fig. 3. Representative autoradiographs of SDS-PAGE showing the presence of the 83 kD protein in *T. brucei*, *T. congolense* and *T. vivax*.

A: Gels are gradient (7.5 to 15% acrylamide) slabs. Lanes 3 and 6: ^{14}C labelled molecular weight standards. The rabbit antisera are those listed in Table 1. Lanes 2 and 4: radiolabelled total trypanosome lysates from clones MITat 1.2 and ILTat 1.2; Lanes 1 and 5 are specific immunoprecipitates obtained with the antiserum from rabbit No. 11. Lanes 7 and 9: total lysates from *T. vivax* stock IL 417 and *T. brucei* clone ILTat 1.2; lanes 8 and 10: specific precipitates obtained with the antiserum from rabbit No. 12. Lane 11: total lysate from MITat 1.2; lanes 12, 13 and 14: precipitates obtained with antisera from rabbits Nos. 13, 14 and a pool of normal rabbit serum.

B: The gel is a 10% acrylamide slab. Lanes 1 and 11: molecular weight standards. Lanes 2, 5 and 8: total lysates from *T. vivax* IL 417, *T. congolense* ILNat 2.1 and *T. brucei* MITat 1.2 respectively; lanes 3, 6, 9: specific precipitates obtained with pooled normal rabbit serum.

Table 3. The packed cell volumes, prepatent periods and time to death of the 7 rabbits cyclically challenged with *T. brucei*

Rabbit number	Immunizing antigen	Initial PCV	Prepatent period (days)	Time to death	PCV at death	% PCV drop
16	83 kD protein	42	9	16	22	47.6
17	83 kD protein	41	10	30	31	24.4
18	83 kD protein	45	7	11	26	42.2
19	83 kD protein	40	7	22	31	22.5
20	83 kD protein	45	7	29	25	44.4
21	adjuvant alone	37	7	22	29	21.6
22	adjuvant alone	42	7	13	37	11.9

Table 4. The packed cell volumes, prepatent periods and time to death of the 7 rabbits cyclically challenged with *T. brucei*

Goat number	Immunizing antigen	Initial PCV	Prepatent period (days)	Time to death	PCV at death	% PCV drop
261	83 kD protein	41	9	25	23	43.9
262	83 kD protein	38	9	23	12	68.4
263	83 kD protein	35	8	28	20	42.9
264	83 kD antigen	35	8	16	26	25.7
265	plasma membrane	34	8	37	16	52.9
266	plasma membrane	37	9	31	28	24.3
267	plasma membrane	26	9	36	18	30.8
268	adjuvant alone	35	9	36	21	40.0
269	adjuvant alone	29	9	32	19	34.5
270	adjuvant alone	33	8	K*	27	18.2

* = Killed on day 75 after challenge

Serum samples from rabbits (16, 17, 18, 19 and 20) immunized with the 83 kD protein showed antibody titres greater than 1:10,000 (Table 2) and stained live *T. brucei* procyclics by IFA. There were no detectable antibodies against the antigen in sera from control rabbits (21, 22) injected with adjuvant alone. When the rabbits were challenged by tsetse infected with *T. brucei* ILTat 2.1, they all showed parasitaemia within 7–10 days (Table 3). At the time of death all animals were anaemic (Table 3).

Sera collected from the 83 kD immunized goats (261, 262, 263 and 264) had antibody titres greater than 1:1000 (Table 2) and stained live *T. vivax* procyclics by IFA. In those goats (265, 266 and 267) immunized with whole membrane fraction the antibody levels ranged from 1:64 to 1:128 (Table 2). Control goats had no detectable antibodies in their sera against either 83 kD protein or whole

membrane fraction. As can be seen in Table 4 all goats, with the exception of goat 270, succumbed to the challenge infection within 16–36 days. Goat 270 underwent a chronic infection and was killed in extremis on day 75.

Discussion

The present investigation has demonstrated that the common 83 kD antigen as well as the plasma membrane preparation were unable to induce protective immunity in goasts and rabbits against tsetse transmitted *T. vivax* and *T. brucei* respectively. The high levels of antibodies detected in the immunized animals proved ineffective in controlling the infection and did not alter the course of the disease.

The reasons for the failure by these antigens to induce protective immunity are not entirely clear. In the case of *T. brucei*, the metacyclics of this trypanosome species have been shown to possess a surface coat (Vickerman, 1974b). The surface coat would have masked the plasma membrane and the 83 kD protein antigenic determinants, thus making these determinants inaccessible to the antibodies in the immunized animals. A similar conclusion was arrived at by Murray et al. (1980) who also failed to induce protection against cyclically transmitted *T. brucei* in mice immunized with purified membrane preparations.

With regard to *T. vivax*, the 83 kD protein in the intact trypanosome appears to be exposed on the membrane since antisera to this protein as well as plasma membranes stained live *T. brucei* and *T. vivax* procyclics but not the bloodstream forms. It would seem therefore that the most plausible explanation of our results is that the metacyclics used for challenge contained organisms possessing a surface coat. The coated *T. vivax* metacyclics could have been responsible for establishing the infection in the immunized animals, having circumvented, like *T. brucei* metacyclics, the antibodies against the 83 kD as well as other plasma membrane antigens. However, the number of coated *T. vivax* metacyclics in tsetse is probably so small that their presence escaped detection despite the extensive examination reported by Tetley et al. (1981).

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