Some Properties of the Immunogens (Protective Antigens) of a Single Variant of *Trypanosoma brucei brucei*

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SUMMARY

Column-separated, clean trypanosomes were subjected to nitrogen cavitation and ultracentrifugation (90 min, 99000 g); the supernatant contained most of the immunogens but a few remained in the well washed residue. Column chromatography yielded a strongly immunogenic protein fraction (SAF1), small doses (0.035 mg protein/dose $\times$ 4) of which protected mice against a maximum challenge of 5×10^4 of the homologous variant organisms for a minimum of 17 weeks. No heterologous (variant or strain) protection was obtained with SAF1 either with increased doses or by homologous challenge followed by heterologous challenge 3 weeks later. Disc electrophoresis of SAF1 showed four anodic components intermediate in mobility between α -macroglobulin and transferrin of normal rat serum. Analytical ultracentrifugation indicated a substantial proportion of 6.5S protein together with 3.0S and 1-1.5S proteins. A smaller soluble antigen fraction (SAF8) consisted of 2.0S protein but preliminary experiment has not shown it to be immunogenic. Infected rat plasma separated after 6 h was as effective as SAF1 in protecting mice against homologous challenge whereas rapidly separated infected rat plasma gave only slight protection. Two identical precipitinogens were detected in infected rat plasma and SAF1 by immunodiffusion. Three additional precipitinogens were present in SAF1 and one other in the infected rat plasma. Antisera raised against various trypanosome fractions, including SAF1, agglutinated the homologous trypanosomes but to a lesser extent than antisera to living trypanosomes.

Incubation of trypanosomes in either SAF1, or infected rat serum or plasma decreased their infectivity which was not altered by 8 h of incubation in normal rat plasma.

INTRODUCTION

During the last decade, many workers (Williamson, 1961, 1963*a*, *b*; Williamson & Desowitz, 1961; Brown & Williamson, 1962*a*, *b*; Seed, 1963; Seed & Weinman, 1963; Brown & Williamson, 1964; Williamson & Brown, 1964; Gill, 1965; Bigalke, 1966; Lanham, 1966*a*; Seed & Gam, 1966; Williamson, Brown & Brown, 1966; Njogu & Humphries, 1967; Le Page, 1968; Le Ray, 1969; Njogu, 1970; Oerlich & Mannweiler, 1970) have examined the properties of fractionated antigens from trypanosomes of the subgenus *Trypanozoon*. However, little attention has been paid to the immunogenic* properties of such fractions. Recently, a column-separation technique (Lanham, 1968) has allowed the preparation of large quantities of pure trypanosomes, fractionation of which has enabled us to study the immunogenic and other properties of some soluble antigens of *Trypanosoma brucei brucei*. The present paper details and extends our preliminary report (Taylor & Lanham, 1972).

* Viz, capable of inducing specific immune protection (functional immunity) against a pathogenic organism (Herbert & Wilkinson, 1971).

METHODS

Trypanosomes. Strain o (Shinyanga III) of *Trypanosoma brucei brucei*, isolated in 1956 by Dr Soltys from cattle in Tanzania, was received at the Lister Institute and was either maintained by blood-passage in rats and mice, or frozen at -196°C . Antigenic variants were obtained from an infected rabbit by subinoculation of blood into mice. Variant b2 was isolated after 10 days and b4 after 17 days; they were either maintained by blood-passage in rats and mice every 2 to 3 days, or stored frozen at -196°C .

Strain 8/18 of *Trypanosoma brucei brucei*, isolated in September 1962 from a pig in Eastern Nigeria, was non-infective to man (Godfrey & Killick-Kendrick, 1967) and subsequently passaged in rats and mice or stored frozen. In January 1968 the strain was received at the Lister Institute from the Nigerian Institute for Trypanosomiasis Research and maintained in guinea-pigs prior to fly-transmission (*Glossina morsitans submorsitans* & *G. austeni*); it was then stored frozen at -196°C .

Nitrogen and protein estimation. Total nitrogen was determined by a Kjeldahl-Nessler method (see Meynell & Meynell, 1970), and was converted to total protein by multiplying by 6.25.

Antigen preparation. Rats were exsanguinated at peak parasitaemia under ether anaesthesia into ice-cold heparin solution (500 i.u./ml 0.95% saline) as an anticoagulant (10 i.u./ml of blood). The blood was diluted 1:3 with ice-cold phosphate-buffered glucose-saline (PSG: 8.092 g Na_2HPO_4 , 0.468 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 2.55 g NaCl and 10 g glucose, made up to 1 l in distilled water; $\text{I} = 0.22$, pH 8.0) and the trypanosomes separated from the blood cells by passage through a DEAE-cellulose (Whatman Chromedia DE 52) column (Lanham, 1968). The trypanosomes were then washed six times in cold PSG (by centrifuging for 10 min at 1600 g at 4°C) before suspension in twice their packed cell volume of distilled water (the usual yield was about 0.5 ml packed trypanosomes/10 ml blood, but could be as high as 1.2 ml). After nitrogen cavitation (1600 p.s.i. for 20 min) at 4°C (Hunter & Commerford, 1961) the resulting suspension of lysed trypanosomes (crude antigen = CA) was stored at least overnight at -25°C , thus ensuring that the few surviving trypanosomes were lysed.

Fractionation of crude antigen (CA). All manipulations were at 4°C . Macroparticles were sedimented from CA (after overnight dialysis against 0.04 M-phosphate buffer, pH 8.0) by centrifuging at 99000 g for 90 min. A sample (48 ml) of the supernatant (nitrogen content 1.95 mg/ml) containing the soluble antigens (SA) was chromatographed on DEAE-cellulose (Whatman Chromedia DE 52, equilibrated with 0.04 M-phosphate buffer at pH 8.0 (5.394 g Na_2HPO_4 , 0.312 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ made up to 1 l in distilled water; conductivity = $0.505 \times 10^4 \mu\text{mho}$ at 20°C) and degassed before packing), employing a 4.5×46 cm column with a sintered disc of porosity 1. The antigens were eluted from the column with a concave gradient of increasing ionic strength (the conductivity of the fractions was measured with a Type MCL (Mark IV) conductivity measuring bridge; Electronic Switchgear (London) Ltd). The starting buffer was 0.04 M-phosphate, pH 8.0, the limiting buffer was 0.8 M-NaCl in starting buffer, and the gradient established with 3 pumping tube elements of a Watson-Marlow Delta micrometering pump. The flow rate was 100 ml/h for the first 600 ml then about 200 ml/h. After discarding the first 100 ml, 411 ml of eluate were collected in 4.9 ml fractions and the remainder in 11.0 ml fractions. Absorbance values of the fractions at 260 nm and 280 nm are shown (Fig. 1); peak fractions were combined as indicated and concentrated up to 40 by ultrafiltration; soluble antigen fraction 1 (SAF 1) showed a characteristic protein absorption spectrum (Fig. 2).

Disc electrophoresis. Polyacrylamide gel was used to analyse antigen preparations according

Fig. 1. Concave gradient chromatography in DEAE-cellulose (DE 52) of the soluble antigens of *Trypanosoma brucei brucei* o variant b2. Absorbance at 260 nm —; absorbance at 280 nm ····; conductivity ----. The fractions corresponding to the separate eluate volumes: fraction 1 (SAF1), 180–438 ml (1a, 180–264 ml; 1b, 265–352 ml; 1c, 353–438 ml); fraction 2, 440–548 ml; fraction 5, 640–713 ml; fraction 6, 714–790 ml; fraction 8, 860–955 ml; fraction 11, 1315–1510 ml; fraction 12, 1511–1604 ml; fraction 13, 1605–1790 ml; fraction 14, 1791–1890 ml; fraction 17, 2050–2242 ml; fraction 19, 2280–2347 ml. Fractions were designated 'nucleic acid' or 'protein' by calculation of their relative absorbancies at 260 and 280 nm, and by reference to the table given in the *Manual of Biochemical Methods* (Conn, 1957).

to the modified method of Davies (1964): buffer, pH 1.9 (A)—48 ml N-HCl, 36.3 g tris, 0.46 ml TEMED, distilled water to 100 ml; acrylamide (B)—20 g acrylamide, 0.8 g bis-acrylamide, distilled water to 100 ml; initiator (C)—0.004 % riboflavin; or (D)—0.1 % persulphate ammonium.

Two volumes of B were mixed with either 1 vol. of C and 4 vol. of distilled water, or 2 vol. of B with 4 vol. of D and 1 vol. of distilled water. The mixture was degassed for 10 min on ice and 1 vol. of A added.

The final concentration of acrylamide was 5 % (w/v) in the gels which were polymerized for 30 min either by a Shandon fluorescent lamp (riboflavin initiator) or in diffuse, low intensity light (ammonium persulphate initiator). The electrode buffer was pH 8.3 to 8.4 (28.8 g glycine, 6.0 g tris, distilled water to 100 ml) and a constant current of 1 mA/tube was applied for the first 5 min followed by 2 mA/tube for 90 min at 4 °C (the electrophoretic front was visualized, when necessary, by the addition of 1 ml of 0.001 % bromophenol blue to the negative electrode buffer); during this time the voltage rose from 50 to a maximum of 190 V. Some of the gels were stained for 1 to 2 h in 1 % naphthalene black (1.0 g naphthalene black in 100 ml 7 % (v/v) acetic acid) rinsed in 7 % (v/v) acetic acid and electrolytically destained for 1.5 h (Ward, 1970) with a change of solvent after half an hour; the bands were then photographed.

Duplicate polyacrylamide preparations were used for agar-gel immunodiffusion tests, carried out in 1 to 1.5 % (w/v) Noble agar (Difco-Bacto) in 0.9 % phosphate-saline, pH 7.0 (0.2 ml of 0.5 M- Na_2HPO_4 in 1500 ml distilled water containing 13.5 g NaCl) and 0.065 % sodium azide. The agar (2.7 mm thick) was overlaid with a polyvinyl chloride mask (0.5 mm thick) having a central trough wide enough to fit the polyacrylamide gel and with narrow

troughs (1 mm wide) 5 mm from it on either side to be filled with antisera; after loading, preparations were kept at room temperature until the antisera were absorbed, then at 4 °C for up to 7 days when the precipitin lines were photographed.

Antisera preparation. All antisera were raised in rabbits and were shown to be free of anti-rat serum components by Ouchterlony immunodiffusion against normal rat serum.

Anti-living-trypanosome serum. *Trypanosome brucei brucei* O variant b2 was column-separated from infected rat blood and washed in PSG six times before intravenous inoculation of 30×10^6 trypanosomes into a rabbit. Blood was removed from the ear or by intracardial puncture after 7 days, allowed to clot for 24 to 48 h at 4 °C, the serum removed by centrifugation (10 min at 500 g) and stored at -25 °C.

Anti-crude-antigen (CA) serum was prepared after the method of Williamson & Brown (1964). Equal volumes of CA in Freund's complete adjuvant (FCA) (Difco) were emulsified in two syringes connected by a single needle (double-barrelled syringe) and stored at -25 °C. A 3.0 ml sample of this emulsion (5.835 mg total nitrogen) was injected intramuscularly into a rabbit (about 2 kg), half into the right fore, and half into the left hind leg, followed by similar injections into the opposite legs 7 days later. After a further 4 weeks, three intravenous injections of 0.6 ml (1.8 ml total) alum-precipitated (Williams & Chase, 1967) CA (1.294 mg total nitrogen) were given at 4 day intervals; blood was taken from an ear vein 1 week after the last injection and the serum stored at -25 °C.

Other anti-trypanosome sera were raised to trypanosome fractions and to formalized trypanosomes with the following modifications. (i) Soluble antigen fraction (SAF 1) (eluate volume 180 to 438 ml in Fig. 1). Doses of antigen were 2.0 ml of Freund's emulsion containing 0.23 mg antigen protein on days 0 and 7, and 0.6 ml alum-precipitated antigen containing 0.069 mg protein on days 35, 39 and 43. (ii) Trypanosome metabolites (TM) were obtained by incubating living trypanosomes (washed six times in PSG) in PSG for 4 min at 37 °C (15×10^8 trypanosomes/ml PSG) and centrifuging (1600 g at 4 °C for 5 min). Doses of supernatant (freshly prepared for each dose) were 4.0 ml of Freund's emulsion containing 0.77 mg total nitrogen, on days 0 and 7, and 0.8 ml of alum-precipitated antigen containing 0.144 mg antigen nitrogen on days 35, 39 and 43. (iii) Formalized trypanosomes (FT). Washed trypanosomes were suspended in six times their volume of formol saline (4.0 ml 38 % (w/v) formalin solution in 96 ml 0.95 % saline) at room temperature for 1 to 2 days and the pH was adjusted to 7.0 by the addition of 0.5 M-sodium bicarbonate when necessary. The trypanosomes were finally washed and resuspended in 0.95 % saline (approximately 15×10^8 /ml) and stored at -25 °C. The suspension was emulsified with an equal vol. of FCA and 3.0 ml of this injected intramuscularly into the rabbit on days 0 and 7; on days 35, 39 and 43, 0.6 ml of alum-precipitated formalized trypanosomes (containing 0.3 ml of formalized trypanosome suspension) were inoculated intravenously.

Ouchterlony immunodiffusion tests. Double-diffusion precipitin tests (Ouchterlony, 1949) were carried out in 1 to 1.5 % (w/v) Noble agar (see Disc electrophoresis, above) which was overlaid with a polyvinyl chloride mask (Feinberg, 1964) 0.5 mm thick having six holes 4 mm diameter, 5 mm apart and 4 mm from a central hole of 7 mm diameter. After loading, the gels were kept at room temperature until the solutions were absorbed and then at 4 °C until fully developed (up to 7 days) when the precipitin lines were photographed.

Agglutination tests. Antisera were examined for their agglutinating properties by the method of Cunningham & Vickerman (1962). PSG was the antisera diluent and the trypanosomes were obtained from infected blood. Suspensions, containing 7 % (v/v) glycerol added after dilution of the heparinized blood 2:1 with PSG, were stored at -196 °C in sealed lymph tubes. Single drops of serial dilutions of the antisera were plated out on siliconized

Table 1. *Infectivity of Trypanosoma brucei brucei* O variant b2 after incubation in soluble antigen fraction I or infected rat plasma or serum

Tube	Contents of incubation tubes					Trypanosomes 15 × 10 ⁸ /ml PSG ^a	Time (h)	Mice infected (six per group) Trypanosomes ^g × 15/mouse						
	PSG ^a (ml)	NRtP ^b (ml)	RIRtP ^c (ml)	IRtS ^d (ml)	SAF 1 ^e (ml)			10 ³	10 ¹	10 ⁰	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴
A	0.4	—	—	—	—	0.1 ml added simultaneously	0	—	—	6	4	2	0	0
B	0.15	0.25	—	—	—			—	—	4	3	2	0	0
C	0.15	—	0.25	—	—			—	—	4	2	2	0	0
D	0.15	—	—	0.25	—			—	—	—	4	4	0	0
E	0.15	0.25 ^f	—	—	—			—	—	—	5	0	0	0
F	0.15	—	—	—	0.25			—	—	—	6	5	1	0
A8	0.4	—	—	—	—	0.1 ml added simultaneously	8	2	0	0	0	0	—	
B8	0.15	0.25	—	—	—			6	6	3	1	1	0	—
C8	0.15	—	0.25	—	—			6	6	1	0	0	0	—
D8	0.15	—	—	0.25	—			6	6	2	0	0	0	—
E8	0.15	0.25 ^f	—	—	—			0	0	0	0	0	0	—
F8	0.15	—	—	—	0.25			0	0	0	0	0	0	—

^a Phosphate-buffered saline-glucose, pH 7.4.^b Normal rat plasma.^c Rapidly prepared infected rat plasma.^d Infected rat serum.^e Soluble antigen fraction I (0.3 % protein, i.e. 3.0 mg/ml PSG, pH 7.4).^f Normal rat plasma diluted one tenth in PSG, pH 7.4, (i.e. approximately 0.3 % protein).^g Serial dilutions (in PSG, pH 7.4) of incubated trypanosomes.

ringed slides (Adams Clay 12 ring microflocculation slides) and a drop of freshly-thawed trypanosome suspension (one lymph tube/slide) added to each dilution. The slides were kept 30 min in a damp chamber at room temperature, coverslips placed over the drops and the tests examined microscopically.

Immunogenic tests. The immunogenic properties of infected rat serum (IRtS) and plasma (IRtP) (see below for preparation), as well as fractions of *Trypanosoma brucei brucei* O variant b2, were determined in mice. IRtS, IRtP, CA, SA, and SAF I were emulsified (in a double-barrelled syringe), 1 vol. with 1 vol. of FCA and 2 vol. of PSG (dosage: rat components = 0.05 ml/0.2 ml; CA = 0.092 mg nitrogen/0.2 ml; SA = 0.2 mg nitrogen/0.2 ml; SAF I = 0.035 to 0.070 mg protein/0.2 ml). Sufficient emulsion was prepared for each experiment and stored at -25°C (if necessary the antigen-FCA mixture was re-emulsified before dosing). Groups of 10 mice were injected intraperitoneally (i.p.) on days 0, 5, 15 and 21; control groups included mice injected with appropriately diluted FCA or not injected. All mice were challenged 12 to 14 days after the last immunizing dose, with 5×10^2 trypanosomes: *T. brucei brucei* O, *T. brucei brucei* O variants b2 and b4, or *T. brucei brucei* 8/18/0.1 ml infected blood, diluted in PSG mouse and examined daily thereafter for infection (microscopic examination of wet blood smears with $\times 40$ objective) and death. In some experiments the mice were rechallenged after 17 to 21 days with homologous or heterologous variants and strains.

Preparation of rat components. These were reduced 2/3 in volume by positive pressure dialysis, before use in the immunogenic tests.

1. Normal and infected rat serum (NRtS and IRtS). Blood from normal or infected rats at peak parasitaemia (infected 3 days previously) was allowed to clot for 6 h at 20°C . The serum was separated by centrifugation (10 min at 500 g), filtered (Millipore APD $0.45\ \mu\text{m}$) to remove any remaining trypanosomes and stored at -25°C .

2. Normal (NRtP) and infected (IRtP) rat plasma. Normal or infected rats at peak parasitaemia (1 or 3 day infections) were bled rapidly into ice-cold heparin solution (500 i.u./ml 0.95% saline) as an anticoagulant (10 i.u./ml blood): (a) Rapidly prepared normal (NRtP) and infected (RIRtP) rat plasma were obtained by centrifugation (15 min at 1600 g at 4°C) and filtration to remove any trypanosomes (preparation time was 35 min). It was either used immediately or stored at -25°C ; (b) slowly prepared infected rat plasma (SIRtP) was obtained as above but the blood was stood for 6 h at 20°C before being centrifuged and filtered. It was stored at -25°C until used.

Infectivity tests. Washed living *Trypanosoma brucei brucei* O variant b2 trypanosomes (see Antigen preparation), harvested from rats at high parasitaemia 1 day after infection, were given a final wash in PSG, pH 7.4 (1.708 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 6.938 g Na_2HPO_4 , 2.55 g NaCl, 10 g glucose, distilled water to 1 l: I = 0.20) before being suspended in either PSG, pH 7.4, alone or in SAF I (see Fig. 1), NRtP, RIRtP, IRtS each diluted in PSG, pH 7.4 (see Table 1 for details: see also above for preparation of rat components). Samples of these trypanosome suspensions were held on ice for immediate use in mouse infectivity tests (Lumsden *et al.* 1963) and the remainder were incubated at 23°C for 8 h before being similarly used (a preliminary experiment showed that 8 h of incubation was required for a significant decrease in infectivity after incubation in PSG, pH 7.4, when compared with incubation in NRtP). After incubation trypanosome suspensions were serially diluted in ice-cold PSG, pH 7.4, yielding five or six final suspensions ranging from 15×10^0 to 15×10^{-4} or 15×10^2 to 15×10^{-3} trypanosomes/0.1 ml; each dilution was injected into a group of six mice (0.1 ml/mouse i.p.). Wet blood films from the mice were examined daily for infection, and the number of deaths per group recorded.

Fig. 2. Polyacrylamide disc electrophoresis of soluble antigens and normal rat serum (gels stained in 1 % naphthalene black in 7 % acetic acid). *a*, Soluble antigens; *b*, soluble antigen fraction 1 (180–438 ml, Fig. 1); *c*, normal rat serum. 1 = α -macroglobulin; 2 = transferrin; 3 = albumin.

RESULTS

Disc electrophoresis. Electrophoresis in polyacrylamide gels separated the soluble antigens (SA) into eight discrete anodic protein bands (Fig. 2). The first fraction of SA after gradient chromatography (SAF 1; Fig. 1, 3) showed four discrete anodic protein bands the mobilities of which were between the α -macroglobulin and transferrin bands of normal rat serum run at the same time. Electrophoresis of sub-fractions of SAF 1 (Fig. 1) showed all four protein components in SAF 1a, only three in SAF 1b (the most positively charged component was missing) and only the most negatively charged component remained in SAF 1c.

The disc electrophoretic patterns of the other fractions of SA (2, 5, 8, 11, 12, 14, 17) are shown in Fig. 3.

Ultracentrifugation. SAF 1 was scanned between 230 to 310 nm and showed a characteristic protein absorption spectrum (Fig. 4). Analytical ultracentrifuging (by Dr J. M. Creeth of this Institute) of a concentrated sample of this fraction (1 % protein) showed two major components (Fig. 5), the faster of which ($s_{20,w} = 6.5S$) accounted for about 60 % of the total. The slower component ($s_{20,w} = 3.0S$) accounted for about 30 % of the total and a still slower ($s_{20,w} = 1.0$ to $1.5S$) approximately 10 %. The incomplete separation of the 6.5S and 3.0S components suggests the presence of a small proportion of a component with an intermediate sedimentation coefficient. The molecular weight of the 6.5S component, estimated from the $s_{20,w}$ values, was 100 to 200×10^3 and that of the 3.0S component, 25 to 50×10^3 .

SAF 8 was a smaller protein fraction concentrated to an absorbance at 280 nm of 2.0 by pressure dialysis at 4 °C for 24 h. It was probably a low molecular weight component of SA,

Fig. 3. Polyacrylamide disc electrophoresis of soluble antigen fractions (SAF) stained in 1% naphthalene black. *a*, SAF2 (440–548 ml, Fig. 1); *b*, SAF6 (714–790 ml, Fig. 1); *c*, SAF8 (860–955 ml, Fig. 1); *d*, SAF11 (1315–1510 ml, Fig. 1); *e*, SAF14 (1791–1890 ml, Fig. 1).

Fig. 4. The characteristic protein absorption spectrum of the soluble antigen fraction 1 (SAF 1) from *Trypanosoma brucei brucei*.

Fig. 5. Ultracentrifugal analysis of soluble antigen fraction 1 from *Trypanosoma brucei brucei* O variant b2 (77 min after attainment of operating speed; Spinco model E, 59 800 rev./min; temp., 25 °C; Schlieren bar angle, 40°; direction of sedimentation left to right; protein concentration, 1.0 % in 0.04 M-phosphate buffer containing 0.1 M-NaCl). S values indicated are sedimentation coefficients corrected to 20 °C in water.

Table 2. *Trypanosoma brucei brucei* O variant b2; agglutination titres with antisera to various homologous antigens

Rabbit antisera against					
Living trypanosomes	Formalized trypanosomes	Crude antigen	Soluble antigen	Soluble antigen fraction 1	Trypanosome metabolites
$\frac{1}{192\,000}$	0	$\frac{1}{1\,530}$	$\frac{1}{4\,000}$	$\frac{1}{1\,287}$	$\frac{1}{990}$

since excessive loss of 280 nm absorbing material occurred during dialysis. Because of this, ultracentrifuging was carried out using a 'synthetic boundary' of the buffer solvent of SAF8 adjusted to the same composition and conductivity. Only one peak was evident and this spread rather extensively during the course of the experiment. The sedimentation coefficient measured from the movement of the peak was approximately $s_{20,w} = 2.0S$ but the precision of this value was low owing to the base-line uncertainty from the use of this non-dialysed fraction.

Agglutinogenicity. Higher agglutination titres were obtained with antisera to living trypanosomes than to any of the various trypanosome fractions, including SAF 1 (Table 2). Antisera raised against formalized trypanosomes did not agglutinate living trypanosomes. Column-separation of trypanosomes, followed by six washes in PSG, did not significantly alter agglutination titres.

Fig. 6. Ouchterlony plate showing the build up, with time, of soluble antigens during plasma preparation; a common precipitin line is visible between these antigens and a component of soluble antigen fraction 1 prepared from pure trypanosomes (SAF 1). *a*, Rapidly prepared infected rat plasma (RIRtP); *b*, slowly prepared infected rat plasma (SIRtP); *c*, soluble antigen fraction 1 (SAF 1); *d*, rabbit anti-SAF 1 serum.

Table 3. *Maximum number of precipitin lines formed during immunodiffusion of Trypanosoma brucei brucei* O variant b2 fractions against antisera to homologous trypanosomes and their fractions

Antigens	Antisera to					
	Living trypanosomes	TM	CA	SA	SAF 1	FT
TM ^a	2	8	4	5	9	2
CA ^b	9	7	10	6	2	4
SA ^c	9	9	9	6	7	4
SAF 1 ^d	3	9	5	8	5	2
RIRtP ^e	0	2	1	2	1	0
SIRtP ^f	1	2	2	6	3	1

^a Trypanosome metabolites.

^b Crude antigen.

^c Soluble antigen.

^d Soluble antigen fraction 1.

^e Rapidly prepared infected rat plasma.

^f Slowly prepared infected rat plasma.

Immunodiffusion

1. Ouchterlony plates. Antisera to living trypanosomes contained only two precipitins against TM but nine against CA or SA and three against SAF 1, antisera to formalized trypanosomes contained two precipitins against TM, four against CA and SA but only two against SAF 1. Antisera to trypanosome metabolites or fractions reacted as shown in Table 3.

Table 4. Efficacy of immunization with *Trypanosoma brucei brucei* O variant b2 soluble antigen fraction I (SAF I) against graded challenges of the homologous variant

Mice dosed with	Trypanosomes per mouse $\times 5$	Mean ^a days to death	Mortality at 10 days
SAF I	10^2	> 10	0/10
	10^3	> 10	0/10
	10^4	> 10	0/10
	10^6	8.0	9/10
	10^8	5.8	10/10
FCA ^b or undosed	10^2	7.0	10/10
	10^8	2.2	10/10

^a In calculating the mean time to death, survivors on the tenth day were given 11 days of life.

^b Freund's complete adjuvant.

Anti-SAF I serum contained identical precipitins against all the fractions of soluble antigens (SA) which indicated that SAF I was probably a contaminant trailing through the column during preparation.

When the anti-SA serum was tested against the infected rat plasmas only two precipitins were detected with RIRtP but six with SIRtP. Similarly with anti-SAF I serum, only one precipitin line developed against RIRtP but three with SIRtP and five with SAF I (Fig. 6). The precipitin line against RIRtP had common identity with one of those in each of SIRtP and SAF I (shown faintly in Fig. 6 but confirmed by stronger lines in other Ouchterlony plates) but the additional lines of SAF I and SIRtP were not identical with each other. With the antisera against trypanosome metabolites, although the same two lines developed against both rat plasmas, these were much enhanced with SIRtP.

2. Immunoelectrophoresis. Three to four precipitinogens were detected after disc electrophoresis of SAF I, SAF Ia, SAF Ib or SAF Ic when tested against rabbit anti-SAF I serum but only two to three precipitinogens were detected with anti-CA serum. However, disc electrophoresis gels of many other SA fractions (SAF 2, 5, 11, 13, 14 and 17) showed a similar precipitinogen pattern when tested against anti-SAF I serum, thus indicating contamination with this fraction during chromatography.

Immunogenicity. Table 4 shows that SAF I protected mice against 5×10^4 homologous trypanosomes/mouse, but was less effective with 5×10^6 or 5×10^8 trypanosomes.

Immunization of mice with either CA, SA or SAF I of *Trypanosoma brucei brucei* O variant b2 (Table 5) protected them against the homologous variant but not against a heterologous variant (b4), the parent strain (*T. brucei brucei* O) or against a recently fly-passaged strain (*T. brucei brucei* 8/18). Subsequent rechallenge of the protected mice (17 to 21 days after the first challenge) showed that the immunity was maintained but only against the homologous variant. The protection given by SAF I (0.035 mg protein/dose) lasted for a minimum of 17 weeks.

Mice immunized with SIRtP or IRtS were protected against homologous challenge almost as well as those mice immunized with SAF I (Table 6). On the other hand very little protection was induced with RIRtP.

Infectivity tests. The significance of the results shown in Table 1 was determined from the tables of Lumsden *et al.* (1963). Thus, there was a significant ($P > 0.05$) decrease in the infectivity of *Trypanosoma brucei brucei* incubated in PSG alone or in combination with RIRtP, IRtS, SAF I or dilute RIRtP; no significant difference in infectivity was obtained in trypanosomes incubated in PSG with NRtP.

Table 5. *Time to death of mice immunized with Trypanosoma brucei O variant b2 soluble antigens when challenged with variants and strains of T. brucei brucei (5×10^3 trypanosomes/mouse)*

Mice dosed with	First challenge variant	Mean ^a days to death	Mortality at 10 days	Second ^a challenge variant	Mean ^a days to death	Mortality ^a at 10 days
Undosed	ob2 ^b ob4 ^c O ^d 8/18 ^e	6.0	5/5	—	—	—
FCA ^f	ob2 ob4 O 8/18	6.0	10/10	—	—	—
SA ^h	ob2	7.4	1/10	ob2	8.5	1/9
SAF1 ⁱ	ob4 O 8/18	9.6 6.7 6.0 6.6	10/10	—	—	—
Undosed	—	—	—	ob2 ob4 O 8/18	6.0	5/5
SAF1 ⁱ	ob2 ob2 ob2 ob2	> 10	0/10	ob2 ob4 O 8/18	> 10 6.7 6.0 7.6	0/10 10/10

^a In calculating mean time to death, survivors on the tenth day were given 11 days of life.

^b *Trypanosoma brucei brucei* O variant b2.

^c *T. brucei brucei* O variant b4.

^d *T. brucei brucei* O.

^e *T. brucei brucei* 8/18.

^f Freund's complete adjuvant.

^g 17 days after first challenge.

^h Soluble antigens — (in other experiments complete, homologous protection was obtained).

ⁱ Soluble antigens fraction I.

N.B. Crude antigen (CA) gave complete, homologous protection in other experiments.

Table 6. Time to death of mice immunized with *Trypanosoma brucei brucei* O variant b2 soluble antigen fraction 1 or infected rat plasma or serum after homologous challenge (5×10^5 trypanosomes/mouse),

Mice dosed with	Mean ^a days to death	Mortality at 10 days
RIRtP ^b	6.2	6/9
SIRtP ^c	9.3	1/9
IRtS ^d	> 10	1/8
SAF 1 ^e	> 10	0/10
NRtS ^f	4.6	10/10
FCA ^g	5.1	10/10
Undosed	4.1	10/10

^a In calculating the mean time to death survivors on the tenth day were given 11 days of life.

^b Rapidly prepared infected rat plasma.

^c Slowly prepared infected rat plasma.

^d Infected rat serum.

^e Soluble antigen fraction 1.

^f Normal rat serum.

^g Freund's complete adjuvant.

DISCUSSION

Most of the immunogens were in the soluble fraction of *Trypanosoma brucei brucei* O variant b2 and after chromatography on DEAE-cellulose, in the first fraction (SAF 1), which was unaffected by frequent freezing (-25°C) and thawing over a 24 month period. As far as we know, this is the first time the variant specific immunogens have been prepared directly from the trypanosomes although Lanham (1967) and Seed (1963) prepared antigens with immunogenic properties from the sera of rats infected with *T. brucei brucei* and *T. brucei rhodesiense* respectively. The purified immunogenic fraction (SAF 1) contained protein but no nucleic acid. The sedimentation value of 6.5S for its major component was similar to the intermediate components (6.0 to 6.7S in fractions V and VI) seen by Njogu (1969) after agar-gel diffusion but it was higher than the values of 5.0S observed by Le Page (1968) for *T. brucei brucei* and the variant specific 4.0S observed by Williamson (1963*a, b*) for *T. brucei rhodesiense*. Of the two minor antigenic components in our SAF 1, one had a sedimentation value of 3.0S and the other of 1.0 to 1.5S; these were similar to Le Page's (1968) values of 2.9S and 1.0 to 2.0S, and Njogu's (1969) values of 3.1 to 3.8S in his fraction VII. These slight but distinct differences could be real since different strains were used; they could also be due to such factors as different methods of preparation and the aggregation of proteins or their subunits.

Some of our immunogens were probably similar to those separated by Lanham (1965, 1966*a*, 1967) from infected rat serum which chromatographically behaved similarly to serum globulin (IgG) and probably also corresponded to the 'exoantigen' described by Weitz (1960*a, b*) from IRtS, which travelled electrophoretically close to the γ -globulin position. These similarities were confirmed by a precipitin line common to IRtS, IRtP and SAF 1 when tested against rabbit antisera to the various fractions of pure trypanosomes, although there were additional lines of non-identity between SAF 1 and the rat components. The precipitin line for RIRtP was not as distinct as that for the SIRtP (Fig. 6), thus corroborating Allsopp, Njogu & Humphries (1971), who found only a trace of released antigen in plasma taken as quickly as possible from an infected rat; increasing amounts appeared on standing in the presence of the trypanosomes. It seems likely therefore that trypanosomes break up

during the preparation of infected plasma, thereby releasing their soluble antigens. This is further supported by the finding that little homologous protection was afforded to mice immunized with rapidly prepared infected rat plasma, whereas those mice immunized with either slowly prepared infected rat plasma or serum, or SAFI were well protected.

The report by Weitz (1960*b*) that 'Exoantigen (contained in IRtS) appears to be essential for the protection of the trypanosomes against adverse environmental conditions *in vitro* and *in vivo*' has not been verified by our experiments. In fact incubation of *Trypanosoma brucei brucei* in infected serum or plasma, or in the purified immunogens, significantly decreased their infectivity to mice whereas their infectivity was unchanged by 8 h of incubation in normal rat plasma. These conflicting results may have been due to use of different variants or to different methods of preparation of the plasma and sera; Weitz kept infected blood at room temperature for varying periods before the plasma or serum were removed.

Weitz (1960*b*) also reported that trypanosomes which have been thoroughly washed in Alsever's solution lose their viability, agglutinability and infectivity; in our hands the infectivity and agglutinability was the same for either unwashed trypanosomes or those washed six times with PSG after column-separation from the host components. Thus the nature of the solution used for washing the trypanosomes is very important and in our experience Alsever's solution is not a good medium for trypanosomes. We have found that even after twelve washes in PSG, trypanosomes examined under the electron microscope (see Taylor & Godfrey, 1970, for preparation technique) appeared normal and their surface coat was intact. In contrast Vickerman (1969, 1970, 1972) reports that extensive washing in saline removes the surface coat. We believe that trypanosomes prepared rapidly in a cold, well-balanced, synthetic medium do not lose their viability, infectivity or agglutinability; morphological deterioration is minimized and in particular there is no loss of surface coat.

In our experiments (Table 2) antisera raised against trypanosome fractions or metabolites had considerably lower agglutination titres than the antisera to living trypanosomes, probably because living trypanosomes prolonged antibody induction. On the other hand, antisera raised against formalized trypanosomes did not agglutinate the homologous variant at all. Formalin reacts with the amino groups of protein and may therefore have altered the structure of the surface agglutinogens.

Although Seed & Gam (1966) predicted that the purified protective antigens of *Trypanosoma brucei gambiense* would not give rise to agglutinating antibody, antiserum to our purified immunogens did agglutinate the homologous trypanosome, albeit to a lesser extent than anti-living trypanosome serum (Table 2). The results may differ because of species differences or because our immunogenic fraction is still a mixture of proteins. Further purification should settle this point.

Contrary to the results of Allsopp *et al.* (1971), who could not detect released precipitinogens after *Trypanosoma brucei brucei* had been incubated in buffer for 6 h, we have found precipitinogens (TM) after *T. brucei brucei* had been incubated in a phosphate buffer for only 4 min. These precipitinogens reacted with all our antisera. The greater release of antigens in our experiments may have been due to mechanical damage during the processing of the trypanosomes. It may be that Allsopp *et al.* (1971) used fewer trypanosomes or perhaps their antisera were too dilute to detect precipitinogens since these were routinely absorbed with normal rat plasma. It is however difficult to account for the non-appearance of precipitinogens after 6 h of incubation when we so readily detected them after only 4 min.

Our purified immunogens also induced precipitating antibody production in rabbits, whereas Seed (1963) was unable to detect precipitinogens in his preparation of protective antigen from *Trypanosoma brucei rhodesiense*.

There appear to be many points of disagreement between the results of all reports on the properties of trypanosomal antigens. Doubtless methods of preparation and strain differences contribute greatly to these contradictory results. Obviously very much more information is required before such results can be extrapolated to the condition of the antigens *in vivo*.

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