SINGLE-DOSE VACCINATION OF MICE AGAINST EXPERIMENTAL INFECTION WITH *TRYPANOSOMA* (*TRYPANOZOON*) *BRUCEI*

W. J. HERBERT* AND W. H. R. LUMSDEN

*Protozoology Research Unit, Royal (Dick) School of Veterinary Studies,
University of Edinburgh, Easter Bush, Roslin, Midlothian*

THE results of early attempts to raise immunity to trypanosome infections in mammals by means of vaccines are confused and difficult to interpret because experiments were necessarily carried out with organisms maintained by serial passage and it was usually impossible to ensure that the trypanosomes used for vaccination were antigenically identical with those used for challenge.

Recent work has been more consistent, usually because greater control over the characteristics of the materials used has been rendered possible by their preservation at low temperatures (Polge and Soltys, 1957; Cunningham, Lumsden and Webber, 1963). Immunisation has been accomplished by the use of trypanosomes killed by various methods, with and without adjuvants, and by the use of released trypanosomal antigens (Soltys, 1957, 1964, 1965; Thillet and Chandler, 1957; Weitz, 1960; Dodin, Fromentin and Gleye, 1962; Johnson, Neal and Gall, 1963; Seed, 1963; Gill, 1965). Most of the immunisation schedules reported as successful have involved the use of repeated inoculations of antigen. Only Soltys (1965) appears to have elicited effective immunity by a single inoculation.

The present experiments have been carried out in extension of Soltys's work, to define the value of single-dose methods of immunisation. Advantage was taken of the availability of replicate samples of a population of *Trypanosoma* (*Trypanozoon*) *brucei*, preserved at -79°C as a stablate (Lumsden and Hardy, 1965), to provide antigen and challenge materials that were strictly comparable in quality over the period of the experiments. Further, the infectivities of the materials were titrated by the method of Lumsden *et al.* (1963) so that challenge inocula could be measured and reproduced in a standard way throughout the course of the work.

MATERIALS AND METHODS

Trypanosome antigens

Except in one experiment, the antigens used for immunisation were derived directly from *Trypanosoma* (*Trypanozoon*) *brucei* Plimmer and Bradford, 1899, subgroup (Hoare, 1957, 1966), stablate TREU-87 (TREU = Trypanosomiasis Research Edinburgh University). The strain of trypanosomes from which this stablate was prepared was derived originally from *Glossina pallidipes* captured in Uganda. TREU-87 is 20th passage material; its ancestry is described in detail by McNeillage, Herbert and Lumsden (1968). In the excepted experiment, the trypanosome antigens were prepared from a clone population (TREU-289)

* Present address: Department of Bacteriology and Immunity, University of Glasgow. Reprints from Dr W. H. R. Lumsden.

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derived from TREU-164 (TREU-164 is a first passage descendant of TREU-87 and has similar antigenic properties to it) or from clone populations derived from first antigenic relapse variants that appeared after the inoculation of TREU-164, at high dilution, into mice (TREU-285, TREU-280, and TREU-295). These four stabilates have been shown by an agglutination test (Cunningham and Grainge, 1963) to be antigenically different and bear the specificities of ETat-1 (TREU-289), ETat-2 (TREU-285), ETat-3 (TREU-280) and ETat-4 (TREU-295) (Lumsden, Herbert and McNeillage, 1967) (ETat = Edinburgh *Trypanozoon* antigenic type).

Three types of antigen were employed for immunisation: released antigens, trypanosome bodies and formalinised whole infected blood. For their preparation, mice were infected with antilog 3·8 ID₆₃ of TREU-87 and were bled from the retro-orbital plexus (Halpern and Pacaud, 1951) on the 2nd or 3rd day. Released antigens were obtained by allowing the blood to clot and collecting the serum. Trypanosome bodies were separated from heparinised blood (5 units heparin per ml, Boots Pure Drug Co., Nottingham) by centrifugation after the addition of a rabbit anti-mouse-red-cell serum. Formalinised whole infected blood was prepared from heparinised blood by adding sufficient of a 0·4 per cent. solution of formaldehyde in 0·85 per cent. NaCl (saline) to the blood to bring the concentration of formaldehyde in the blood to 0·05 per cent. The mixture was then allowed to stand on the bench for 15 min. Before use, a check was made with a microscope to ensure that no motile organisms were present.

Measurement of antigens. Some of the antigens used were quantitated by the method of Sewell (1967) with a rabbit anti-stablate TREU-87 serum. The units used are arbitrary.

Adjuvants. Antigens were incorporated into water-in-oil adjuvants and multiple emulsion adjuvants by the methods described by Herbert (1967). The oil phases of the emulsions were prepared from light liquid paraffin (Manchester Refinery (Sales) Ltd, London) and Arlacel A (Honeywill-Atlas Ltd, Carshalton, Surrey), which had been found to be non-toxic by the criteria of Berlin (1962). Aluminium hydroxide adjuvant was prepared by adding 250 ml of 5 per cent. aluminium sulphate to 100 ml of 5 per cent. sodium hydroxide. The mixture was then centrifuged at 250*g* for 3 min., the supernate removed, and the precipitate resuspended to the original volume with saline. This suspension was stored at 4°C till used. To adsorb trypanosome antigens on the adjuvant, equal volumes of the suspension of aluminium hydroxide and mouse serum containing released trypanosome antigens were mixed and incubated at 37°C for 30 min. The whole mixture was then used for vaccination.

Thiomersal. To ensure sterility of the vaccines, thiomersal was added to them to give a concentration of 1 g thiomersal to 10,000 ml of the antigen preparation.

Vaccines

Vaccines used to immunise the mice were prepared from the antigens as follows.

(1) *Water-in-oil emulsion containing trypanosome bodies.* Trypanosome bodies recovered from 0·5 ml of the blood of mice 2 days after infection with TREU-87 were suspended in 0·25 ml saline. The suspension was then alternated between -20°C and +37°C six times to disrupt the organisms. Thiomersal was added and the suspension was incorporated into a water-in-oil emulsion. The final volume of emulsion was 3 ml.

(2) *Water-in-oil emulsion containing released antigens.* Serum was collected from mice that had been infected as described in (1) above, thiomersal was added and the serum was incorporated into a water-in-oil emulsion; the serum composed half the volume of the finished emulsion.

(3) *Multiple emulsion containing trypanosome bodies.* Trypanosome bodies recovered from 1·5 ml of the blood of mice 3 days after infection with TREU-87 were suspended in 1·5 ml saline. The suspension was alternated four times between -20°C and +37°C to disrupt the organisms. Thiomersal was added and the suspension was incorporated into a multiple emulsion. The final volume of emulsion was 6·0 ml.

(4) *Released antigens adsorbed to aluminium hydroxide.* Serum was collected from mice that had been infected with TREU-87 3 days previously. After the addition of thiomersal,

the serum was adsorbed to aluminium hydroxide as described above. The finished vaccine contained half its volume as infected serum.

(5) *Formalinised infected whole blood.* This was prepared as detailed above.

(6) *Formalinised plasma.* This was obtained from formalinised infected whole blood by centrifugation at 2500*g* for 10 min. to remove blood cells and trypanosomes. The supernatant plasma was collected and diluted with an equal volume of saline before use.

Immunisation experiments

Animals. Random-bred Swiss white mice of the CD-TO strain, obtained from Messrs A. Tuck & Son, Rayleigh, Essex, were used. Those in the immunised groups were inoculated subcutaneously, dorsolaterally over the ribs, with the vaccines prepared as water-in-oil or multiple emulsions. The formalinised and the adsorbed antigens were given intravenously into the tail veins.

Challenge. Vaccinated and control groups of mice were challenged by the intraperitoneal inoculation of antilog 1·8 ID63 of stabilate TREU-87 in 0·1 ml of a balanced salts solution buffered (phosphate system) at pH 8·0 (Lumsden, Cunningham *et al.*, 1965). In the excepted experiment (see above), mice were challenged with antilog 2·0 ID63 of TREU-289, -285, -280, or -295 (representing, respectively, the antigenic types ETat-1, ETat-2, ETat-3 and ETat-4), or with a mixture containing antilog 2·0 ID63 of each of these stabilates.

Observation of mice. Tail blood from the mice was examined microscopically for trypanosomes on the 4th day after challenge and on at least every other day thereafter. This was done in order to distinguish deaths from trypanosomiasis from deaths due to intercurrent infection.

Drug cure. Berenil (Hoechst Pharmaceuticals Ltd, Brentford, Middlesex) was used to cure infections. Dr S. E. G. Smith tells us it contains diminazene aceturate, 31 per cent., and phenyldimethylpyrazolone, 69 per cent.

Infectivity titration of the stabilates was carried out as described by Lumsden *et al.* (1963). Total counts were also made of the organisms in each stabilate. TREU-87, -289, -285, -280, and -295 contained, respectively, antilog 6·9, 7·0, 8·3, 7·7 and 8·0 ID63 per ml, and antilog 8·7, 8·5, 8·9, 8·8 and 8·6 organisms per ml.

Probabilities that the results obtained could have occurred by chance have been calculated by the exact method for four-fold contingency tables given by Fisher (1950).

RESULTS

Immunity after drug cure of trypanosomal infection

Nineteen mice were each given intraperitoneal inoculations of antilog 1·8 ID63 of stabilate TREU-87. A group of 31 mice for use as controls was selected at the same time (table I). All the infected mice had shown trypanosomes in their blood by the 7th day when they were treated with Berenil at the rate of 25 mg active principle per kg body weight. Four mice died on the day of treatment or later, the rest survived. Groups of these surviving mice were challenged at intervals between 42 and 370 days after treatment. All were found to be immune. Control mice almost all succumbed to the same challenge.

Immunity after vaccination with the aid of adjuvants

Water-in-oil emulsions. Each of 18 mice was given a subcutaneous inoculation of 0·1 ml of the water-in-oil emulsion vaccine containing disrupted trypanosome bodies. On days 21, 42 and 161 after vaccination, groups of the mice were challenged. The results were as shown in table II. All vaccinated

TABLE I
Immunity after cure of trypanosomal infection with a drug

Interval between treatment and challenge (days)	No. of mice surviving for at least 14 days after challenge/no. of mice challenged in		P ‡
	infected and treated group	uninfected and untreated group (controls)	
42	5/5	1/6	0.01
126	4/4 *	1/7	0.01
253	4/4 †	0/6	< 0.01
370	2/2	0/12	< 0.01

Immunisation of 15 mice was by infection with stabilate TREU-87 and treatment with Berenil 7 days later. Challenge of 15 immunised and 31 control mice was by intraperitoneal injection of antilog 1.8 ID63 of stabilate TREU-87.

* These mice were found still to be immune when re-challenged on the 253rd day.

† These mice were found still to be immune when re-challenged on the 370th day.

‡ P = Probability that difference between the results recorded in columns 2 and 3 occurred by chance.

TABLE II
Immunity after vaccination with water-in-oil emulsions

Interval between vaccination and challenge (days)	No. of mice surviving for at least 14 days after challenge/no. of mice challenged in group vaccinated with		
	trypanosome bodies	released antigen	nil (controls)
21	5/7 (P = 0.05)	...	1/7
37	...	2/7	1/7
42	6/7 (P = < 0.01)	...	0/7 *
119	...	4/6 (P = 0.02)	0/7
154	...	4/7 (P = 0.04)	0/7
161	4/4 (P = < 0.01)	...	0/5

Vaccination was by subcutaneous injection of a single dose of a water-in-oil emulsion containing either disrupted trypanosome bodies containing c. 0.3 unit of antigen (18 mice) or serum containing released antigens (20 mice) derived from *Trypanosoma brucei* stabilate TREU-87. Challenge was by intraperitoneal injection of antilog 1.8 ID63 of stabilate TREU-87. Probabilities (P) are not given for non-significant results.

* One infected mouse died only on the 15th day after challenge.

groups showed a significant level of protection as compared with unvaccinated control groups.

The vaccine containing released trypanosome antigens in a water-in-oil emulsion was also tested. Twenty mice were each given 0.1 ml of this vaccine by subcutaneous injection. Groups of the vaccinated animals were challenged on days 37, 119 and 154 after inoculation and the results were as shown in table II. As compared with unvaccinated control groups, a significant level of protection was found only in the last two out of the three groups challenged.

The vaccination experiments with water-in-oil emulsions were done before the method for the estimation of the antigen content of the vaccines was developed. However, from comparison with other results it is known that the dose of antigen was very small, of the order of 0.3 unit.

TABLE III
Immunity after vaccination with multiple emulsions

Interval between vaccination and challenge (days)	No. of mice surviving for at least 14 days after challenge/no. of mice challenged in		P
	vaccinated group	unvaccinated (control) group	
21	3/6	2/7	NS †
77	5/5 *	0/9	< 0.01
182	6/6	0/5	< 0.01

Vaccination of 17 mice was by a single subcutaneous inoculation of disrupted trypanosome bodies derived from *Trypanosoma brucei* stabilate TREU-87 equivalent to 0.75 unit of antigen contained in a multiple emulsion. Twenty-one control mice were inoculated with goat casein in a multiple emulsion. Challenge was by intraperitoneal injection of antilog 1.8 ID63 of stabilate TREU-87.

* One mouse died before the 14th day, but no trypanosomes were seen in its blood.

† NS = Not significant.

Multiple emulsions. Each of a group of 17 mice was given a subcutaneous injection of 0.2 ml of the vaccine containing disrupted trypanosome bodies contained in a multiple emulsion. On assay, the dose of antigen given to each mouse was found to have been 0.75 unit. Control mice were given injections of a multiple emulsion containing goat casein. Groups of mice were challenged on days 21, 77 and 182 after vaccination and the results are recorded in table III. Only the last two out of the three groups challenged showed a significant level of protection.

Aluminium hydroxide. Sixteen mice were given an intravenous injection of 0.2 ml of the vaccine consisting of released trypanosome antigens adsorbed to aluminium hydroxide. Groups of these mice were challenged 30 min., 2 days and 7 days after vaccination and control groups of untreated mice were challenged at the same times. Marginally significant protection was shown by the 30-min. group and this was perhaps due to passive immunity. Mice of the 2-day group were not significantly protected, but significant protection, perhaps ascribable to

active immunity, appeared in the 7-day group. The immunity of the 7-day group was, however, found to have been largely lost by the 159th day after vaccination, when the mice were re-challenged (table IV).

TABLE IV
Immunity after vaccination with antigen adsorbed to aluminium hydroxide

Interval between vaccination and challenge	No. of mice surviving for at least 14 days after challenge/no. of mice challenged in		P
	vaccinated group	unvaccinated (control) group	
30 min.	2/3	0/6	0.08
2 days	3/7	0/12	0.50
7 days	6/6 *	2/6	0.05

Vaccination was by intravenous injection of released antigens derived from *Trypanosoma brucei* stabilate TREU-87, adsorbed to aluminium hydroxide. Challenge of 16 vaccinated mice and 24 unvaccinated control mice was by intraperitoneal injection of 1.8 ID₆₃ of stabilate TREU-87.

* When re-challenged on the 159th day after vaccination, only 2 of the 5 mice remaining survived.

TABLE V
Immunity after vaccination with formalinised antigens

Interval between vaccination and challenge (days)	No. of mice surviving for at least 14 days after challenge/no. of mice challenged in group vaccinated with				
	whole blood		plasma		nil (controls)
	IV	SC	IV	SC	
7	6/7 *	0/7	6/7 *	2/7	0/7
14	7/7 *	...	...	...	2/7
28	7/7 *	1/7	2/6	1/7	0/8
56	7/7 *	...	...	...	0/7
112	6/6 *	...	...	...	0/7

Vaccination was by a single intravenous (IV) or subcutaneous (SC) injection of antigen derived from *Trypanosoma brucei* stabilate TREU-87, either as formalinised whole blood or as plasma from formalinised blood. Challenge of 75 vaccinated and 36 control mice was by intraperitoneal injection of antilog 1.8 ID₆₃ of stabilate TREU-87.

* P = 0.01 or less; probabilities are not given for non-significant comparisons.

Immunity after vaccination with formaldehyde-treated antigens

Groups of mice were given intravenous or subcutaneous injections of formalinised infected whole blood or the plasma derived from it (table V). Each mouse was given a dose of 0.1 ml, adjusted to contain 32 units of antigen. Groups of the vaccinated mice were challenged at intervals (table V). Though

no significant protection was detected in the mice given either whole blood or plasma by the subcutaneous route, those given whole blood intravenously were fully protected up to 112 days after vaccination. The mice given plasma alone by the intravenous route showed a transient protection only.

When re-challenged 177 days after the original inoculation, all the 6 surviving mice that had been given formalinised whole blood intravenously were found still to be protected.

*Simultaneous immunisation
against trypanosomes of four different antigenic types*

Whole blood containing trypanosomes with the antigenic specificities ETat-1, ETat-2, ETat-3 and ETat-4 was obtained from mice that 3 days before had been given an inoculation of a stabilate of one of these antigenic types. In

TABLE VI
*Immunity after vaccination against four different antigenic types of
Trypanosoma brucei*

Antigenic type(s) in vaccine	No. of mice surviving for at least 14 days after challenge/no. of mice challenged with				
	ETat-1	ETat-2	ETat-3	ETat-4	mixture of all four antigenic types
ETat-1 . . .	6/6	1/6	...	...	...
ETat-2 . . .	...	2/6 *	0/1	...	...
ETat-3 . . .	...	...	6/6	0/6	...
ETat-4 . . .	0/6	...	...	6/6	...
Mixture of these four antigenic types	6/6	6/6	6/6	6/6	6/6
Nil (control mice) .	2/6	0/6	0/6	1/6	0/6

Vaccination was by intravenous injection of formalinised whole infected blood containing trypanosomes of the antigenic specificities shown. Challenge was done 14 days later by intra-peritoneal injection of antilog 2.0 ID₆₃ of trypanosomes from stabilates of the antigenic specificities ETat-1, ETat-2, ETat-3 and ETat-4, or with a mixture containing antilog 2.0 ID₆₃ of each of these four antigenic types.

* Only 7 mice were available for this comparison. The inconclusive result was considered to be due to an intercurrent cause of death; the four mice died 2 days earlier than typical for challenge with ETat-2.

each case the blood was collected from mice that showed a similar intensity of parasitaemia. The blood samples were treated with formaldehyde as described under *Materials and methods*.

Mice in four groups were given intravenous injections of formalinised infected blood containing trypanosomes of one or other of the four antigenic types; the dose was 0.1 ml of a mixture of equal volumes of blood and balanced salts solution. Mice in a fifth group were given an intravenous injection of 0.2 ml of a mixture of equal parts of undiluted formalinised blood of the four antigenic types. The dose of antigen administered was, therefore, similar for each of the four types. The inoculations were started 50 min. after the addition

of formaldehyde to the last batch of blood (ETat-4). They were carried out in the order: mixture, then ETat-1, ETat-2, ETat-3 and, lastly, ETat-4. They took 3 hr to complete.

Fourteen days later the mice were challenged with antilog 2.0 ID₆₃ of stabilates with the antigenic specificities ETat-1, ETat-2, ETat-3 and ETat-4, or with a mixture of these stabilates containing antilog 2.0 ID₆₃ of each. The results are given in table VI. Protection against homologous challenge was complete (except in the case of ETat-2, where it is unlikely that the deaths were caused by trypanosomiasis). No protection was evident against heterologous challenge. The mice vaccinated with a mixture of antigens were resistant both to challenge with the individual antigenic types and to challenge with a mixture of the antigens.

DISCUSSION

The method of immunising animals against trypanosomal infection by curing an induced infection with a drug is sometimes open to the criticism that any protection demonstrated to subsequent challenge may be due to residual effects of the drug. In the present experiments (table I) this possibility may be excluded as Lumsden, Herbert and Hardy (1965) have shown that when the drug (Berenil) was used at a higher dosage than that employed in the present experiments, its effects lasted less than 42 days. In our infection-and-cure experiments the mice were still fully protected at that time and the protection persisted for at least 370 days (table I).

Soltys (1965) showed that a single subcutaneous dose of trypanosomes inactivated with β -propiolactone effectively protected mice against subsequent homologous challenge. Protection was demonstrable 7 days after vaccination and was complete at 3 wk. The present work shows that an effective level of protection against homologous challenge may be induced by a single dose of antigen administered in one of several different ways, but that differences occurred in the rate of development of immunity to a protective level. With oil-emulsion adjuvant vaccines inoculated subcutaneously, small quantities of antigen (tables II, III) were effective, but the immunity usually took some weeks to rise to a protective level. This is in agreement with the results of Herbert (1968), who showed that the antibody response of mice to protein inoculated in water-in-oil or in multiple emulsions did not reach a peak till about the 50th day after inoculation. Protective immunity developed much more rapidly, in 7 days, after the intravenous inoculation of formalinised antigens (table V), but in this case the dose of antigen administered to each mouse (80 units) was very large in comparison with those given with oil adjuvants. The immunity produced by formalinised antigens was long lasting when whole infected blood was used, but only transient when the vaccine was composed of released antigens.

The method used to prepare the antigen, whether breaking up the organisms by repeated freezing and thawing, or formalinisation, did not appear to affect its antigenicity for immunisation of mice. Soltys (1965) obtained unsatisfactory protection with a formalinised vaccine, but he used a concentration of formaldehyde (0.2 per cent.) ten times higher than that used in the present work. We

found that the intravenous inoculation of formalinised antigens provided a simple and rapid method of assessing the results of simultaneous immunisation against trypanosomes of several different antigenic types (table VI). A mixed vaccine protected against challenge with trypanosomes corresponding to each of its components, either severally or together, but cross protection between any of the four antigenic types used was not demonstrated.

SUMMARY

Protective immunity against intraperitoneal challenge with *Trypanosoma brucei* of homologous antigenic type was produced in mice by the induction of infection and its cure with a drug. It was also produced by single doses of a variety of vaccines, including killed organisms, released antigens and formalinised whole infected blood or plasma administered in the form of crude, water-in-oil or multiple emulsions by the subcutaneous or intravenous route. Vaccines of mixed antigenic types protected mice against challenge with trypanosomes corresponding to each of its components either severally or together, but cross protection between antigenic types was not demonstrated.

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