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Immunodepression during *Trypanosoma brucei gambiense* infections in the field vole, *Microtus montanus*

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SUMMARY

The effects of *Trypanosoma brucei gambiense* infections upon immune responses were examined in an outbred laboratory colony of field voles, *Microtus montanus*. Antibody levels to challenge with heterologous erythrocytes and bovine serum albumin were significantly depressed in infected animals. Trypanosome infections impaired both primary and secondary humoral responses, although previously established specific antibody levels were not affected by infection. Specific antibody-producing capabilities of previously infected, trypanocidal drug-treated voles were found to be comparable to uninfected controls, within 3 days following chemotherapy. Cell-mediated hypersensitivity responses to oxazolone were also significantly depressed by trypanosome infection; responses to the initial sensitization of oxazolone and to secondary challenge treatments were depressed compared to uninfected controls. Possible mechanisms of trypanosome-induced immunodepression are discussed.

INTRODUCTION

The induction and expression of cell-mediated immunity and the expression of humoral immunity have been found to be impaired during Gambian trypanosomiasis in humans (Greenwood, Whittle & Molyneux, 1973). It is also well established that several species of African trypanosomes in laboratory rodents produce immunosuppressive effects (Goodwin *et al.*, 1972; Murray *et al.*, 1973; Longstaffe, Freeman & Hudson, 1973). Although the mechanisms of this trypanosome-induced immunologic defectiveness are being investigated in *Trypanosoma brucei*-infected rats and mice, these host–parasite models are not totally applicable to the *T. gambiense*–human relationship. *Trypanosoma brucei* infections in laboratory rodents usually result in acute, relatively short-term infections characterized by heavy parasitaemias and severe disturbances in reticuloendothelial function (Goble & Singer, 1960). In contrast, *T. gambiense* infections in humans may exhibit low levels of parasitaemia and chronicity extending over several years.

Seed & Negus (1970) have described the characteristics of *Trypanosoma brucei gambiense* infections in a small microtine vole, *Microtus montanus*. In chronic infections the scanty peripheral parasitaemia, relapsing antigenic waves, neurologic signs and absence of extensive skin lesions suggest that *M. montanus* may represent an excellent host model for human trypanosomiasis. This present investigation was undertaken to characterize the effects of *T. b. gambiense* infections upon some humoral and cellular immune responses of *M. montanus*. We also describe the effects of trypanosome infection upon previously induced levels of specific antibody and upon humoral immunological functions in previously infected, drug-cured voles.

MATERIALS AND METHODS

Animals. Microtus montanus were obtained from an outbred colony maintained at Tulane University in New Orleans' Louisiana. The stock, care and maintenance of the colony has been described by Seed & Negus (1970). All animals used in

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these experiments were sexually mature and maintained on a lighting regime of 12 hr light : 12 hr dark. Animals were immunized, infected and killed between 10.00 and 14.00 hours to obviate variations resulting from possible circadian rhythms.

Parasites. The Wellcome TS strain of *T. b. gambiense* was used throughout these investigations. It has been continuously passaged in rodents since its isolation from a human patient in 1921. The strain was maintained by passage every second day through 25 g white mice. Blood trypanosomes were harvested from mice as described by Seed & Baquero (1965) and suspended in glucose–Ringer's phosphate buffer (Seed & Gam, 1966). Each vole was infected by a single intraperitoneal injection of 1×10^6 organisms suspended in glucose–Ringer's phosphate buffer. *Microtus montanus* have been demonstrated to be susceptible to *T. b. gambiense* and approximately 22% of infected animals undergo chronic infections analogous to that observed in humans (Seed & Negus, 1970). Except where otherwise noted, infected animals exhibited parasitemia for a minimum of 14 days prior to immunologic study.

Immunization and serology. Antibody responses to heterologous (human type B+) erythrocytes were induced in infected and control animals via intraperitoneal injections of 10^7 thrice washed red blood cells suspended in phosphate-buffered saline (PBS), pH 7·2. Animals were killed (chloroform overdose), then immediately exsanguinated via cardiac puncture. Unless otherwise noted, animals were killed for blood collection 7 days following the last immunization. Prior to use the serum was heated to 56°C for 30 min.

To examine the effects of previous infection upon humoral immune responses the following tests were performed. In one experiment voles infected for over 21 days and uninfected animals received two intraperitoneal injections of 1 mg of Suramin (Bayer 205), a trypanocidal drug, in sterile saline 6 days apart. Two-thirds of the animals were immunized with human erythrocytes 7 days after the second immunization. One-third of the animals (five uninfected and five 21 day infected) which received drug treatment were maintained as controls and not immunized. In a second experiment animals infected for at least 14 days and uninfected voles were given a single antigen (human RBC) administration either 3, 6 or 9 days following the second drug treatment and were bled 7 days following immunization.

Haemagglutination titres were performed by adding an equal volume of 2% washed human type B+ erythrocytes to doubling-dilutions of inactivated vole sera in microtitrator plates (Limbro Incorporated). The pattern of agglutination was read before and after gentle swirling and after 24 hr incubation at room temperature. Haemagglutination titres were performed twice for each serum sample.

Immunocytoadherence tests were performed as follows. After exsanguination, spleens were removed and teased apart in sterile, cold (4°C) Eagle's minimum essential medium (MEM). The suspensions were filtered through two layers of gauze, and centrifuged for 10 min at 100 *g*. Erythrocytes were lysed by osmotic shock by resuspending cells for 15 sec in distilled, sterile water. The suspension was then centrifuged at high speed in a clinical centrifuge for 1 min, then resuspended in MEM. After twice washing in MEM, spleen cells were resuspended to a concentration of $1{\cdot}0 \times 10^7$ cells/ml in MEM. Cells were maintained at 4°C during all manipulations.

One half of a ml of the spleen cell suspension in MEM and 0·5 ml of a standardized human type B+ erythrocyte suspension ($6{\cdot}0 \times 10^6$ cells/ml) were incubated at room temperature for 20 min with gentle shaking every 5 min. Twenty-five micro litres of this mixture was pipetted onto a microscope slide, previously delineated with an area of 20×20 mm with a wax pencil. A cover glass was added and the preparation was examined microscopically at × 100 magnification. Spleen cells with at least three erythrocytes adhering were classified as rosettes. Four preparations were examined for each suspension, constituting a total volume of 100 micro litres (or about 5000 spleen cells). This technique is similar to the immunocytoadherence method of Biozzi *et al.* (1966) and Cornille (1969).

Infected and control animals were tested for their ability to produce antibodies to bovine serum albumin (BSA) (Calbiochem). Animals were immunized intramuscularly with 0·5 mg BSA in Freund's complete adjuvant on day 1. On day 7 one-third of the animals were infected with trypanosomes. On day 14 another one-third were infected and all animals received a booster dose (same as above) of antigen in Freund's incomplete adjuvant. All animals were exsanguinated by cardiac puncture 7 days after the last immunization. Precipitin titres were determined by the Ouchterlony technique. Doubling-dilutions of vole serum were placed in wells cut into 1% Ionagar no. 2 (Consolidated Laboratories, Incorporated) opposite BSA (1 mg/ml) and the plates allowed to incubate for 24 hr at room temperature. Precipitin titres were determined by recording the highest serum dilution which resulted in a detectable precipitin band, and were performed twice for each serum sample.

Cell-mediated immunity. Cell-mediated immunity was examined by investigating the ability of infected and control voles to respond to oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one) (Aldrich Chemical Company, Incorporated) according to the ear thickening technique of DeSousa & Parrott (1969). Both ears of each animal were given a primary sensitization of two drops of 10% oxazolone in absolute ethanol. Ears were measured with a dial gauge micrometer prior to each treatment, then again 24 hr after each treatment. Secondary applications were made using 1% oxazolone in olive oil to each ear, 14 days after primary sensitization. Ear measurements were also performed on uninfected and infected non-treated animals in order to determine the effects of measurements and infection upon unsensitized animals.

Statistical analyses. Haemagglutination and precipitation antibody titres are reported as $\log_{10}$ values of the geometric group means ± standard deviations (s.d.). Comparisons between group means were made by use of Student's *t*-test. Differences were considered statistically significant at the 95% confidence level ($P < 0{\cdot}05$).

RESULTS

Microtus montanus were found to possess 'natural' haemagglutinins to human type B+ erythrocytes in

TABLE 1. The humoral response of *Microtus montanus* infected with *Trypanosoma brucei gambiense* to human type B+ erythrocytes

Expt	Serological test	Group	Condition	No. of animals	Immunization	Results	Significance
I	Haemagglutination*	A	Controls	8	Primary§	2·38±0·18	(A) *vs* (B) $P<0·001$
		B	Infected†	12	Primary	0·81±0·30	(B) *vs* (D) n.s.
		C	Controls	8	Primary+secondary¶	3·12±0·25	(A) *vs* (C) $P<0·001$
		D	Infected	10	Primary+secondary	1·07±0·42	(C) *vs* (D) $P<0·001$
II	Immunocyto-adherence‡	E	Controls	4	Primary	353±106	(E) *vs* (F) $P<0·05$
		F	Infected	5	Primary	136±71	(F) *vs* (H) n.s.
		G	Controls	4	Primary+secondary	975±216	(E) *vs* (G) $P<0·01$
		H	Infected	4	Primary+secondary	162±82	(G) *vs* (H) $P<0·001$

* Haemagglutination results expressed as $\log_{10}$ values of geometric means of titres ± s.d.
† All infected animals were infected for a minimum of 7 days prior to primary immunization.
‡ Immunocytoadherence results recorded as mean numbers of rosettes per 5000 spleen cells ± s.d.
§ Primary: animals received 10^7 human RBC via intraperitoneal injection on day 1 and bled on day 8.
¶ Primary+secondary: animals received 10^7 human RBCs by intraperitoneal injection on days 1 and 8 and bled on day 15.

very low levels (<2). In voles infected with *Trypanosoma brucei gambiense* for about 14 days, haemagglutinin levels rose slightly, but were still very low (<4). Uninfected animals responded to both primary and primary and secondary injections of human type B+ erythrocytes with significant antibody responses (Table 1). Trypanosome-infected voles demonstrated depressed haemagglutinin titres after primary ($P<0·001$) or primary and secondary ($P<0·001$) erythrocyte injections compared to uninfected controls. Furthermore, in infected animals no significant rises in antibody levels were exhibited following secondary antigen injections, compared to levels found in infected animals receiving only a primary immunization.

Prior to immunization neither uninfected nor trypanosome-infected animals exhibited significant numbers of human type B+ erythrocyte-adhering spleen cells (less than twenty rosettes per 5000 spleen cells). Following immunization, the number of rosettes rose in both uninfected and infected voles (Table

TABLE 2. Effect of *Trypanosoma brucei gambiense* infections in *Microtus montanus* on pre-existing states of humoral immunity to human type B+erythrocytes

Group	No. of animals	Experimental schedule				Haemagglutinin titre‡	Significance
		Day 1	Day 8	Day 15	Day 22		
A	14	Imm*		Bled		2·29±0·42	(A) *vs* (B); (C) *vs* (D) n.s.
B	16	Imm	Inf†	Bled		2·12±0·48	(A) *vs* (C); (B) *vs* (D) $P<0·001$
C	12	Imm	Imm		Bled	3·04±0·25	
D	14	Imm	Imm	Inf	Bled	3·00±0·29	(E) *vs* (A) or (B) n.s.
E	14	Imm	Imm and Inf		Bled	2·27±0·46	(E) *vs* (C) or (D) $P<0·05$

* Imm: animals immunized with 10^8 human type B+ erythrocytes by intraperitoneal injection.
† Inf: animals infected with 10^6 organisms by intraperitoneal route.
‡ Titres expressed as $\log_{10}$ values of the geometric means ± s.d.

1). However, the mean number of spleen cells demonstrating immunocytoadherence was significantly depressed in infected animals compared to controls following either primary ($P<0{\cdot}05$) or primary and secondary ($P<0{\cdot}001$) immunizations. Again, infected animals exhibited no significant increase in immunocytoadherence following secondary immunization.

Trypanosome inoculation subsequent to the formation of antibody to human erythrocytes did not appear to affect previously acquired states of humoral immunity (Table 2). Groups A and B animals demonstrated that infection 7 days after primary immunization did not significantly affect haemagglutinin levels. Similar results were observed when animals were infected 7 days after secondary immunization (see groups C and D). The results for group E animals suggested that infecting between primary and secondary immunizations significantly inhibited ($P<0{\cdot}05$) secondary responses (compare groups E and C or D), yet did not significantly alter previously induced, primary antibody responses (compare groups E and A or B).

Haemagglutinin production to human erythrocytes in uninfected and 21 day infected voles were examined following chemotherapeutic treatment with Suramin (Table 3, Experiment 1). Unimmunized controls (uninfected and 21 day infected animals) possessed low background haemagglutinin titres.

TABLE 3. Effect of previous *Trypanosoma brucei gambiense* infection in *Microtus montanus* on humoral response to human type B+erythrocytes

Experiment 1

Group	Condition*	No. of animals	Immunized†	Haemagglutinin titre‡
A	Uninfected	5	−	0·03±0·09
B	Uninfected	10	+	2·99±0·30
C	21 day infected	5	−	0·21±0·15
D	21 day infected	8	+	2·89±0·35

Experiment 2

Group	Condition*	No. of animals	Days between last drug treatment and immunization§	Haemagglutinin titre‡
E	14 day infected, no drug treatment	9	−	0·88±0·24¶
F	Uninfected	6	3	2·21±0·45
G	14-day infected	6	3	2·00±0·31
H	Uninfected	6	6	2·31±0·36
I	14 day infected	6	6	2·31±0·15
J	Uninfected	6	9	2·06±0·44
K	14 day infected	6	9	2·36±0·35

* All groups except group E treated with two 1 mg intraperitoneal doses of Suramin (Bayer 205) 6 days apart.

† Immunized animals received 10^7 human RBCs by intraperitoneal injections 7 and 14 days following last Suramin treatment and bled 7 days after second antigen administration.

‡ Titres expressed as $\log_{10}$ values of the geometric means± s.d.

§ All animals (including group E) received 10^7 human RBC by intraperitoneal injection and bled 7 days later.

¶ Mean titre for group E significantly less ($P<0{\cdot}001$) than mean values for groups F–K.

Following immunization, the haemagglutinin titres in uninfected and 21 day infected, drug-treated voles were not significantly different (compare groups C and D).

The time lapse involved in restoration of normal immune responsiveness following chemotherapeutic cure of trypanosome infection was examined (Table 3, Experiment 2). Antibody responses to a single challenge with erythrocytes were significantly ($P<0{\cdot}01$) higher, within 3 days of trypanocidal chemotherapy, in 14 day infected, drug-treated voles than in untreated, 14 day infected animals. Two drug doses were administered 6 days apart, so it is possible that infections were partially or totally eliminated 6 days prior to values shown in Table 3. A comparison of haemagglutinin titres in Tables 1 and 3 suggests that Suramin is neither enhancive nor suppressive upon antibody responses to human erythrocytes in *M. montanus*.

TABLE 4. The humoral response of *Microtus montanus* infected with *Trypanosoma brucei gambiense* to bovine serum albumin (BSA)*

Group	Condition	Duration of infection	No. of animals	Precipitation titres†	Significance
A	Uninfected controls	—	12	1·4±0·28	(A) *vs* (B) $P<0{\cdot}001$
B	Infected	7 days	10	0·60±0·35	(A) *vs* (C) $P<0{\cdot}001$
C	Infected	14 days	9	0·52±0·34	(B) *vs* (C) n.s.

* All animals received 0·5 mg BSA intramuscularly in Freund's complete adjuvant on day 1 and 0·5 mg BSA intramuscularly in Freund's incomplete adjuvant on day 15, then were bled on day 22.

† Titres expressed as $\log_{10}$ values of the geometric means± s.d.

Anti-BSA responses were examined in uninfected control and trypanosome-infected *M. montanus* (Table 4). Animals infected for 7 days prior to secondary immunization (group C) had similar responses to those infected on the same day as secondary immunization (group B). In both groups, there were significant ($P<0{\cdot}001$) depressions in precipitin titres compared to uninfected controls.

Experiments designed to examine the effects of trypanosome infections on cell-mediated immune responses of voles to oxazolone were performed (Table 5). Examining only the 'treated' values indicated that no significant differences were measured between uninfected and infected animals after either primary sensitization or secondary challenge. Non-treated controls were examined and 'adjusted' values

TABLE 5. The changes in ear thicknesses in uninfected and *T. b. gambiense* infected *Microtus montanus* in response to oxazolone treatment*

	Mean increases in ear thicknesses± s.d. (in mm)			
	Treated	Non-treated	Adjusted‡	Significance
Primary sensitization (1°)				
A. Uninfected controls	(10)† 0·29±0·12	(12) 0·01±0·02	(10) 0·28±0·12	(A) *vs* (B) $P<0{\cdot}02$
B. Infected 3 days prior to 1°	(10) 0·18±0·11	(10) 0·08±0·10	(10) 0·10±0·15	
Secondary challenge (2°)				
C. Uninfected controls	(10) 0·46±0·10	(12) 0·01±0·02	(10) 0·45±0·10	(C) *vs* (D) $P<0{\cdot}001$
D. Infected 3 days prior to 1°	(11) 0·34±0·18	(10) 0·18±0·08	(11) 0·16±0·20	(C) *vs* (E) $P<0{\cdot}05$
E. Infected between 1° and 2°	(10) 0·46±0·11	(10) 0·14±0·10	(10) 0·32±0·15	

* Measurements performed just prior to and 24 hr after oxazolone treatment according to the method of DeSousa & Parrott (1969).

† Number in parentheses represents number of animals tested.

‡ Adjusted values computed by subtracting non-treated mean values from treated mean values; standard deviations of adjusted values were calculated as $\sqrt{(\text{s.d. treated})^2+(\text{s.d. non-treated})^2}$.

were calculated (mean treated values minus mean non-treated values) to ensure that changes in ear thickness were due solely to contact hypersensitivity to oxazolone sensitization. Using these 'adjusted values', it can be seen that, although infected animals responded to oxazolone, their responses were depressed compared to uninfected controls, after both a primary sensitization ($P<0{\cdot}02$) and secondary challenge ($P<0{\cdot}001$). By comparing the mean adjusted value for those animals infected between primary and secondary oxazolone application (group E) to the mean adjusted value for uninfected controls receiving both treatments (group C), it can be seen that trypanosome infection seemed to impair secondary cell-mediated responses ($P<0{\cdot}05$). Indeed, the adjusted mean value for group E is not statistically different from the adjusted mean value of animals receiving only a single primary sensitization (group A).

DISCUSSION

The results of the present investigation suggest that *Trypanosoma brucei gambiense* infections in *Microtus montanus* depress haemagglutinin titres (humoral levels and cellular production) to heterologous erythrocytes and precipitin titres to soluble bovine serum albumin. Also, using adjusted values to compensate for oedematous swelling during infection, oxazolone-induced contact hypersensitivity responses were depressed in voles infected with *T. b. gambiense* compared with values for uninfected controls. Similar impaired cell-mediated responses have been reported to occur during *T. brucei* infections in mice, when oxazolone was used to induce sensitivity (Urquhart *et al.*, 1973; Murray *et al.*, 1974). However, several other groups of researchers have reported that in mice and guinea-pigs infected with *T. brucei*, normal responses to oxazolone were demonstrated (Freeman *et al.*, 1973; Murray *et al.*, 1973; Longstaffe, 1974). These discrepancies may be due to the different strains of parasites or hosts used, or in the failure by some groups of investigators to utilize 'adjusted' values to compensate for non-specific increases in background levels of cell-mediated reactions during infection.

The results of this study also suggest that *T. b. gambiense* infections in *M. montanus* do not significantly alter previously existing states of specific humoral immunity to heterologous erythrocytes. This information, coupled with the observation that infected animals and humans respond vigorously to trypanosomal antigens, suggests that trypanosomal-induced immunodepression does not manifest itself via a generalized impairment in protein or immunoglobulin synthesis nor via increased rates of immunoglobulin catabolism.

Finally, it was observed that humoral responses quite rapidly achieved uninfected states in previously infected voles following trypanocidal chemotherapy (Suramin). *Microtus montanus* immunized with human erythrocytes as early as 3 days following drug treatment responded with haemagglutinin titres comparable to uninfected control levels, suggesting that live trypanosomes may be necessary for the observed immunodepressive effects. These results are similar to those of Murray *et al.* (1973), who found that antibody responses to sheep erythrocytes and cell-mediated reactions to oxazolone were restored to normal within several days following chemotherapy in *T. brucei*-infected mice. The observations that histologic alterations in the immune system of *T. brucei*-infected rodents are sustained for some weeks following trypanocidal therapy (Murray *et al.*, 1974), yet immunologic capabilities are rapidly restored, reinforces the suggestion that an active infection may be necessary for immunodepression. The preliminary report of Longstaffe (1974), who found that *T. brucei* organisms added to cultures of normal lymphocytes depressed mitogenic responses to phytohaemagglutinin, is also consistent with this view. The direct immunodepressive properties of *Trypanosoma brucei gambiense* need to be further examined to see if live cells are necessary, or whether a cell fraction or secretory product can produce depressed responses in immunocompetent cells *in vitro*.

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