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Trypanosoma musculi Infection in B-Cell-Deficient Mice

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The course of infection with *Trypanosoma musculi* was assessed in mice deprived of B-lymphocytes by administration, from birth, of rabbit antiserum to mouse immunoglobulin M (IgM). Initial control of parasitemia leading to the first crisis and establishment of the plateau phase was unaffected by lack of B-lymphocyte function, although multiplicative forms persisted throughout the infection in anti-IgM-treated mice, instead of disappearing after the first crisis as in intact mice. Elimination of trypanosomes after the second crisis was not observed in anti-IgM-treated mice, which maintained high numbers of parasites in the blood and peritoneal cavity, resulting in some mortality. A temporary reduction in parasitemia was achieved in anti-IgM-treated mice by transfusion of immune plasma. Immunodepression, as measured by splenic mitogen responsiveness, and splenomegaly were both observed in anti-IgM-treated as well as in intact mice, indicating that these features of murine trypanosomiasis are independent of B-lymphocyte function. Since in *T. musculi* infection parasitemia can be controlled initially but not eliminated in mice lacking B-cell function, the only crucial protection provided by antibody would appear to be in curing the infection after the second crisis.

Trypanosoma musculi, a natural parasite of mice, produces a self-limiting infection of about 25 days duration which is characterized by a series of distinct phases (23). After a short prepatent period, rapidly increasing parasitemia (growth phase) ensues, with multiplicative forms (epimastigotes and dividing parasites) present in the blood. The parasitemia then stabilizes (plateau phase) after the first crisis, and multiplicative forms disappear, leaving a monomorphic population of long, slender, adult trypomastigotes. Complete clearance of trypanosomes from the blood (elimination phase) is seen after termination of the plateau phase at the second crisis. Parasites are found only in the *vasa recta* of the kidneys after this time, and mice are immune to reinfection (24). Dividing forms of *T. musculi* are seen in the peritoneal cavity throughout the infection but disappear when the parasites are eliminated from the blood (13). In T-cell-deprived mice, the curve reaches a plateau but at a higher level of parasitemia than in normal mice, and there is no elimination phase (4, 18, 20, 23).

Initial control of parasitemia in *T. musculi* infection was ascribed in earlier work by Viens et al. (20, 23) to the joint action of (i) a thymus-dependent "ablastin" which inhibits parasite reproduction (analogous to an immunoglobulin G (IgG) antibody with this property [7] seen in *Trypanosoma lewisi* infection) and (ii) a thymus-independent trypanocidal antibody (IgM) which removes newly formed parasites. More recently, these authors have suggested that trypanocidal mechanisms alone can account for the establishment and maintenance of the plateau phase (22). Others, however, have presented evidence for the production of an ablastic antibody, presumed to be IgG, in *T. musculi* infection (5). The mechanism responsible for elimination of parasites after the second crisis appeared not to depend on a direct trypanocidal antibody but, nevertheless, to involve sensitized theta-negative lymphocytes (17, 21, 24). Passive transfer of immune serum did not influence the speed of recovery, but immune theta-negative spleen or peritoneal lymphocytes provided protection in T-cell-deprived mice (17, 21).

The present experiments were carried out to clarify the role of antibody in controlling parasitemia during the course of this infection. For this purpose, the infection was followed in mice deprived of B-lymphocytes by the administration, from birth, of rabbit antiserum to mouse IgM. In such mice, there is selective impairment of B-cell development, leading to a pan-specific suppression of the synthesis of all classes of immunoglobulin (8, 14, 16), whereas T-cell function remains intact (8, 15). The results reported herein have demonstrated that initial control of the parasitemia is relatively unaffected by lack of B-cell function, whereas the parasites cannot be eliminated from the blood under such conditions.

(A preliminary report of these findings has already been published [12].)

MATERIALS AND METHODS

Abbreviations. p.i., postinfection; IgM, immunoglobulin M; IgG, immunoglobulin G; NRS, normal rabbit serum; NT, no treatment; LPS, lipopolysaccharide; ConA, concanavalin A; PHA, phytohemagglutinin.

Experimental animals. Specific-pathogen-free 12- to 14-day-pregnant C57BL/6 female mice were purchased from Charles River Breeding Laboratories, St. Constant, Quebec, and housed in sterilized cages in a sterile hood to provide filtered air. They received sterilized food, water, and bedding. The offspring were weaned at 4 weeks of age and maintained under the same conditions throughout the experiments.

Albino male rabbits, weighing 1.5 kg each, were purchased from Charles River.

Infection of mice with parasites. An inoculum of 10^4 viable *T. musculi*, Partinico II strain (23), was administered intraperitoneally to mice under light ether anesthesia.

Measurement of infection. Individual heparinized blood samples were taken every 2 to 3 days p.i. or at longer intervals where indicated from the retroorbital sinus. If the parasitemia was low, infection was followed by counting

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parasites on wet blood films. Five-lambda blood was dispersed with a cover slip (22 by 40 mm) on a glass slide, and the parasites were enumerated microscopically with a 25× objective and 10× ocular. When more than five parasites per high power field were present, hemacytometer counts were made of infected blood diluted 1:100 with saline containing 0.002% Formalin. The number of parasites per milliliter of blood was calculated and expressed as $\log_{10}$. The percentage of epimastigotes and dividing forms (indicative of reproductive activity) was estimated by examination of 100 trypanosomes on blood films (23) stained with Diff-Quik (Harleco, Gibbstown, N.Y.).

Measurement of antitrypanosomal antibodies. Sera obtained during infection and after recovery were assayed for specific antitrypanosomal IgM, IgG1, and IgG2 antibodies using indirect fluorescence antibody titration (23). The endpoint was read as the highest dilution of serum giving a strongly positive fluorescence. This was expressed as the antibody titer of the serum.

Preparation of anti-IgM antiserum. Rabbits were immunized with purified mouse IgM myeloma protein, MOPC 104E (Litton Bionetics Inc., Kensington, Md.) in two subcutaneous injections of 100 μg in Freund complete adjuvant 2 weeks apart, followed by a third injection of 100 μg of IgM in saline. Bleeding was performed at weekly intervals thereafter, and the rabbits were exsanguinated at week 3. The pooled antisera were partially purified by 50% ammonium sulfate precipitation, followed by DEAE cellulose (Whatman, England) column chromatography, to obtain the IgG fraction (hereafter referred to as anti-IgM). The preparation was concentrated by ultrafiltration through a PM 30 diaflo-ultrafilter (Amicon Corp., Lexington, Mass.), rendered isotonic by washing with Dulbecco phosphate-buffered saline (GIBCO, Grand Island, N.Y.) and adjusted to 35 to 40 mg of protein per ml, after which it was passed through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) and stored in small portions at −20°C. By immunodiffusion analysis in agar, the anti-IgM preparations gave precipitin bands against purified IgM in dilutions of 1:32 to 1:64. NRS (GIBCO) was processed in an identical fashion.

Immune plasma. Mice that had recovered from *T. musculi* infection were heparinized and exsanguinated by cardiac puncture 24 days p.i. The plasma was separated by centrifugation and stored at −20°C. Normal mouse plasma was prepared similarly.

Preparation of anti-IgM-treated mice. Mice were injected intraperitoneally with a standard dose (3.5 to 4 mg) of anti-IgM thrice weekly, starting within 24 h of birth. Control mice received either NRS on the same schedule or NT. At 6 weeks of age the B-cell-suppressed status of the anti-IgM-treated mice was monitored by testing a small serum sample (obtained from tail vein blood taken 48 h after the last antiserum injection) for the absence of mouse IgM and presence of an excess of rabbit anti-IgM. These criteria have previously been shown to correlate with a state of suppression of immunoglobulin synthesis (3). Since it was equally important to demonstrate the presence of normal T-cell function in anti-IgM-treated mice, a small number of these animals and NRS-treated mice were randomly selected from those to be used for experiments and tested for skin allograft reactivity. The mice received *H-2* incompatible A/J (Jackson Laboratories, Bar Harbor, Maine) strain full-thickness skin grafts by standard techniques, and the endpoint of graft rejection was scored as the time at which the graft was 90% scabbed over (11). In all cases, skin allograft rejection time was the same (9 to 10 days) for the anti-IgM-treated and control groups of mice, demonstrating by this criterion that T-cell function was intact.

Splenic responses to T- and B-cell mitogens. Spleen cells were tested for responsiveness to the mitogens *Escherichia coli* LPS (Difco Laboratories, Detroit, Mich.), ConA (Pharmacia, Uppsala, Sweden), and PHA (Wellcome Laboratories, England) by assessing the incorporation of tritiated thymidine by cells incubated with each mitogen. The method followed was that described by Kirchner et al. (10). A range of mitogen doses was used, and the peak response, expressed as mean counts per minute of triplicate samples, is reported.

RESULTS

Course of *T. musculi* infection in anti-IgM-treated and control mice. Groups of five mice, anti-IgM treated, NRS treated, or given NT, were inoculated at 6 weeks of age with 10^4 *T. musculi*, and blood parasitemia was followed throughout the course of infection (Fig. 1). Initially, there was a delay in the rise of parasitemia in the anti-IgM-treated mice, but by day 9 p.i., the establishment of the plateau phase was seen to occur at the same level in all groups. The elimination of parasites from the blood in control NRS-treated and NT mice between days 16 and 21 p.i. was not observed in the anti-IgM-treated mice, which retained high parasitemias until death occurred or the experiment was terminated.

In control mice, multiplicative forms were not seen after day 11 p.i., whereas in anti-IgM-treated mice, epimastigotes and dividing forms persisted throughout the infection (Fig. 1). In addition, the peritoneal cavity of anti-IgM-treated-mice became distended with ascitic fluid, which, upon microscopic examination, was found to contain high numbers of trypanosomes in various stages of division.

This experiment was repeated twice more with similar findings, except that, in one case, anti-IgM treatment was terminated at the start of the infection. Under this protocol the mice eventually recovered from the infection by day 70 p.i., after escape from suppression of antibody production, as evidenced by detection of IgM and IgG antibodies in the serum by day 60 p.i. (results not shown).

Production of antitrypanosomal antibodies in anti-IgM-treated and control infected mice. In the experiment shown in Fig. 1, the production of antitrypanosomal IgM, IgG1, and IgG2 antibodies was also measured in each group of mice. In control NRS-treated and NT mice, IgM antibodies were detected by days 3 and 5 p.i., respectively, and IgG1 and IgG2 antibodies were detected by day 9 p.i. The titers ranged between 1:8 and 1:256. In contrast, no specific antitrypanosomal antibodies were detected in the anti-IgM-treated hosts at any time during the entire course of the experiment.

Transfer of immune plasma to anti-IgM-treated infected mice. A small number of anti-IgM-treated *T. musculi*-infected mice were injected intravenously with immune plasma with two 0.5-ml doses 24 h apart, starting at 38 days p.i. Other mice received normal mouse plasma as a control.

Transfer of immune, but not normal, plasma to anti-IgM-treated mice resulted in a dramatic transitory reduction in the blood parasitemia in two of three mice (Fig. 2). Normal mouse plasma had no effect. In addition, the parasites in the peritoneal cavity (obtained by needle biopsy) were observed to decrease tremendously in number; in one mouse this was complete by 24 h after transfer of the first dose of immune plasma.

In vitro splenic response to mitogens in anti-IgM-treated and control infected mice. To monitor the immune status of

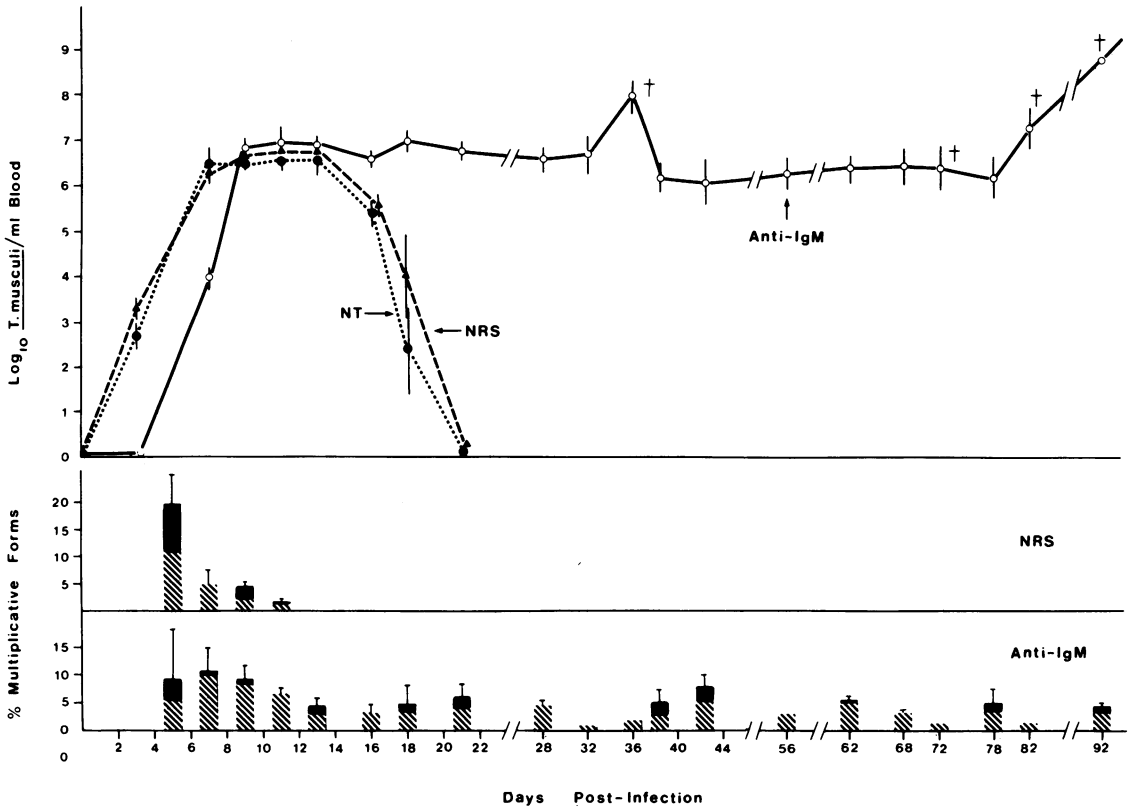

FIG. 1. Course of infection with *T. musculi* in anti-IgM-treated (○), NRS-treated (▲), and NT (●) mice inoculated with 10^4 trypanosomes on day zero. Percentage of multiplicative forms (epimastigotes [▨] and dividing parasites [■]) are shown for anti-IgM- and NRS-treated groups on the bar graphs; values for NT (not shown) were similar to those of the NRS-treated group. Each point represents the mean of five mice ± standard error of the mean. †, Death of individual mice.

anti-IgM-treated and control mice during the course of *T. musculi* infection, the spleen cells of infected mice were tested in vitro for their responsiveness to ConA, PHA, and LPS at 0, 14, and 21 days p.i. The experiment was performed concurrently with that shown in Fig. 1, using mice randomly selected from the same experimental and control batches and infected with 10^4 trypanosomes.

The mitogen responses obtained with the optimum dose of each mitogen are shown in Table 1. It can be observed that there is no response to the B-cell mitogen, LPS, by spleen cells from anti-IgM-treated mice. These cells do, however, respond to the T-cell mitogens, PHA and ConA. The values at day zero differ from those of spleen cells obtained from control (NRS treated and NT) mice, but this is hardly surprising since the cellular composition of the spleen in the anti-IgM-treated mice, lacking B-cells, is not normal; indeed, similar differences in mitogen responses between anti-IgM- and NRS-treated mice have been reported by others (8). The essential point is that the splenic responses to PHA and ConA were positive in the anti-IgM-treated mice at day zero of infection.

During the course of the infection, the mitogen responses of the spleen cells become suppressed. In the control groups, maximal suppression occurred at day 14, during the plateau phase of parasitemia, and lessened by day 21, after the elimination phase. In the anti-IgM-treated mice, immunosuppression (which can be observed in the ConA and PHA responses) developed similarly by day 14 but in this case was maintained or even increased (in the case of the PHA response) by day 21. In these mice, of course, the parasites were not eliminated.

Spleen size in anti-IgM-treated and control infected mice. In the preceding experiment, splenomegaly was observed in the spleens of *T. musculi*-infected mice. In control mice, splenic enlargement was maximal on day 14 p.i., whereas in anti-IgM-treated mice it was greatest on day 21 p.i. (values not shown). Four weeks after infection, the total number of nucleated spleen cells was measured in randomly selected mice taken from the same batches as those used in the experiments described above. The values were 261×10^{-6} and 101×10^{-6} nucleated cells per spleen for the anti-IgM-treated and NRS-treated groups, respectively, i.e., two to three times greater in the former group.

DISCUSSION

The course of infection with *T. musculi* falls into three phases, namely, the initial growth phase, the plateau phase, and the elimination phase. It is apparent that the protective immunity which develops in the host during the course of the infection involves a plurality of mechanisms. The purpose of the present experiments, using anti-IgM-treated mice which lack demonstrable B-cell function, was to clarify the role played by antibody with regard to each phase of the infection.

The growth of the parasite population during the initial phase up to and including the first crisis can be controlled by inhibiting parasite reproduction, by destroying newly formed

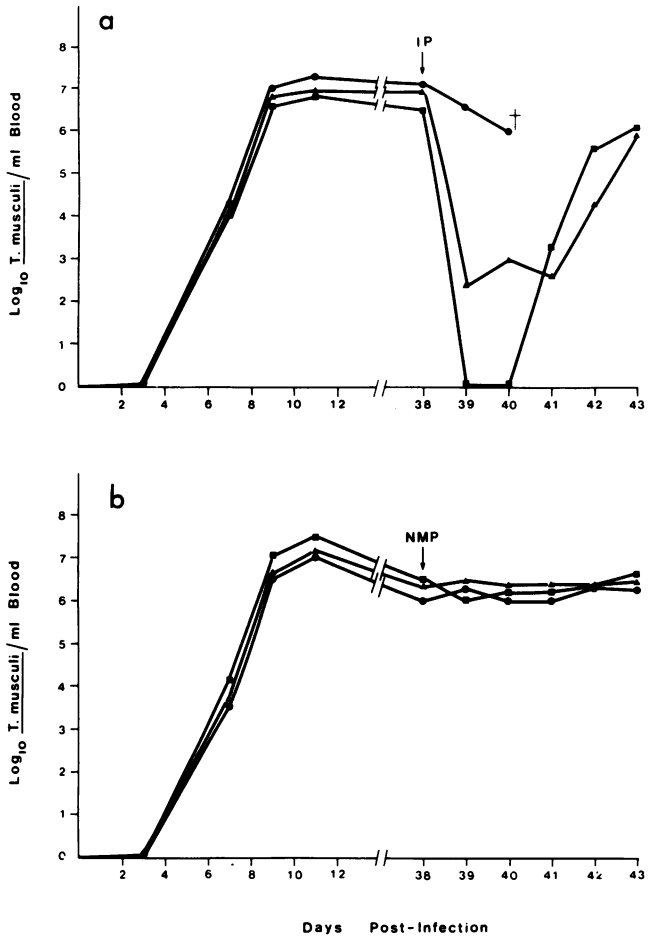

FIG. 2. Effect of transfusing anti-IgM-treated *T. musculi*-infected mice with (a) immune plasma from recovered (24 day p.i.) donors or (b) normal plasma. Two 0.5-ml intravenous injections were given 24 h apart; the arrow indicates the first dose. Individual values are shown for each mouse. †, Death.

parasites, or by both mechanisms. Thus, it has previously been proposed that the initial control of growth is mediated by two humoral factors: one, an ablastic (reproduction inhibition) factor, possibly antibody in nature (5, 20), and two, an early trypanocidal antibody which is cytotoxic to the young forms of the parasite (20). Our findings have suggested that, indeed, antibodies of this nature are produced, since epimastigotes and dividing forms persist in significant numbers over the whole period of infection in B-cell-deprived hosts, whereas they disappear from the circulation after the first crisis in intact mice (Fig. 1). However, the interesting observation is that, in B-cell-deprived mice in which there are no detectable antitrypanosomal antibodies, the first crisis still occurs normally and the parasitemia stabilizes initially at the same plateau level as that seen in control hosts. Thus, it is evident that a mechanism other than acquired humoral immunity must be responsible primarily for controlling parasite growth in the initial phase of the infection. Antibody may assist by removing young forms and inhibiting reproduction to some extent but cannot be said to play a crucial role in bringing about the first crisis.

In the final stage of infection, when the parasites are abruptly eliminated after the second crisis, antibody appears

TABLE 1. In vitro reactivity of spleen cells[a] of anti-IgM-treated and control mice to mitogens at various times after infection with *T. musculi*

Mitogen (μg)	[^{3}H]thymidine incorporated[b] (cpm)									
	Anti-IgM treated			NRS treated			NT			
	0[c]	14	21	0	14	21	0	14	21	
None	3,782 ± 1,390	3,146 ± 222	4,002 ± 1,017	6,720 ± 1,219	6,520 ± 120	3,852 ± 504	5,739 ± 938	7,182 ± 730	9,055 ± 1,008	
LPS (5)	1,862 ± 79	2,279 ± 181	2,406 ± 170	57,073 ± 6,416	12,080 ± 1,218	30,505 ± 3,450	73,250 ± 14,992	26,608 ± 4,100	42,414 ± 2,450	
PHA (2.5)	44,400 ± 6,821	24,479 ± 3,114	5,643 ± 324	163,403 ± 1,756	30,504 ± 13,320	39,680 ± 6,765	188,632 ± 582	46,140 ± 2,774	102,047 ± 1,711	
ConA (5)	79,192 ± 1,033	3,910 ± 323	4,596 ± 188	245,673 ± 18,666	32,107 ± 5,830	116,453 ± 1,814	408,556 ± 25,909	51,989 ± 3,019	154,439 ± 5,933	

[a] Pooled cells from three mice per group.
[b] Mean of triplicate samples ± standard error of the mean.
[c] Day of infection. For day zero point, mice were infected 1 h before cell harvest, but similar results (not shown) were obtained from noninfected groups of mice.

to play a crucial role, since elimination of parasitemia does not occur in B-cell-deprived mice (Fig. 1) but can be induced, at least temporarily, by passive transfer of immune plasma (Fig. 2). This finding supports the observation that a theta-negative cell, presumably a B-cell, plays a role in this phase of the response (17, 21). More recent evidence showing that passive transfer of immune plasma eliminates parasitemia in normal infected mice supports this hypothesis (D. Wechster and P. A. L. Kongshavn, submitted for publication). Since neither T-cell- (23) nor B-cell (Fig. 1)-deprived mice are able to eliminate *T. musculi* from the blood, it seems clear that a T-cell-dependent antibody must be involved in mediating this phase of the host response. We have recently provided evidence that platelets may also be implicated in this mechanism, as the effector cell (23a). It is envisioned that an antibody-dependent cell-mediated cytotoxic type of mechanism involving antibody and an effector cell could be responsible for eliminating the parasites and curing mice of infection. The platelet is one possibility; however, it is quite possible that another cell, such as a granulocyte or mononuclear phagocyte, could be the effector cell.

The present findings resemble to some extent those reported by Campbell et al. (6), who showed that anti-IgM-treated mice infected with *Trypanosoma rhodesiense* failed to reduce the first peak of parasites and died after a persistently high parasitemia. It should also be mentioned that, in these experimetns, a prolonged prepatent period was observed in anti-IgM-treated mice similar to that seen by us (Fig. 1), a finding which we attribute to an increase in natural (innate) host resistance of mice injected with anti-IgM from birth (3).

It has been reported previously that splenomegaly is a characteristic feature of *T. musculi* infection in intact mice (1, 19). Studies in nude mice have shown that *T. musculi*-induced splenomegaly is dependent on a functioning T-lymphocyte system, whereas other data have provided indirect evidence that this phenomenon is B-cell independent (19). Our results have demonstrated that, indeed, splenomegaly occurs in the absence of a functioning B-lymphocyte system and is thus apparently a B-cell-independent phenomenon.

Finally, in performing mitogen assays to assess the immune status of the mice used in our experiments, it was also observed that nonspecific immunodepression, a feature of *T. musculi* infection in intact mice (1, 2), develops in B-cell-deprived infected mice (Table 1). In contrast to normal (NRS-treated and NT) mice, in which immunodepression peaks on day 14 p.i. and is reversing itself by day 21 p.i., this effect becomes more marked on day 21 p.i. in anti-IgM-treated mice. The mechanism inducing immunodepression has not been established. However, unlike intact mice, parasites are still present in the circulation on day 21 p.i. in B-cell-deprived mice, which could be taken as a suggestion that parasite products are directly responsible for inducing the state of immunodepression, as has been postulated (1, 2). Alternatively, macrophage or lymphoid suppressor cells in the spleen could also be the agents responsible (1), although recent in vitro evidence has been presented which argues against this possibility (2). In any event, the results of our experiments show that functional B-cells per se are not required to induce immunodepression.

In conclusion, it appears that the host response to infection with *T. musculi* during the initial growth phase and first crisis is controlled by a non-antibody-dependent mechanism, whereas the second crisis and phase of elimination of the trypanosome is apparently dependent on an antibody-mediated mechanism.

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