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Depressed Antibody Responses to a Thymus-Dependent Antigen in Toxoplasmosis

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The immunodepressive effect of *Toxoplasma gondii* infection in mice was studied, using sheep erythrocytes (SRBC) as the testing antigen and serum hemagglutinins, hemolysins, and both direct and indirect splenic plaque-forming cells (PFC) to SRBC as assays. In the primary antibody response, immunoglobulin M (IgM), hemagglutinins, and hemolysins and both IgM- and IgG-secreting PFC were depressed in animals immunized after infection. Maximum immunodepression occurred during the first 3 weeks of *Toxoplasma* infection. When the secondary antibody response was studied, results varied. Mice immunized with SRBC after being infected with *T. gondii* had a depression in both IgM and IgG PFC. Mice immunized with SRBC before being infected with *T. gondii* and then given a challenge dose of SRBC had a delay, but not an actual depression, in IgG hemagglutinins and hemolysins and IgG-secreting PFC. These studies show that the immunodepression associated with *Toxoplasma* infection is complicated, and they provide no definitive explanation for the mechanism.

Previous studies have shown that mice infected with *Toxoplasma gondii* and subsequently immunized with sheep erythrocytes (SRBC) have depressed spleen plaque-forming cells (PFC) and serum hemagglutinins and hemolysin responses (7, 18). This depression was believed to be due to antigenic competition, with the *T. gondii* infection acting as a primary and continuing antigen. The depressed response to the secondary antigen, SRBC, occurred as early as 7 days after infection and persisted for the entire period of the study (5 weeks) (18). The immunodepression coincided with the maximal increase in spleen size and in *Toxoplasma* antibodies. Huldt et al. (7) have shown that indirect PFC after a single dose of SRBC are depressed in *Toxoplasma*-infected mice.

The present study was designed to answer several questions suggested by the results of the previous experiments: (i) Are both the immunoglobulin G (IgG) and IgM responses to subsequent antigens depressed by *Toxoplasma* infections? (ii) Are both the primary and secondary responses depressed? (iii) How long does the immunodepression last? (iv) What is the mechanism of this immunodepression?

MATERIALS AND METHODS

Mice. C3H/He Dublin inbred male mice (Flow Laboratories), 6 to 8 weeks old when purchased, were used throughout these studies.

Infection. Half the mice were injected intraperitoneally with mouse brain emulsion containing 100 (one group of studies) or 150 cysts of the Gleadle strain of *Toxoplasma gondii*. (The Gleadle strain was kindly supplied by Douglas Fleck, St. George's Hospital, London. It was originally isolated from a human lymph node and has been passaged by the intraperitoneal injection of brain emulsion from infected to noninfected mice. In these animals, at the dosage given in this experiment, it causes a mild infection with persistent cysts in the brain.) This dosage was believed always to cause infection since animals receiving cyst-containing brain emulsion always appeared slightly ill, with reduced activity, increased spleen size, less weight gain, and poor fur quality in comparison with animals receiving uninfected brain emulsion. All animals tested after at least 3 weeks of infection had *Toxoplasma* cysts in their brains and *Toxoplasma* antibodies (indirect fluorescent-antibody test) in their sera. The other half received a similar volume of brain emulsion from noninfected mice.

Immunization with SRBC. To study the immune response to SRBC during infection with *T. gondii*, four infected and four noninfected mice were immunized with 0.2 ml of 6×10^8 to 8×10^8 SRBC.

(i) Primary immune response. When the primary immune response was studied, the animals received a single intraperitoneal inoculation of SRBC on the appropriate day before or after receiving the brain emulsion (−3 days to +133 days, with *Toxoplasma* infection as day 0). After 4 days (direct) or 8 days (indirect), PFC assays were performed.

(ii) Secondary immune response. Two immunization schedules were used in the secondary immune

response studies. In one series of experiments the mice received two initial inoculations of SRBC at 2-week intervals before being infected (−28 and −14 days). A third immunization of SRBC was then given at the appropriate times, and both direct (on the 4th day after the last SRBC immunization) and indirect (on the 4th and 8th days after immunization) PFC assays were performed. The other sequence for studying the secondary response consisted of infecting the mice on day 0, followed by two immunizations with SRBC at 2-week intervals (+14 and +28 days). The animals were then given a third inoculation of SRBC on the appropriate days, with indirect PFC performed 4 days later.

Two mice, one infected with *T. gondii* and the other not infected, were included in each study as nonsensitized controls. The numbers of PFC were very low in these animals, confirming a low background for the technique.

Procedure for collecting spleen cells and sera. At either 4 or 8 days after the only (primary response studies) or the last (secondary response studies) injection of SRBC, groups of four *Toxoplasma*-infected and four noninfected mice were killed and splenic PFC were assayed by the technique of Jerne and Nordin (8, 9), as modified by Merchant (13, 14).

The mice were sacrificed and their peripheral blood was removed by means of a Pasteur pipette after transection of the aortic arch. The blood was allowed to clot at room temperature, and the serum was separated and kept frozen at −20°C until used. Spleens were removed aseptically and placed in a sterile plastic tissue culture dish containing 10 ml of Hanks balanced salt solution (Hanks) with phenol red as a pH indicator and buffered with sodium bicarbonate.

Procedure for preparing cells. Spleen cells were obtained by gently teasing the capsule, using a rubber-tipped policeman. Cell clumps were dispersed by gentle successive aspiration through 20- and 26-gauge needles. The spleen cell suspensions were washed, and nucleated cell counts were performed. A sample was then diluted with Hanks to contain 2×10^6 nucleated spleen cells per 0.2 ml.

Procedure for performing PFC assay. The direct and indirect PFC assays were performed in quadruplicate as described previously (13, 14). Frozen guinea pig serum, diluted 1:10, was used as a complement source. A 1:100 dilution of rabbit anti-mouse IgG was added after the first incubation period in the indirect PFC assays. Incubation periods were for 1 h at 37°C.

Serological titrations. All sera were inactivated at 56°C for 30 min and were individually titrated against SRBC, using a microtiter technique. Reciprocals of titers were expressed as the $\log_2$ of the last well showing macroscopic agglutination or lysis. A 25-μl amount of one of the following was added to each well of a 96-well V-shaped microtiter plate: (i) phosphate-buffered saline (PBS); (ii) 0.1 M 2-mercaptoethanol (2-ME) in PBS; (iii) 1:20 dilution of guinea pig complement in PBS; or (iv) 0.1 M 2-ME containing complement diluted 1:20 in PBS. After this, 25 μl of the test sera was added to the first well of a row of 12 wells. The sera and diluting solution were mixed twice with the pipette, and 25 μl was serially transferred to the next well in the row. Each was tested with the four diluting liquids.

The microtiter plates were then covered with a plastic lid and incubated for 3 h at room temperature. After this, 50 μl of a 1% suspension of SRBC in PBS was added to each well. This procedure resulted in 12 double dilutions, with lowest 1:4 and highest 1:8,192. In some secondary antibody response studies, further serum dilutions were performed. The plates were covered and placed at 4°C for reading the next morning. The wells with PBS and PBS + 2-ME were read for hemagglutination, whereas the wells with PBS + complement and PBS + complement + 2-ME were read for hemolysis.

Statistical analysis of data. The average PFC counts were calculated from the four test plates done for each mouse. Then the means and standard errors for the PFC counts and the hemagglutinin and hemolysin titers for the four mice in each *Toxoplasma*-infected and noninfected group were compared, using Student's *t* test.

RESULTS

Primary antibody response. Direct PFC assays performed 4 days after a single inoculation of SRBC demonstrated a marked depression of antibody response in *Toxoplasma*-infected mice in comparison with noninfected control mice (Fig. 1). This was most apparent when ana-

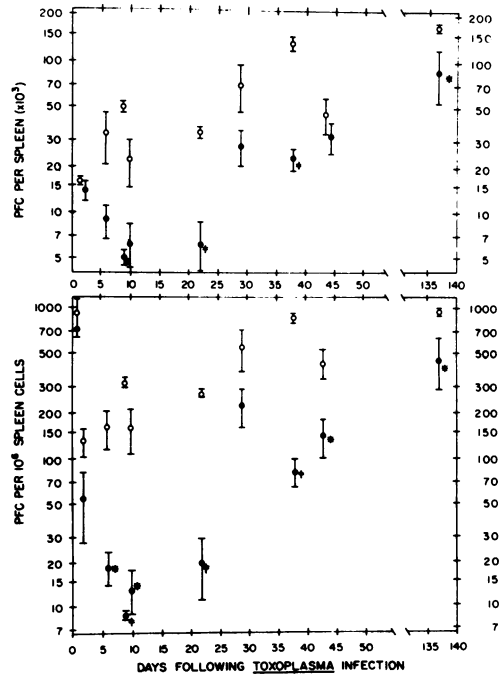

FIG. 1. *Mean ± standard error of the direct (IgM) PFC response 4 days after immunization with SRBC. Mean value of T. gondii-infected group (●) statistically differs from mean of uninfected controls (○). Symbols: *, $P < 0.05$; +, $P < 0.01$; ‡, $P < 0.001$.*

lyzed as plaques per 10^6 spleen cells, but also occurred when compared as plaques per spleen. Noninfected mice usually had 150 to 900 plaques per 10^6 spleen cells and 20×10^3 to 150×10^3 plaques per spleen. There was no significant reduction in PFC during the first 2 days of *T. gondii* infection (SRBC inoculated on day −2 or before). By day 6, however, the *Toxoplasma*-infected animals had a marked reduction. This depression was greatest during the first 3 weeks, but persisted for the entire period of the study (4.5 months).

Indirect PFC assays performed 8 days after a single injection of SRBC showed a marked depression in *Toxoplasma*-infected mice (Fig. 2). Like the direct plaque assay results, indirect plaque responsiveness was not reduced during the first 2 or 3 days of infection, but thereafter a depression occurred in the *Toxoplasma*-infected mice which persisted for the entire 140 days of the study. Maximum depression also occurred during the first 3 weeks of the study. Indirect plaques in the noninfected control mice usually were 150 to 350 per 10^6 spleen cells, or 20×10^3 to 60×10^3 per spleen. In four studies performed 7 to 10 days after infection with *T. gondii* (SRBC inoculated on day −1 to day +2), the infected groups averaged only 8 to 15 plaques per 10^6 spleen cells or 4,000 to 8,500 plaques per spleen.

Secondary antibody response. As expected, the quantity of direct PFC detected during a secondary response to SRBC was very low, with the number of these IgM plaques increasing with time after the initial sensitization with SRBC. There was a statistically significant reduction in plaques in several groups of animals up to 90 days after infection with *T. gondii*.

As described in Materials and Methods, the IgG secondary antibody response was studied using two immunization schedules. *Toxoplasma*-infected animals immunized thrice

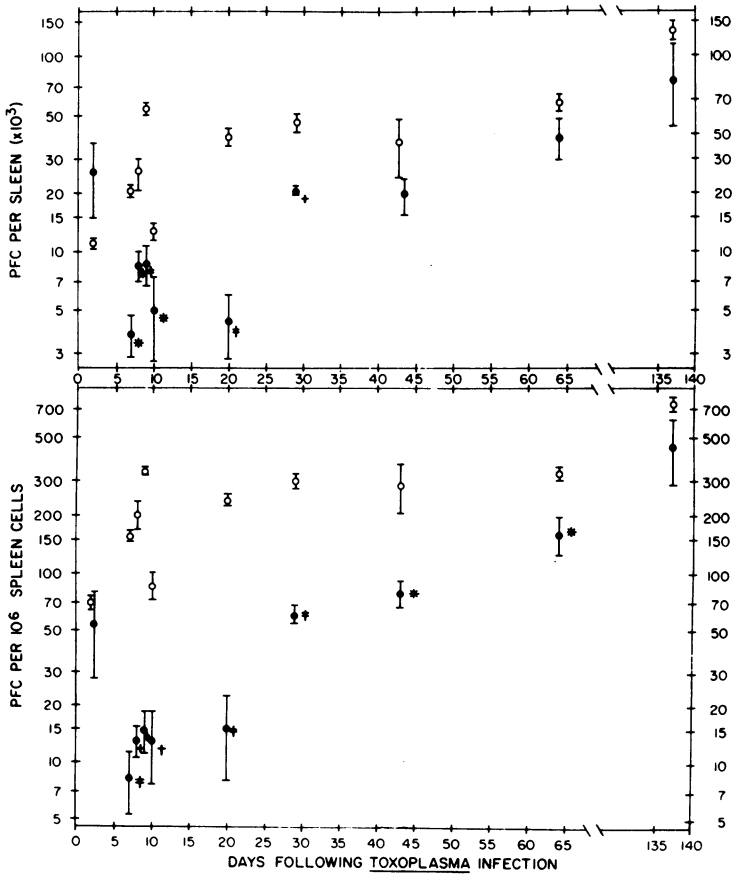

FIG. 2. *Mean ± standard error of indirect (IgG) PFC response 8 days after immunization with SRBC. Mean of T. gondii-infected group (●) statistically differs from mean of uninfected controls (○). Symbols are as in Fig. 1.*

with SRBC after being infected had statistically significant reductions in numbers of PFC, performed 4 days after a challenge dose of SRBC, for as long as 3 months after infection with *T. gondii*. Four studies were performed with the infected groups having been inoculated with *Toxoplasma* between 45 and 90 days previously. Noninfected mice averaged between 1,250 to 2,000 indirect PFC per 10^6 spleen cells (190×10^3 to 420×10^3 per spleen), whereas *Toxoplasma*-infected groups averaged 50 to 450 indirect PFC per 10^6 spleen cells (16×10^3 to 140×10^3 per spleen). In the other sequence, mice were given two immunizing inoculations of SRBC before receiving infected or noninfected brain emulsion. A group of mice studied on the 14th day of infection had a marked reduction ($P < 0.001$) in 4-day indirect PFC (Fig. 3A). Other SRBC-preimmunized groups were studied 8 days after receiving a challenge dose of SRBC. Control groups had lower numbers of PFC on the 8th as compared with the 4th day, and the *T. gondii*-infected group had significant increases in PFC (Fig. 3B).

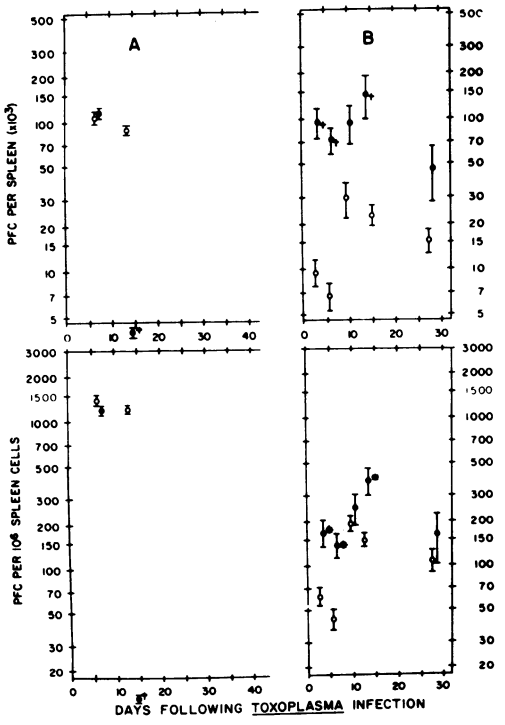

FIG. 3. *Mean ± standard error of indirect (IgG) PFC response 4 (A) and 8 (B) days after priming inoculation with SRBC. These animals were immunized twice with SRBC before receiving noninfected or T. gondii-infected brain emulsion. Mean of T. gondii-infected group (●) statistically differs from mean of noninfected controls (○). Symbols are as in Fig. 1.*

Hemagglutinins and hemolysins. Serum antibodies to SRBC were measured by performing both hemagglutinin and hemolysin titrations. Total antibodies and IgG only (2-ME-treated sera) were studied. In the primary antibody response studies, decreases in hemagglutinin and hemolysin titers were noted in the sera from *T. gondii*-infected mice taken both 4 and 8 days after a single inoculation of SRBC (Fig. 4). There was no IgG antibody response in the 4-day sera and very little in the 8-day sera.

As expected, the major portion of the secondary antibody response to SRBC was 2-ME resistant, and thus was IgG. Four-day hemagglutinins and hemolysins were depressed in the *Toxoplasma*-infected groups in most studies for as long as 3 months after infection. Eight-day hemagglutinin titers were significantly depressed in the *Toxoplasma*-infected groups receiving booster doses of SRBC 5 and 2 days before infection. Thereafter, the infected groups had higher antibody titers, this increase being statistically significant in the group given a booster of SRBC on the 17th day of infection ($P < 0.05$). The 8-day hemolysin titers followed the same pattern: slightly depressed in the infected groups receiving booster doses before and on the 2nd day of infection, but elevated ($P < 0.01$) in the group given SRBC on the 17th day.

DISCUSSION

These studies confirm previous work (7, 18) indicating that, under certain conditions, the PFC and serum antibody responses to immunizations with SRBC are depressed in mice infected with *T. gondii*. However, under other conditions, different responses were noted (Table 1).

A depression in primary antibody response to SRBC did not occur in animals immunized before the infection. The depression in both direct and indirect plaques was maximal during the first 3 weeks, but persisted for at least 3 months. A depression in IgM antibody response to SRBC after infections has been reported on many occasions. For example, viruses (15, 17, 21), malaria (1, 5, 11, 16), trypanosomes (M. K. Behbehani, Ph.D. thesis, University of London, London, England, 1972; 12) and *Toxoplasma* (7, 18) all depress serum antibodies and direct PFC to SRBC.

Chronic malaria depresses human antibodies after immunizations. McGregor and Barr (12) reported that children without malaria had a greater antibody response to tetanus toxoid

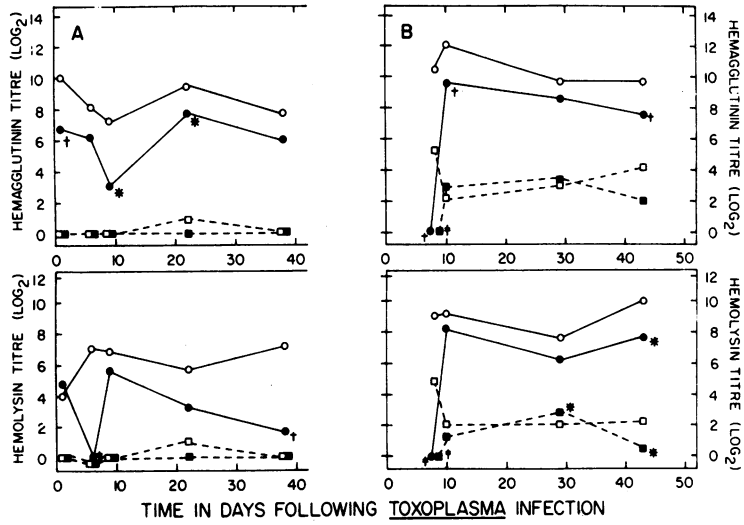

FIG. 4. *Mean 4-day (A) and 8-day (B) hemagglutinin and hemolysin titers in mice receiving a single inoculation of SRBC. Circles represent total antibodies, and squares designate only IgG antibodies (2-ME-treated sera). Mean titers of T. gondii-infected group (● and ■) statistically differ from those of non-infected (○ and □). Symbols are as in Fig. 1.*

TABLE 1. *Results of serum antibody determinations and PFC assays in Toxoplasma-infected mice*

Response[a]	Sequence[b]	Results[c]			
		Antibodies		PFC	
		IgM	IgG	IgM	IgG
Primary	Toxo △ SRBC ▲	↓	–	↓↓	↓↓
Secondary (before)	SRBC ▲▲ Toxo △ SRBC ▲	—	→	↓	→
Secondary (after)	Toxo △ SRBC ▲▲▲	–	↓	↓	↓↓

[a] Type of immune response to SRBC immunization.

[b] Sequence of infection with *Toxoplasma* (Toxo) and immunization with SRBC, including number of inoculations with SRBC.

[c] Response in *Toxoplasma*-infected groups in comparison with uninfected controls. Symbols: ↓, Decreased; →, delayed; –, too little response for interpretation.

than did malaria-infected children. Greenwood et al. (4) confirmed these findings and also showed a diminished antibody response to the O (but not to the H) antigen of *Salmonella typhi* in children with acute malaria. Although not specifically tested, the depression in titers was most likely in IgG antibodies. The same group (6) reported a depressed delayed hypersensitivity to purified protein derivative and to *Candida* and streptococcal antigens, and a reduced antibody response to immunization with *S. typhi* vaccine in 38 Africans infected with *Trypanosoma gambiense*. A depressed antibody response after tetanus toxoid immunization has also been reported in mice infected with malaria parasites (20).

Depression of the IgG antibody response by infections has been demonstrated less frequently than depression in IgM antibodies. Huldt et al. (7) demonstrated depressed quantities of indirect PFC in spleens of mice infected with *Toxoplasma* for 3 to 6 weeks and for 5 months in the same order of magnitude as the depression in numbers of direct PFC. *Toxoplasma*-infected mice also had depressed antibody titers to killed polio vaccine (7). Bomford and Wedderburn (2), studying concomitant infections with Moloney leukemia virus and *Plasmodium berghei yoelii* in mice, showed that malaria suppressed the serum-neutralizing antibody to the virus. Treatment of the sera with 2-ME demonstrated that the depression was

TABLE 2. *Results of serum antibody determinations and PFC assays in mice infected with Friend virus (FV)*[a]

Response	Sequence	Results		
		Antibodies (total)	PFC	
			IgM	IgG
Primary	FV (△) SRBC (▲)	↓	↓↓	↓↓↓
Secondary	SRBC (▲) FV (△) SRBC (▲)	↓	↓↓↓↓	↓↓↓↓↓

[a] Taken from Salaman and Wedderburn (15) and Wedderburn and Salaman (21)

primarily in IgG antibodies. They interpreted these findings as suggesting that the malarial infection was specifically preventing T (thymus-dependent) lymphocytes from responding to the virus, since IgG response is more T cell dependent than that of IgM.

Similar studies have been performed in mice concomitantly infected with *P. berghei yoelii* and *T. gondii* (2). Dually infected animals had depressions in both malaria and *Toxoplasma* antibodies. Since the sera for testing were taken at least 13 days after the infections, the majority of the antibodies probably were IgG. Malarial antibodies were suppressed from 13 to 16 days after the infection, but not on days 24 and 25. This is similar to the findings reported in this study and suggests that the *Toxoplasma* infection causes a delay in IgG antibody production or release rather than an actual suppression.

Wedderburn and Salaman (21), assaying both direct and indirect hemolytic PFC in both the primary and secondary responses to SRBC in BALB/c mice infected with Friend virus, reported the findings shown in Table 2. Friend virus infections caused a much greater immunodepression than *Toxoplasma* infections. The greatest reduction in hemolytic antibody-producing cells was with the indirect plaque assays and with the secondary response, both chiefly IgG responses.

These studies have answered the first three questions proposed in the introduction. Both IgG and IgM responses are depressed by *Toxoplasma* infections. Depression occurs after either single or multiple doses of antigen. This immunodepression is prolonged, persisting for at least 4.5 months after infection.

However, the mechanism has not been identified. It may be secondary to an interference with helper T cell function. Although bursa-equivalent lymphocytes (B cells) are responsible for antibody production, thymus-dependent lymphocytes (T cells) have a regulatory effect upon the response of B cells to most antigens (10). Stimulated T cells have the following effects on the response of B cells to antigen: (i) for most antigens, they control the differentiation of antigen-stimulated specific B cells into antibody-secreting cells; (ii) they influence the switch from the production of IgM to IgG antibodies; (iii) they determine the rate of selection of specific cells by antigen in the immune response as reflected in the change in affinity of humoral antibody with time; (iv) under certain conditions, they may have a suppressive effect on antibody responses by B cells. Aberrations in all four of these regulatory functions have been documented or suggested to occur with *Toxoplasma* or other infections.

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