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Nonspecific Suppression of Primary Antibody Responses and Presence of Plastic-Adherent Suppressor Cells in *Toxoplasma gondii*-Infected Mice

YASUHIRO SUZUKI*, NAOHIRO WATANABE, AND AKIO KOBAYASHI

Department of Parasitology, Jikei University School of Medicine, Minato-ku, Tokoyo 105, Japan

Received 18 March 1981/Accepted 20 June 1981

The effect of *Toxoplasma* infection on primary antibody responses to both T-dependent and T-independent antigens was examined in mice. Drastic suppression of primary responses to sheep erythrocytes (SRBC) occurred when mice were immunized 7 days after infection. The suppression was observed in both 2-mercaptoethanol-sensitive and -resistant hemagglutinin responses. Anti-dinitrophenol (DNP) immunoglobulin E and G1 responses to DNP-conjugated keyhole limpet hemocyanin were also suppressed by infection. It was suggested that the suppressive effect is nonspecific for the antigens and immunoglobulin classes produced. Anti-DNP responses to DNP-Ficoll, a T-independent antigen, were suppressed by infection, but the suppressive effect was weaker than that on the responses to SRBC. This suggests that both T and B cells are suppressed by infection. In vitro responses of infected mouse spleen cells to SRBC and DNP-Ficoll confirmed the results of in vivo experiments. In addition, the presence of plastic-adherent suppressor cells was demonstrated in the spleen cells of infected mice, which suppressed the ability of normal mouse spleen cells to mount an SRBC-specific plaque-forming cell response. These plastic-adherent suppressor cells appeared to be a major cause of nonspecific suppression of primary antibody responses in *Toxoplasma*-infected mice.

The occurrence of immunosuppression to unrelated antigens has been reported in humans and animals with protozoan infections (reviewed in reference 2). This phenomenon could represent a mechanism by which parasites survive in the host, since the immunosuppression would also affect the immune responses to parasites themselves. Mice infected with *Toxoplasma gondii* showed depressed antibody responses to sheep erythrocytes (SRBC) (5, 18, 19) and to killed polio vaccine (5). Moreover, blastogenic responses of infected mouse spleen cells to T-cell mitogens (concanavalin A and phytohemagglutinin) and a B-cell mitogen (lipopolysaccharide) were diminished (17). The mechanism of the reported phenomenon, however, is still obscure. The present paper reports the effect of *Toxoplasma* infection on primary antibody responses to both T-dependent and T-independent antigens in vivo and in vitro and the cellular basis of the immunosuppression.

MATERIALS AND METHODS

Animals. Inbred female C57BL/6 mice were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan), and mice 10 to 15 weeks old were used in all experiments. Female Wistar rats weighing 200 to 250 g and outbred ddY mice were purchased from the same breeder and used for passive cutaneous anaphylaxis (PCA) reactions.

***T. gondii* infection.** Brains of C57BL/6 mice chronically infected with the avirulent Fukaya strain of *T. gondii* (kindly supplied by Y. Tsunematsu, Teikyo University) were triturated in pH 7.2 phosphate-buffered saline with a mortar and pestle. The brains from two or three infected mice were pooled and treated with 0.25% trypsin for 10 min. Released bradyzoites were washed three times in phosphate-buffered saline, centrifuged at 700 × *g* for 10 min, and resuspended in phosphate-buffered saline at 10^4 organisms/ml. C57BL/6 mice were inoculated with 0.5 ml of the suspension intraperitoneally.

Antigens. SRBC (Nippon Bio-Test Laboratories, Inc. Tokyo), dinitrophenylated keyhole limpet hemocyanin (DNP_{15}-KLH), DNP_{60}-AECM-Ficoll (Biosearch, San Rafael, Calif.) and DNP_{50}-AECM-Ficoll (kindly supplied by T. Tadakuma, Keio University) were used for immunization. KLH was purchased from Pacific Bio Marine Laboratories, Inc. (Venice, Calif.). Ovalbumin (OA) and bovine serum albumin (BSA) were obtained from ICN Pharmaceuticals, Inc. (Cleveland, Ohio). DNP_{14}-OA and DNP_{44}-BSA were used as challenging antigens for the PCA reaction. DNP_{15}-KLH, DNP_{14}-OA, and DNP_{44}-BSA were prepared by the methods described by Eisen et al. (4); subscripts refer to the average number of DNP groups per mol-

ecule of a carrier protein.

Antibody titration. The titer of hemagglutinin antibody was measured by a micro-agglutination test. An equal volume of 0.6% washed SRBC was added to doubling dilutions of sera made in microtiter trays, and the pattern of agglutination was read after the trays had stood at room temperature overnight. For the titration of 2-mercaptoethanol (2-ME) -resistant hemagglutinin, sera were incubated with equal volumes of 0.1 M 2-ME in phosphate-buffered saline at 37°C for 1 h before the addition of SRBC. The DNP-specific hemagglutinin titer was measured by using DNP-BSA-coated SRBC prepared by the method described by Bloch et al. (1). The titers of anti-DNP immunoglobulin G1 (IgG1) and IgE antibodies were determined by PCA reactions (12). For IgG1 antibody titration, ddY mice were injected with test sera intradermally and challenged intravenously with 500 μg of DNP-OA 1.5 h later (13). Wister rats were used for IgE antibody titration (11), and 1 mg of DNP-BSA was the challenging antigen with a 2-h sensitization period (13). Complement-fixing antibodies were titrated by passive lysis, using DNP-BSA-coated SRBC (1).

Spleen cell culture. Single-cell suspensions from spleens of normal or infected mice were prepared by the method described by Pierce et al. (14). The washed spleen cells were suspended at a concentration of 5×10^6/ml in completely supplemented Eagle minimal essential medium containing Hanks salts, 50 U of penicillin per ml, 50 μg of streptomycin per ml, 5×10^{-5} M 2-ME, and 10% fetal calf serum (Flow Laboratories, Inc., Stanmore, New South Wales, Australia). Portions of 200 μl (10^6 cells) were dispensed into the wells of Microtest II culture plates (Falcon Plastics, Oxnard, Calif.). As an immunogen, 20 μl of appropriately diluted DNP_{50}-AECM-Ficoll or SRBC suspension was added. Experimental groups were composed of three identical wells. The plates were incubated for 4 days at 37°C in 5% CO_2 in air.

Assays for PFC. At the termination of the culture period, cells from each experimental group were harvested by aspiration, pooled, and sedimented by centrifugation. The sedimented cells were suspended in original volume of Hanks balanced salt solution. The number of plaque-forming cells (PFC) was determined by the method described by Pierce et al. (14). Four hundred microliters of 0.6% agarose (Nakarai Chemicals Ltd., Kyoto) in Hanks balanced salt solution, 50 μl of an 8% suspension of SRBC, and 100 μl of the cultured cell suspension were mixed at 47°C and poured onto microscope slides previously coated with 0.1% agarose in water. Duplicate slides, prepared from each group, were incubated for 1.5 h at 37°C in a humid atmosphere. Guinea pig complement (Toshiba Chemicals, Tokyo) diluted 1/20 in Hanks balanced salt solution was added, and incubation was continued for another 1.5 h. For assays for DNP-specific PFC, trinitrophenylated SRBC prepared by the method of Rittenberg and Pratt (15) were used as target cells.

RESULTS

Suppression of primary antibody responses to T-dependent antigens by *T. gondii* infection in vivo. To examine the effect of *Toxoplasma* infection on primary antibody responses to SRBC during the course of infection, groups of mice were immunized with 10^8 SRBC various days before or after infection, and their antibody responses were followed for several weeks. Figure 1 shows hemagglutinin titers on the 7th day after immunization, since the maximal hemagglutinin titers were obtained 7 days after immunization in most groups. The hemagglutinin responses of mice immunized 2 days before, immediately after, or 3 days after infection were not suppressed. Immunization on day 7 of infection resulted in a drastic suppression of both 2-ME-sensitive and -resistant hemagglutinin responses. In the infected group, the total hemagglutinin response was suppressed to 3% of the uninfected control response ($P < 0.001$), and 2-ME-resistant hemagglutinin could not be detected ($P < 0.001$). Ability to produce hemagglutinin was gradually restored during infection; when mice were immunized 56 days after infection, the total hemagglutinin response was comparable to that of the control. However, the 2-ME-resistant hemagglutinin response did not recover until 98 days after infection. Thereafter, mice were immunized with antigens 7 days after infection.

Next, the effect of *Toxoplasma* infection on anti-DNP IgE and IgG1 antibody responses to DNP-KLH was examined. Mice were immunized with 10 μg of DNP-KLH in 1 mg of $Al(OH)_3$ 7 days after infection and were bled weekly after the immunization. In uninfected control mice, the maximal anti-DNP IgE and IgG1 titers were obtained 2 weeks after immu-

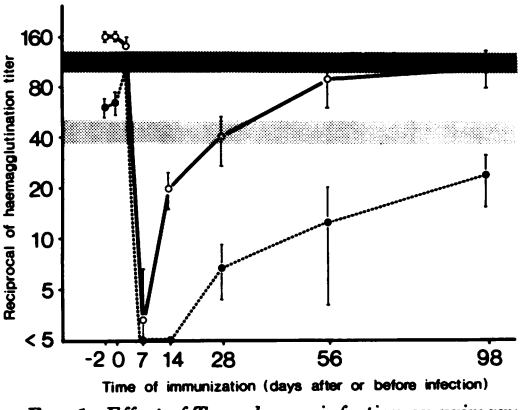

FIG. 1. *Effect of Toxoplasma infection on primary antibody responses to SRBC in mice. Each point represents the mean ± standard error of 5 to 10 mice. Toxoplasma infection was performed on day 0. Dotted areas show total and 2-ME-resistant hemagglutinin titer of uninfected control mice, respectively. Symbols: ○, total hemagglutinin titer; ●, 2-ME-resistant hemagglutinin titer of each group of infected mice on the 7th day of immunization.*

nization: 1:20 for IgE and 1:80 for IgG1. On the other hand, in the infected mice, both anti-DNP IgE and IgG1 titers were less than 1:5 for 5 weeks after the immunization. These results indicate that *Toxoplasma* infection also suppressed both IgE and IgG1 antibody responses to DNP-KLH.

Suppression of primary antibody responses to T-independent antigen by *T. gondii* infection in vivo. To determine whether *Toxoplasma* infection also suppresses antibody responses to T-independent antigen, the effect of infection on primary responses to DNP-Ficoll was examined. Mice were immunized with 100 μg of DNP-Ficoll intraperitoneally 7 days after infection. Table 1 shows anti-DNP hemagglutinin titers on the 7th day after immunization. Both 2-ME-sensitive and -resistant antibody responses to DNP-Ficoll were significantly suppressed by infection. The total anti-DNP hemagglutinin titer was suppressed to 13% of the uninfected control titer. The suppression of the responses to DNP-Ficoll (Table 1) was less than that to SRBC (Fig. 1).

Primary antibody responses of spleen cells from *T. gondii*-infected mice in vitro. Reactivity of infected mouse spleen cells to SRBC and DNP-Ficoll was studied in an in vitro culture system. Spleen cells were harvested 7 days after infection and cultured for 4 days with appropriate concentrations of SRBC or DNP-Ficoll. Responses of cultured cells to these antigens were measured by PFC assay. Table 2 shows the results of anti-SRBC response. Normal mouse spleen cells responded maximally when 4×10^5 SRBC were added and developed about 150 PFC per culture at this SRBC concentration. On the other hand, responses of infected mouse spleen cells were strongly suppressed at all concentrations of SRBC used ($P < 0.05$) and were almost the same as the background response of cells cultured without SRBC.

Table 3 shows responses to DNP-Ficoll. Normal mouse spleen cells responded maximally at 10 to 100 ng of DNP-Ficoll per ml and developed about 300 DNP-specific PFC per culture. The responses of infected mouse spleen cells to DNP-Ficoll were suppressed, and their responses were one-fourth to one-sixth of those of normal cell controls ($P < 0.05$). These results were consistent with the results of in vivo experiments in that the suppressive effect was greater on the response to SRBC than to DNP-Ficoll.

Presence of suppressor cells in spleen cells from *T. gondii*-infected mice. The in vitro system was used to determine whether a suppressor cell population is present in infected mouse spleen cells. Normal mouse spleen cells were mixed with infected mouse spleen cells in various ratios, and responses of the mixed cell cultures to SRBC were measured (Fig. 2). If infected mouse spleen cells do not affect the response of normal spleen cells and do not respond to SRBC, the PFC responses of mixed cells will decrease (Fig. 2, a dotted line) as the percentage of infected mouse spleen cells is increased. However, PFC responses of cultured cells were drastically suppressed in the presence of even a small portion of infected mouse spleen cells. For instance, the PFC response of cells mixed with both infected and normal mouse

TABLE 1. *Suppressive effect of Toxoplasma infection on primary antibody responses to DNP-Ficoll in mice*

Group[a]	Anti-DNP hemagglutinin titer[b] (±SE)[c]	
	Total	2-ME resistant
Control	384 ± 55	27 ± 5
Infected[d]	49 ± 13	4 ± 2
P	<0.001	<0.05

[a] Five mice per group were used in each experiment.
[b] Titers on the 7th day of immunization.
[c] SE, Standard error of the mean for three experiments.
[d] Mice were inoculated with 5×10^3 bradyzoites of *T. gondii* and immunized with 100 μg of DNP-Ficoll 7 days after infection.

TABLE 2. *Suppressed activity of spleen cells from Toxoplasma-infected mice in primary anti-SRBC antibody response in vitro*

No. of SRBC added	PFC/culture (±SD)[a]	
	Normal[b]	Infected[c]
0	4 ± 0	7 ± 7
4×10^4	86 ± 20	11 ± 1
4×10^5	159 ± 27	11 ± 7
4×10^6	103 ± 1	10 ± 3

[a] SD, Standard deviation of the mean of duplicate assays.
[b] 10^6 normal mouse spleen cells were cultured in each well for 4 days.
[c] 10^6 spleen cells from mice infected 7 days previously were cultured in each well for 4 days.

TABLE 3. *Suppressed activity of spleen cells from Toxoplasma-infected mice in primary anti-DNP antibody response to DNP-Ficoll in vitro*

Final concn of DNP-Ficoll (ng/ml)	DNP-specific PFC/culture (±SE)[a]	
	Normal[b]	Infected[c]
0	13 ± 7	2 ± 1
1	139 ± 11	36 ± 4
10	291 ± 29	54 ± 2
100	300 ± 56	51 ± 9

[a] SE, Standard error of the mean for three experiments.
[b,c] See Table 2.

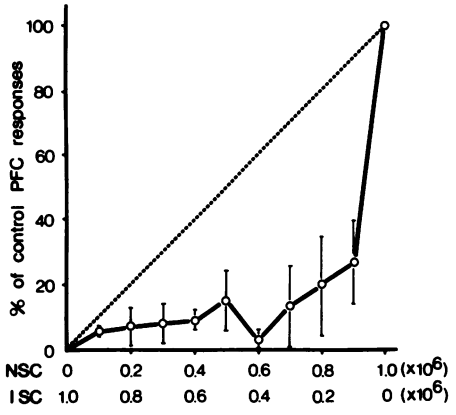

FIG. 2. *Suppressive effect of infected mouse spleen cells on primary anti-SRBC antibody response of normal spleen cells in vitro. Normal mouse spleen cells were mixed with infected mouse spleen cells in various ratios, and the PFC responses of the mixed cells were measured after 4 days in culture. Each point represents the mean ± standard error of mean. Abbreviations: NSC, normal mouse spleen cells; ISC, infected mouse spleen cells.*

spleen cells at a 1:9 ratio was 24% of the response of the normal mouse spleen cell control ($P < 0.05$). These results demonstrate the existence of suppressor cells in infected mouse spleen cells which can actively affect normal mouse spleen cells and suppress their antibody responses.

Plastic-adherent suppressor cells in spleen cells from *T. gondii*-infected mice. To investigate the nature of suppressor cells in infected mouse spleen cells, further studies were performed by separating cells by their propensity to adhere to plastic. Either plastic-adherent or nonadherent cells prepared from 10^6 infected mouse spleen cells were added to 10^6 normal mouse spleen cells, and the effects of these cells on the responses of normal mouse spleen cells to SRBC were examined. The adherent population had the suppressor activity (Fig. 3). Addition of adherent cells from infected mouse spleen cells caused a 62% suppression in the PFC response of normal mouse spleen cells ($P < 0.001$). On the other hand, nonadherent cells from infected mouse spleen cells did not suppress the PFC responses. As a control, both adherent and nonadherent cells from normal mouse spleen cells were tested. These cells enhanced, rather than suppressed, the antibody responses of normal mouse spleen cells. The suppressive effect of adherent cells from infected mouse spleen cells depended on the number of those cells added (Fig. 4). Moreover, it was also demonstrated that if a sufficient number of adherent cells from infected mouse spleen cells was added, the response of normal mouse spleen cells was completely suppressed.

DISCUSSION

Depression and recovery of immune responses were observed in primary antibody responses to SRBC in mice infected with avirulent *T. gondii*. When mice were immunized with SRBC shortly before or after infection, suppressed anti-SRBC responses were not observed. Mice immunized 7 days after infection induced drastic suppression of anti-SRBC responses. The suppression was observed in both 2-ME-sensitive and -resistant hemagglutinin responses. This suggests that both IgM and IgG antibody production to SRBC were suppressed by infection. These results confirmed the results of previous studies (19).

The effect of *Toxoplasma* infection on primary antibody responses to another T-dependent antigen was examined. Anti-DNP IgE and IgG1 antibody responses to DNP-KLH were also strongly suppressed in mice 7 days after infection. Since the responses to both the particulate (SRBC) and soluble (DNP-KLH) antigens were suppressed by infection, it is suggested that suppressed antibody responses will occur independ-

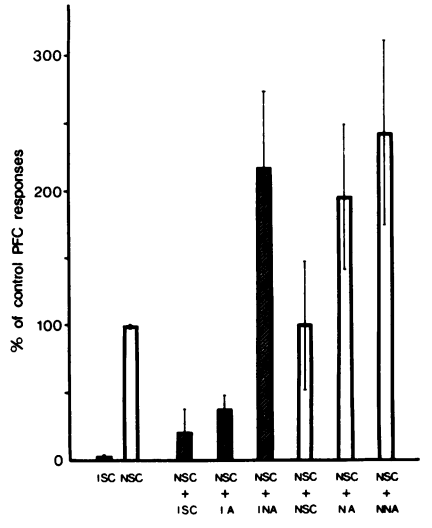

FIG. 3. *Suppressor activity of plastic-adherent spleen cells from Toxoplasma-infected mice in primary antibody responses to SRBC in vitro. Normal or infected mouse spleen cells (10^6) were separated into plastic-adherent and nonadherent cells. The separated cells were added to 10^6 normal mouse spleen cells and cultured with SRBC for 4 days. Each bar represents the mean ± standard error of mean. Abbreviations: NSC, normal mouse spleen cells; ISC, infected mouse spleen cells; NA, adherent cells of NSC; NNA, nonadherent cells of NSC; IA, adherent cells of ISC; INA, nonadherent cells of ISC.*

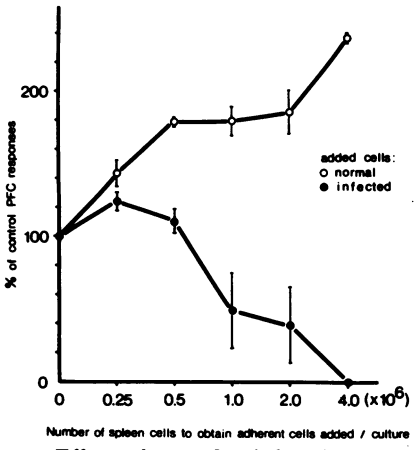

FIG. 4. *Effects of normal or infected mouse adherent spleen cells on primary anti-SRBC antibody response of normal spleen cells in vitro. Plastic-adherent cells from various numbers of normal or infected mouse spleen cells were added to 10^6 normal mouse spleen cells and cultured with SRBC for 4 days. Each point represents the mean ± standard error of mean.*

ently of immunizing antigens. Moreover, it is also suggested that the suppressive effect is nonspecific for immunoglobulin classes because suppression was observed in all immunoglobulin classes tested, i.e., anti-SRBC IgM and IgG and anti-DNP IgE and IgG1. As for the immunoglobulin classes other than IgM and IgG2 in antibody responses during immunosuppression by protozoan infections, Mendes et al. (10) reported recently that IgG1 and IgE responses to OA were depressed in *Trypanosoma cruzi*-infected mice. It is conceivable that nonspecificity for immunoglobulin classes may be a common characteristic of immunosuppressions induced by some protozoan infections.

The responses to a T-independent antigen, DNP-Ficoll, were also suppressed by *Toxoplasma* infection. This indicates that the responses of B cells were suppressed by infection. In addition, the stronger suppression of responses to SRBC than to DNP-Ficoll suggests that not only B cells but also T cells were suppressed in infected mice.

The responses of spleen cells from infected mice to SRBC and DNP-Ficoll were examined in an in vitro culture system. Their responses truly reflected the results of the in vivo experiments. Therefore, the mechanism of suppression was studied by using this in vitro primary antibody response system.

The possibility that suppressor cells may have been present was examined. Mixing infected mouse spleen cells with normal mouse spleen cells induced drastic suppression of anti-SRBC responses of normal mouse spleen cells. These results rule out the possibility that the suppressed responses of infected mouse spleen cells were caused merely by a loss in activity of some of the immunocompetent cells, such as an antigen presenting activity of macrophages, and indicate the presence of certain suppressor cells in infected mouse spleen cells.

The suppressor cells were plastic adherent in nature. The plastic-adherent cells of infected mouse spleen cells had strong suppressor activity, though a slight decrease in suppressive activity was observed as compared with that of unseparated spleen cells. In contrast, nonadherent cells could not suppress the response of normal mouse spleen cells. The suppressive effect depended on the number of adherent cells added, and a sufficient number could totally suppress anti-SRBC responses of normal mouse spleen cells. The plastic-adherent suppressor cells induced in the spleen by *Toxoplasma* infection play a major role in the immunosuppression of infected mice.

According to a morphological study of the adherent cells of infected mouse spleen cells by Giemsa staining, macrophages accounted for about 90% and the remaining cells were mostly polymorphonuclear leukocytes (Suzuki et al., unpublished data). In addition, the splenic macrophages from infected mice were large and rich in cytoplasm. Ruskin et al. (16) have reported that peritoneal macrophages are activated by *Toxoplasma* infection in mice. Furthermore, it has been reported that activated macrophages induced by the injection of BCG, *Propionibacterium acnes* or some tumors can suppress various immune responses of both T and B cells (7–9, 20). Thus, it is conceivable that the suppressor cells in splenic adherent cells of *Toxoplasma*-infected mice are macrophages and that they will suppress the functions of both T and B cells and nonspecifically interfere with primary antibody responses to T-dependent and T-independent antigens. In other protozoan infections, some studies have reported that suppressor macrophages are effectors of immunosuppression in primary antibody responses to SRBC in vitro (3, 21, 22) and that the suppressor macrophages induced by *Trypanosoma rhodesiense* infection need cell-cell contact with lymphocytes in expressing their potential (22). In our preliminary experiment, culture fluid of the spleen cells from *Toxoplasma*-infected mice did not have suppressive activity (Suzuki et al., unpublished data). Mechanisms by which the suppressor macrophages induced by *Toxoplasma* infection can interfere with the antibody responses are under study.

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