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Kinetics of Murine Delayed-Type Hypersensitivity Response to *Eimeria falciformis* (Apicomplexa: Eimeriidae)

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Mice recovering from a primary infection with an intestinal protozoan parasite, *Eimeria falciformis* (Apicomplexa: Eimeriidae), showed a classic delayed-type hypersensitivity (DTH) reaction to oocyst antigen challenge. This reaction was characterized by a biphasic pattern of footpad swelling. The first swelling peaked at 2 h after antigen challenge, whereas the second swelling peaked at 24 to 48 h after challenge. The DTH reaction was transferable with a T-cell-enriched spleen cell population from mice that had recovered from *E. falciformis* infection. Cytotoxic depletion of immune T cells with anti-L3T4 antibody and complement abrogated DTH transfer, indicating that L3T4-positive T cells were required. A T-cell-enriched spleen cell population from acutely infected mice suppressed the transfer of DTH with immune cells from recovered animals, implicating the existence of infection-induced immunoregulatory cells controlling the parasite-specific immune response during infection. Immune spleen cells also transferred resistance to infection as measured by oocyst production and death rate of recipients. Together, these results indicate that the DTH reaction, induced by infection with *E. falciformis*, is mediated by L3T4-positive T cells and is associated with resistance to infection.

Coccidian infection of the intestinal epithelium is accompanied by the production of parasite-specific antibodies and cell-mediated immunity (CMI) (20, 21, 30). Immunity to *Eimeria falciformis* (Apicomplexa: Eimeriidae), a murine coccidian parasite, is thymus dependent, as evidenced by elevated oocyst production during primary infection and the susceptibility to reinfection in athymic mice (25). With other coccidian infections, adoptive transfer of immune thoracic-duct lymphocytes, spleen cells, or peripheral blood lymphocytes results in significant reduction of oocyst production in challenged recipients (18, 32). Also, B-cell depletion by hormonal bursectomy does not affect the development of immunity in chickens (20), and immune serum does not to protect naïve animals (27). Thus CMI, mediated by T cells, appears to be central for the development of acquired resistance to *Eimeria* infections in these experiments. However, the importance of humoral immunity has also been noted (4, 6, 33). The role of secretory immunoglobulin A (IgA) may be essential (5, 6), since IgA can directly neutralize parasites at the infection site of the intestine. A clear understanding of CMI and antibody production is needed. T cells may perform direct cytotoxic killing, or they may induce secondary effects such as delayed-type hypersensitivity (DTH) and granuloma formation. T cells may also regulate the immune system through the release of lymphokines (11, 14) which influence antibody production, especially IgA, which may be important for the resistance to coccidian parasites (5, 6).

The DTH response to coccidian parasites was first reported to occur in humans and guinea pigs infected with *Toxoplasma gondii* (7). This response served as a useful diagnostic test for human toxoplasmosis. DTH in eimerian infections of several animal species has been investigated. Klesius et al. (15) studied the DTH response to particulate oocyst antigen (OAg) in rabbits infected with *Eimeria stiedai* and found a long-lasting positive reaction. Klesius et al. (16) also studied *Eimeria bovis* infection in cattle and found that the DTH response was comparable to tuberculin DTH reaction. Rose (29) tested antigens prepared from sporozoites, merozoites, and oocysts from a chicken coccidium, *Eimeria tenella*, and observed that OAg produced the greatest DTH response in recovered animals, but caused minimal reactivity in coccidium-free animals. The particulate OAg-specific DTH reaction in chickens immunized with several avian coccidian species (Coccivac, a commercial vaccine; Sterwin Laboratories, Inc., Opelika, Ala.) is positively correlated with resistance (8). Recently, we have observed that *E. falciformis* infection induces production of several classes of parasite-specific antibodies in serum, particularly of IgM, IgA, and IgG subclasses. The predominant antibody produced is strain dependent: C3H/He mice produce more IgG2a, whereas C57BL/10 mice produce primarily IgG3 (Y. Shi, J. L. Mahrt, R. J. Mogil, and D. R. Green, unpublished data). Clare et al. (2) found that the chicken major histocompatibility complex influences DTH to OAg of *E. tenella* and suggested that responder status may be influenced by the specific association between OAgs and a particular major histocompatibility complex gene product. Our recent studies also showed that susceptibility to murine *E. falciformis* infection is linked to the major histocompatibility complex and is modified by the non-major histocompatibility complex genotype of the host (24).

Since Gray and Jennings (9) established the use of mouse footpads in testing DTH reactivity, it has become a widely used assay for detecting DTH. Although DTH is the best-established measurement of the CMI response to coccidian infection, the identity of the cells which initiate DTH is unknown. We have established a mouse coccidian parasite (*E. falciformis*) system to further elucidate the relationship of DTH to CMI in coccidian infections. The experiments described herein show that the OAg-induced DTH is a classic DTH response as seen by the footpad swelling pattern. In addition, adoptive cell transfer experiments show that DTH to OAg is mediated by L3T4-positive, immuno-

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globulin-negative spleen cells. Immune spleen cells also transferred resistance to infection in terms of reducing parasite reproduction (oocyst production) and death rate of recipients. During the peak of acute infection, DTH to OAg is suppressed, and spleen cells from these animals are capable of inhibiting the adoptive transfer of DTH by immune cells to normal recipients. These results are discussed in relation to the role of CMI in immunity to coccidian infection.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice were obtained from Charles River Canada, Inc., St.-Constant, Quebec, Canada. Mice were allowed to rest for 1 week following shipment, and their feces were checked for the presence of helminth eggs or coccidian oocysts by using a Sheather sugar (500 g of sucrose and 6.5 g of phenol in 320 ml of water) flotation method (17). Mice were age matched (8 to 10 weeks) and kept in an isolating hood with negative air pressure to prevent spreading of infective oocysts. Food and water were provided ad libitum.

Parasite. Oocysts of *E. falciformis* were originally obtained from C. A. Speer, Veterinary Research Station, Montana State University, Bozeman. The oocysts were purified by flotation and petri dish adhesion with Sheather sugar solution. After purification the oocysts were sporulated in aerated 2.5% (wt/vol) $K_2Cr_2O_7$ solution. Sporulated oocysts were stored for less than 2 months in aqueous 2.5% $K_2Cr_2O_7$ at 4°C. Oocysts were washed three times by centrifugation with distilled water before use.

Infection. Mice were anesthetized with CO_2 before infection. Sporulated oocysts suspended in 0.3 ml of water were inoculated by intubation with a 1-ml syringe and a 23-gauge needle connected to 2 cm of polyethylene tubing. Dosages varied for different experiments and are specified in the corresponding sections.

Antigen preparation and DTH testing. OAg for DTH testing was prepared at a concentration of 10^6 sporulated oocysts per ml of sterile phosphate-buffered saline or Hanks balanced salt solution (HBSS) (Sigma Chemical Co., St. Louis, Mo.). Oocysts were ruptured in a French pressure cell at 15,000 lb/in^2 (15) or by sonication and were microscopically checked for breakage. The prepared OAg was stored at −20°C.

For DTH testing, OAg at a concentration of 8,000 oocysts in 25 μl of phosphate-buffered saline or HBSS was injected into the right footpad (preliminary experiments showed that 8,000 oocysts was the optimal dosage to induce footpad swelling). The left footpad was injected with 25 μl of phosphate-buffered saline or HBSS as the control. The footpad thickness was measured with a caliper (no. 7308, Mitutoyo, Tokyo, Japan). The pressure of caliper plunges on the footpad was controlled by looking at the disappearance of a beam of light. Repeated measurements introduced an error of ±0.05 mm. The antigen-induced footpad thickness was derived from the following formula: $(R_x - L_x) - (R_0 - L_0)$, where R is the thickness of the right footpad; L is the thickness of the left footpad; x is the time (in hours) at which the DTH was measured, and 0 represents time zero.

Spleen cell preparation. Immune spleen cells were obtained from mice which had been infected twice (first with 500 sporulated oocysts and reinfected, 30 days after the primary infection, with 20,000 sporulated oocysts). Suppressor cells were harvested from spleens of mice 10 days after primary infection with 500 sporulated oocysts. Thy-1-negative spleen cells were prepared by treating spleen cell suspensions (10^7/ml) with monoclonal anti-Thy-1.2 antibody (Du Pont, NEN Research Products) in HBSS for 45 min. Cells were centrifuged at 250 × *g* for 10 min at room temperature and suspended in rabbit complement (diluted 1:10 with HBSS) (SCI-CAN Diagnostics, Edmonton, Alberta, Canada) at 10^7/ml. This suspension was incubated at 37°C for 30 min, washed in HBSS, and assessed for viability with trypan blue before use. Immunoglobulin-negative (T-cell-enriched) cells were purified in glass bead columns which were coated with mouse immunoglobulin, and then conjugated with goat anti-mouse immunoglobulin (SCI-CAN Diagnostics, Edmonton, Alberta, Canada) by the method described by Wigzell (34). Monoclonal anti-L3T4 (GK1.5; obtained from D. R. Green, University of Alberta) plus complement was used for cytotoxic depletion of L3T4-positive cells from T-cell-enriched spleen cells (obtained by immunoglobulin-positive cell depletion as described above).

Adoptive transfer. Naïve recipients received an intravenous injection of 3×10^7 unfractionated immune spleen cells or the corresponding equivalent of Thy-1-negative, immunoglobulin-negative, or L3T4-negative cells. At 4 to 8 h after receiving cells, animals were infected with 500 sporulated oocysts.

Assessment of fecal oocyst production. Fecal collection began 7 days postinfection (p.i.) and continued at 2-day intervals. Aluminum cake pans containing 2.5% $K_2Cr_2O_7$ were used. Collections were continued until the end of oocyst excretion (15 to 16 days p.i.). Total fecal collections were blended, samples were suspended in Sheather sugar solution, and oocysts were counted in McMaster chambers. Near the end of the patent period, oocysts were counted by the direct centrifuge flotation technique, which was more sensitive to low oocyst output.

Statistics. The Student *t* test was used to test differences in DTH reactivity among groups. Oocyst production was compared between different groups by using a two-way analysis of variance with treatment and fecal collection date as the two factors.

RESULTS

Pattern of murine DTH to *E. falciformis* infection. Hyperimmune mice showed a biphasic pattern of DTH reaction to

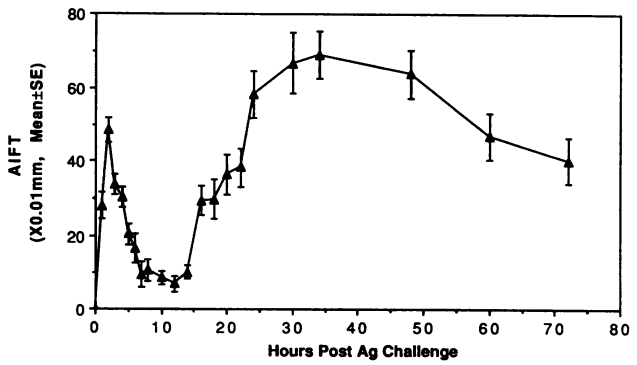

FIG. 1. Time course of DTH skin reactivity of C57BL/6 mice to OAg 1 week after recovery from a second infection with *E. falciformis* (data from eight mice). Nonspecific footpad swelling due to parasitic antigen was always less than 15 units. AIFT, Antigen-induced footpad thickness; Ag, antigen.

TABLE 1. Time course of adoptive transfer of C57BL/6 mouse DTH to *E. falciformis* with immune spleen cells

Group	No. of mice	Cell transfer[a]	DTH testing time[b]	Mean 24-h DTH level (10^{-2} mm)	P[c]
A	4	+	4 h	41.3 ± 6.4	
B	4	−	4 h	11.1 ± 3.6	<0.01[d]
C	4	+	10 days	17.3 ± 3.3	<0.05[e]
D	3	−	10 days	17.0 ± 2.3	>0.05[f]
E	4	+	22 days	94.7 ± 12.4	<0.001[g]
F	5	−	22 days	52.3 ± 4.6	<0.001[h]

[a] A total of 3×10^7 immune spleen cells were injected into each mouse of groups A, C, and E.

[b] Animals were infected with 300 sporulated oocysts 4 h after cell transfer and tested for DTH 4 h, 10 days, or 22 days after cell transfer by injection of 8,000 ruptured oocysts into the footpad. Footpad swelling was measured 24 h after footpad challenge.

[c] The comparison of DTH reactivity between groups was tested by the Student t test: [d]between A and B; [e]between A and C; [f]between C and D; [g]between C and E; [h]between E and F.

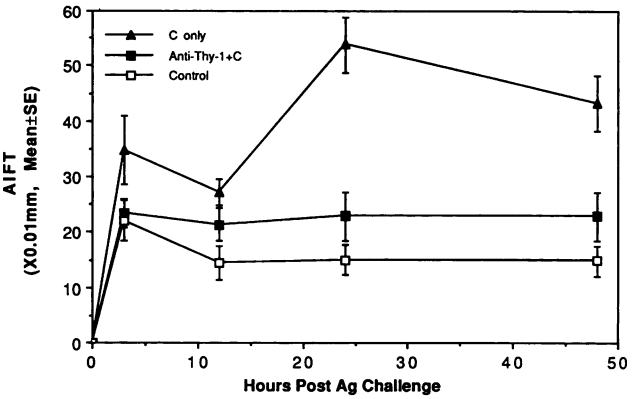

FIG. 2. Effect of removing Thy-1-positive immune spleen cells on the transfer of DTH to *E. falciformis*. Immune spleen cells were treated with anti-Thy-1 antibody plus complement (C) or complement only prior to cell transfer. AIFT, Antigen-induced footpad thickness; Ag, antigen.

E. falciformis OAg (Fig. 1). For this experiment, eight C57BL/6 mice were each infected with a primary inoculation of 500 sporulated oocysts followed by a second challenge of 20,000 oocysts on day 30 p.i. Starting 22 days later, the DTH response was measured at hourly intervals (0 to 8 h), bihourly (10 to 24 h), and then at 30, 34, 48, 60, and 72 h after OAg challenge into the footpad. The first swelling peak occurred 2 h after antigen challenge and then receded. The second peak of swelling occurred 24 to 48 h after antigen challenge. This response pattern fits the classical DTH pattern as described previously by Loveren et al. (23) in which the 24- to 48-h DTH reactivity was preceded by an early T-cell- and tissue-mast-cell-dependent swelling reaction that peaked 2 h after antigen challenge. This pattern is clearly different from an antibody-mediated Arthus reaction in which a single peak swelling is observed 4 to 10 h after antigen challenge (28).

DTH reactivity was still detectable 3 months after a single infection with 500 oocysts ([48.3 ± 3.7] × 10^{-2} mm, mean ± standard error [SE]). Animals which had been reinfected twice with 20,000 oocysts after recovery from the primary infection with 500 oocysts showed higher DTH reactivity ([77.0 ± 10.3] × 10^{-2} mm). Injection of sheep erythrocytes as a control induced very low footpad swelling at 48 h ([8.7 ± 2.0] × 10^{-2} mm), thus indicating that the DTH is antigen specific in immune animals.

Transfer of DTH with immune spleen cells. To determine whether OAg-induced DTH can be adoptively transferred with immune cells, we transferred unfractionated immune spleen cells from hyperimmune donors to syngenic mice. The recipient mice were infected with 300 sporulated oocysts 4 h after cell transfer. DTH reactivity to OAg was measured by injecting OAg into the footpad at different time points after cell transfer (Table 1). At 4 h after cell transfer, recipients showed significant DTH reactivity ($P < 0.01$) (Table 1, group A versus group B). During the peak oocyst production period (10 days p.i.), the DTH response in mice receiving immune cells was not different from that in the controls (group C versus group D). In mice receiving immune spleen cells, the DTH was significantly lower on day 10 than when tested 4 h after cell transfer (group A versus group C), as well as when tested on day 22 after infection (group E versus group C). At 22 days after infection, recipients of immune cells showed a significantly higher DTH than control mice did (group E versus group F). Oocyst production was also assessed, and comparison was made between immune-cell recipients and control mice. The oocyst production of mice (groups A, C, and E) receiving unfractionated immune cells was (7.668 ± 0.765) × 10^6 (mean and standard error), while control mice (groups B, D, and F) reveiving no cells produced (13.210 ± 0.302) × 10^6 oocysts ($P < 0.001$). Three mice died in the three control groups, while all mice from the three transferred groups survived. These results show that (i) the murine DTH reaction to *E. falciformis* is transferable with immune cells; (ii) during the peak oocyst production period (10 days p.i.), DTH reactivity is temporarily suppressed; (iii) the transfer of immune spleen cells improves the p.i. DTH reaction (day 22); and (iv) the transferred cells can partially protect recipient mice as measured by oocyst production and death rate after infection.

Phenotype of the cells mediating DTH to OAg. To evaluate the contribution of immune spleen T cells in the adoptive transfer of DTH, we treated immune spleen cells with monoclonal anti-Thy-1 antibody plus complement to obtain a Thy-1-negative (B-cell-enriched) population. Thy-1-negative spleen cell recipients showed no DTH response (Fig. 2). In contrast, Thy-1-positive cells (i.e., cells treated with only complement) transferred strong DTH reactivity to OAg. Spleen cells from hyperimmune mice were also passed through columns to deplete immunoglobulin-positive cells. The immunoglobulin-negative (T-cell-enriched) cells were injected intravenously into syngeneic, naïve mice. At 24 h after cell transfer, DTH was measured. The results (Fig. 3) demonstrate that the T-cell-enriched population transferred the DTH reactivity. Together, these experiments demonstrate that immune Thy-1-positive, immunoglobulin-negative cells mediate the adoptive transfer of DTH.

To further characterize the T cells that mediate DTH, we treated immunoglobulin-negative spleen cells with anti-L3T4 antibody and complement. The recipients of immune L3T4-negative, immunoglobulin-negative cells showed no DTH response, whereas recipients of complement-treated immunoglobulin-negative cells exhibited a strong DTH response (Fig. 3). Thus, the L3T4-positive cells were clearly responsible for transfer of DTH. However, whether the L3T4-positive T-cell subset acts alone or in cooperation with other

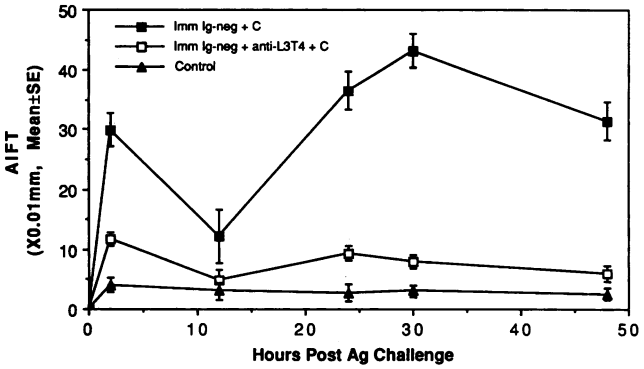

FIG. 3. Transfer of DTH to OAg by L3T4-positive immune spleen cells. Immune immunoglobulin-negative spleen cells were treated with anti-L3T4 antibody (GK1.5) plus complement (C) or C only prior to cell transfer. Each mouse received 3×10^7 immunoglobulin-negative immune spleen cells treated with C or anti-L3T4 antibody plus C. AIFT, Antigen-induced footpad thickness; Ag, antigen; Ig, immunoglobulin.

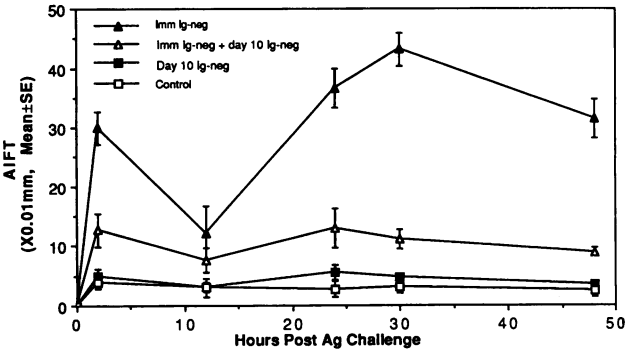

FIG. 5. Suppressive effects of T-cell-enriched spleen cells from acutely infected mice (10 days after infection) on the adoptive transfer of DTH by T-cell-enriched immunoglobulin-negative immune spleen cells. AIFT, Antigen-induced footpad thickness; Ag, antigen; Ig, immunoglobulin.

cell types to mediate the DTH response has yet to be determined.

Abrogation of DTH by lymphocytes from acutely infected mice. Immune-cell recipients temporarily lose their DTH reactivity 10 days after infection. To investigate the possibility that the parasite induced suppressor cells in these mice, spleen cells prepared from mice 10 days after the first infection were transferred together with immune cells. Mice which received only immune cells showed a strong DTH response, whereas those receiving immune cells plus spleen cells from acutely infected mice showed no DTH (Fig. 4). When immunoglobulin-negative immune cells were cotransferred with immunoglobulin-negative cells from mice infected for 10 days, the DTH transferred with immunoglobulin-negative immune cells was abrogated (Fig. 5). Thus, immune cells from acutely infected mice suppress the DTH transferred by immune T cells.

DISCUSSION

In the present study we analyzed the DTH response in mice infected with the intestinal parasite *E. falciformis*.

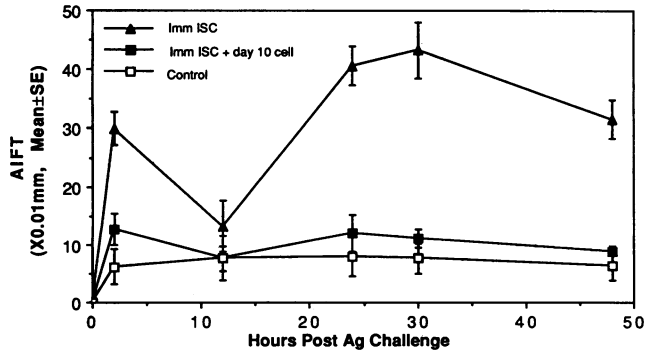

FIG. 4. Abrogation of the DTH response by lymphocytes from acutely infected mice. Groups of mice received either 3×10^7 immune spleen cells (ISC) or immune spleen cells plus 3×10^7 spleen cells obtained from mice 10 days after a primary infection. AIFT, Antigen-induced footpad thickness; Ag, antigen.

Immune mice exhibited a classic DTH reactivity when challenged with *E. falciformis* OAg. The OAg-induced footpad swelling reaction peaked at 2 h and also at 24 to 48 h after footpad antigen challenge. This DTH response was specific for eimerian antigens, as shown by the negative response to sheep erythrocytes. DTH reactivity was adoptively transferred with immune spleen cells. Anti-Thy-1 antibody plus complement treatment of immune cells abrogated DTH transfer, whereas the removal of immunoglobulin-positive B cells from the cell population did not reduce the level of transferred DTH. Therefore, we conclude that T cells mediate the DTH to OAg. Furthermore, immunoglobulin-negative cells depleted of L3T4-positive cells were unable to transfer DTH to OAg, thus demonstrating that L3T4-positive T lymphocytes are the major cell subset mediating the classical DTH to OAg.

During the acute stage of infection (i.e., peak oocyst production), mice were unable to mount a DTH response to OAg. This suppression also prevented the expression of adoptive transfer of DTH by immune cells in infected animals. Furthermore, the ability of immune spleen cells to adoptively transfer DTH to naïve recipients was inhibited when spleen cells from acutely infected mice were cotransferred. The cells responsible for the inhibition of DTH were immunoglobulin negative. Experiments are in progress to characterize this suppressor-cell population.

The DTH response consists of a cascade of events, a 2-h peak and a 24- to 48-h peak (23). The first peak is mediated by a Ly-1-positive, non-H-2 restricted T-cell population and mast cells, which induce the release of serotonin, thereby increasing local vascular permeability. This allows a second, Ly-1-positive, H-2-restricted T-cell population to enter the reaction site and release lymphokines responsible for the delayed peak (22). The DTH reactivity induced by *E. falciformis* infection from our study was consistent with this described pattern.

CMI is considered the basis for immunity to coccidiosis (30). This has been shown by adoptive transfer of syngeneic lymphocytes to athymic or bursectomized animals (18, 25, 26, 32). Vaccine-induced immunity to *Eimeria* spp. in chickens also correlates with DTH (8). Our experiments show that CMI, as measured by DTH, is associated with increased resistance against coccidiosis as evidenced by the ability of the transferred immune lymphocytes to reduce oocyst production as well as prevent the death of recipients.

In other parasitic infections, such as *Leishmania* and *Schistosoma* infections, DTH reactivity is regulated by suppressor T cells (3, 19). In our adoptive-transfer experiments, it is apparent that 10 days after infection, *Eimeria* spp. induce suppression to oocyst-specific DTH. During the acute period of infection, proliferating parasites may release antigens which induce T suppressor cells. Further studies are needed to clarify the role of parasite-derived antigens in the induction and suppression of DTH and the way in which this suppression affects the subsequent establishment of immunity in the host. Green and Ptak (10) have suggested that antigen overload and tissue damage caused by proliferating pathogens can suppress immunity. These mechanisms may be applicable to mice infected with coccidian parasites, and they should be investigated further. Immunosuppression during protozoan infections may be an important adaptive mechanism for evasion of host immunity (1). Howard et al. (12, 13) found that *Leishmania tropica*-specific T suppressor cells can suppress DTH and resistance in susceptible hosts. Yano et al. (35) demonstrated that nonspecific suppressor T cells can reduce in vitro lymphocyte proliferation during acute *T. gondii* infection. The specificity of the immune suppression in our experiments requires further investigation. Our experiments, however, demonstrate that the presence of acute-phase suppressor cells does not preclude resolution of infection.

Further studies should concentrate on the mechanisms by which antigen-specific L3T4-positive cells mediate their effects. Determination of which lymphokines are produced during infection may reveal whether L3T4-positive cells participate directly or indirectly through macrophage activation for intracellular or extracellular killing of coccidian parasites. In addition, analysis of parasitic antigens involved in the induction of acute-phase suppressor cells would greatly aid in our understanding of the dynamics of host-parasite interactions.

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