Antigen-Specific T-Helper Cells Abrogate Suppression in Trypanosoma cruzi-Infected Mice

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The ability of antigen-specific T-helper (Th) cells to enhance direct plaque-forming cell responses in spleen cells from Trypanosoma cruzi-infected C57BL/6 mice was investigated at various times during the course of infection from day 7 to day 230. The injection of antigen-specific Th cells in vivo or the addition of antigen-specific Th cells in vitro was effective in enhancing direct plaque-forming cell responses, except at the time of the most intense suppression during the acute phase of infection (i.e., day 28). The ability of antigen-specific Th cells to overcome nonspecific immunosuppression was due not only to the activity of antigen-specific Th cells added to Mishell-Dutton cultures but also to activation of resident T cells. Thus, antigen-specific Th cells and resident T cells act in concert to produce enhanced direct plaque-forming cell responses. The effect of plastic-adherent spleen cells from infected mice on the ability of antigen-specific Th cells to stimulate anti-sheep erythrocyte responses of normal spleen cells was examined because macrophages have been shown to have an immunoregulatory role during the course of experimental American trypanosomiasis. Increasing numbers of macrophages from infected mice caused increased immunosuppression of normal spleen cells that could not be overcome with the addition of primed Th cells. It can be concluded from these data that antigen-specific Th cells can potentiate immune responses in mice infected with T. cruzi but that highly active suppressor macrophages can inhibit the expression of these primed Th cells.

Trypanosoma cruzi, a flagellated protozoan parasite, causes acute and chronic infections in mammalian hosts and is the agent of Chagas' disease. Various studies have shown that T. cruzi-infected mammals have altered immune responses, including suppression of both humoral and cellular responses (2, 7, 12). Studies by Tarleton and Kuhn (15) and Reed et al. (11) showed that the addition of interleukin-2 (IL-2) to cultures of spleen cells from infected mice could restore antibody responsiveness to heterologous antigens. Additionally, Tarleton and Kuhn (15) showed that parasite-specific immune responses are suppressed during infection and can be enhanced by the addition of IL-2 in vitro to cultures of spleen cells from infected mice. Injection of IL-2 into infected mice has also been shown to enhance parasite-specific responses in vivo as reflected by reduced parasitemia levels and increased longevity (1). These data suggest that alteration in immune regulation in T. cruzi-infected mice, which results in suppression of humoral and cellular immune responses, is due to an inability of T-helper (Th) cells from infected mice to function normally. Cunningham and Kuhn (2) found that the suppression of immune responses in T. cruzi infections is mediated by a suppressor macrophage population. This was demonstrated by replacement of macrophages from spleen cells of infected mice with macrophages from normal spleens, which resulted in enhancement of previously suppressed direct plaque-forming cell (DPFC) responses (2). These studies suggest that the site where macrophages mediate suppression in T. cruzi-infected mice is the Th-cell population.

In the present study, the ability of antigen-specific Th cells to restore immune responses when injected into infected animals or added in vitro to infected spleen cells was examined. The addition of antigen-specific Th cells to infected spleen cells in vitro or injection of antigen-specific Th cells into T. cruzi-infected animals has proven to be a successful manipulation for enhancing immune responsiveness. The restoration of antigen-specific DPFC responsiveness was evident on all days of infection tested except those days when the most intense suppression existed (i.e., 3 to 4 weeks postinfection). Therefore, under certain conditions suppressor macrophages could suppress immune responses even when primed Th cells were present.

MATERIALS AND METHODS

Mice. C57BL/6 female mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at 4 to 6 weeks of age. The mice were provided food and water ad libitum.

Infection. Mice were given 10⁴ blood-form trypomastigotes of the Brazil strain of T. cruzi (9) intraperitoneally in 0.1 ml of Dulbecco phosphate-buffered saline (DPBS). C57BL/6 mice given this dose of parasites develop an intense parasitemia that peaks at about 4 weeks of infection, with the greatest degree of immunosuppression observed at this time (6).

Media. Modified Click medium (10) was used in the Mishell-Dutton culture system. Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) was used in the Jerne plaque assay. Modified Click medium was supplemented with 5 U of IL-2 (a gift of Hoffman-LaRoche Inc., Nutley, N.J.) per ml and used for maintenance of the Th cell lines.

TNP coating of SRBC and trypomastigotes. Trypomastigotes of T. cruzi were harvested from SVBLKH fibroblast cultures and washed three to five times in DPBS. The SVBLKH fibroblast cell line is a simian virus 40-transformed cell line derived from C57BL/6 mice. The trypomastigotes were then fixed in 1% Formalin in DPBS for 1 h at room temperature. After this incubation, the parasites were
washed five times in DPBS, counted, and adjusted to 2 × 10^8 to 4 × 10^8 trypomastigotes per ml. The trypomastigotes were reacted with 0.0135 M 2,4,6-trinitrobenzene sulfonic acid (TNP) in 0.28 M cacodylate buffer (10) in a foil-covered tube on a tumbler for 10 min. The TNP-coated T. cruzi (TNP-T. cruzi) were then washed five times in DPBS and stored at 4°C. SRBC were coated with TNP by the method of Mishell and Shigi (10).

T_h-cell lines. The T_h-cell lines were derived from spleen cells from C57BL/6 mice that had been immunized intraperitoneally three times, at weekly intervals, with 0.1 ml of 10% SRBC or 10^5 heat-killed (2 h at 56°C) trypomastigotes derived from infected fibroblast cultures. Splenic leukocytes were isolated bymincing the spleen and osmotically lysing the erythrocytes. The spleen cells were separated into nonadherent and adherent fractions by using nylon wool columns (5). The nonadherent cells were harvested from the column, spun down at 250 × g for 5 min, and suspended in a 1:20 dilution of anti-Lyt 2.2 + antiserum (Accurate Chemical and Scientific Corp., Westbury, N.Y.) in cytotoxicity medium (0.3% bovine serum albumin in RPMI 1640). The cell-antibody mixture was incubated for 1 h at 4°C. The cells were again washed in cytotoxicity medium, suspended in a 1:10 dilution of rabbit-Low-10x complement (Accurate Chemical and Scientific Corp.), and incubated at 37°C for 1 h. Treatment of T cells with anti-Lyt 2.2 antibody and complement resulted in a 93% pure population of L3T4 + cells, as determined by trypan blue dye exclusion. Viable cells were counted and adjusted to 5 × 10^6 cells per ml of medium supplemented with 5 U of IL-2 per ml. The cells were placed in wells of a 24-well plate (Flow Laboratories, Inc., McLean, Va.) with feeder cells that had been treated with mitomycin C at a concentration of 25 μg per 10^7 cells in a volume of 1 ml (10) and SRBC or heat-killed trypomastigotes as an antigen on day 1 of an 8-day cycle. Spleen cells from C57BL/6 mice that had previously been immunized at weekly intervals for 3 consecutive weeks with either SRBC or heat-killed trypomastigotes, as appropriate, were used as feeder cells. Mitomycin C-fixed feeder cells were added to T_h-cell lines at a concentration of 2 × 10^6 cells per well containing 2 × 10^6 antigen-specific T_h cells. On day 4, the cells received fresh IL-2-supplemented medium; on day 8, the T_h-cell lines received new feeder cells and antigen in medium supplemented with IL-2. The cells were maintained on this 8-day cycle. The SRBC-specific T_h-cell lines and parasite-specific T_h-cell lines helped induce specific antibody only when the appropriate antigen was employed in the Mishell-Dutton culture system.

Mishell-Dutton culture. A single-cell suspension of spleen cells was isolated from C57BL/6 mice. SRBC-specific and T. cruzi-specific T_h-cell lines were injected intraperitoneally into normal or infected mice for in vivo immunization of spleen cells used in the Mishell-Dutton culture system. Spleen cells were obtained from normal or infected C57BL/6 mice, and T_h-cell lines were added for in vitro immunization in Mishell-Dutton cultures. The erythrocytes were osmotically lysed with distilled water. Leukocytes were counted and adjusted to 5 × 10^6 cells per ml in modified Click medium, and 1-ml samples of the suspension were placed in wells of a 24-well plate (Flow Laboratories). Various doses of SRBC (Colorado Serum Co., Denver, Colo.) ranging from 10 to 50 μl of a 1% suspension or TNP-T. cruzi ranging from 10^2 to 10^6 trypomastigotes per well were set up in triplicate cultures and incubated for 5 days at 37°C in a humidified 5% CO2 environment (10).

Jerne plaque assay. Triplicate cultures were harvested from the Mishell-Dutton cultures and assayed individually by using a slide modification of the Jerne et al. plaque assay (4) either undiluted or at a dilution of 1:20. Cells were harvested from Mishell-Dutton cultures, washed, and then added to an agaro-SRBC or agaro-TNP-SRBC mixture. The cells and agaro mixture were poured onto precoated slides and allowed to solidify. The slides were then exposed to a 1:30 dilution of guinea pig complement (GIBCO) and allowed to incubate for 2 h at 37°C. The slides were fixed, and the number of DFPC was determined.

Statistics. DFPC data were normalized by a log2 transformation before analysis of variance and comparison of means by the Student-Newman-Keuls test (17). Differences were considered significant at P < 0.05. Error bars in the figures represent ± one standard deviation.

RESULTS
A series of experiments was done to determine the effect of SRBC-specific T_h cells on immune responses of spleen cells from infected mice. As expected, supplying antigen-specific T_h cells to normal spleen cells in vitro resulted in an increase in the number of DFPC (Fig. 1a). Cultures of spleen cells from animals infected for 7 and 14 days (Fig. 1b and c) also showed a proportional enhancement of DFPC when compared with that seen with normal spleen cells as doses of anti-SRBC T_h cells were increased. On day 21 of infection, responses of infected spleen cells were only marginally increased at higher T_h-cell doses in comparison with responses of spleen cells from earlier days of infection (Fig. 1d). In contrast, spleen cells from animals infected for 28 days (Fig. 1e) did not develop significant numbers of DFPC, even when large numbers of anti-SRBC T_h cells were supplied. The addition of as many as 10^6 antigen-specific T_h cells to spleen cells from animals infected for 28 days could not induce DFPC responsiveness (data not shown). As the infection period progressed to days 35, 42, 49, and 56 of infection (Fig. 1f, g, h, and i), higher T_h-cell doses were necessary to obtain nearly normal splenic anti-SRBC DFPC responses. Spleen cells from mice infected for 126 days (Fig. 1j) were not as responsive to the addition of T_h cells as those infected for 35, 42, 49, and 56 days, but on days 160 and 230 of infection (Fig. 1k and l) the addition of antigen-specific T_h cells resulted in enhanced DFPC responses, especially at higher doses of T_h cells. Therefore, the results from these experiments show that the immune activity of the antigen-specific T_h cells can enhance antibody responses of infected spleen cells, except on day 28 of infection.

The ability of SRBC-specific T_h cells to enhance immune responses when injected into infected mice was then examined. T_h cells were injected at intervals postinfection in various doses, and spleen cells from these animals were assayed in Mishell-Dutton cultures on day 49 (Fig. 2). Results of injection of anti-SRBC T_h cells on day 7 of infection (Fig. 2a) showed that 10^5 T_h cells were significantly different in their effectiveness in promoting enhanced DFPC responses. In addition, a dose of 10^5 primed T_h cells injected on day 14 appeared to induce a slightly enhanced DFPC response compared with that of the group that received 10^2 SRBC-specific T_h cells (Fig. 2b). Both groups of experimental animals that received antigen-specific T_h cells had significantly enhanced DFPC responses over the group that received DBS. In contrast, the results obtained with injection of primed SRBC-specific T_h cells on day 21 of infection showed no significant difference between experimental
FIG. 1. Effect of in vitro addition of anti-SRBC Th cells to spleen cells from normal (a) or infected (b, day 7; c, day 14; d, day 21; e, day 28; f, day 35; g, day 42; h, day 48; i, day 56; j, day 126; k, day 160; l, day 230) animals as determined by the ability to enhance DPFC responses. Experiments conducted with infected and noninfected mice were performed with three mice per group.

groups that received $10^2$ or $10^3$ antigen-specific Th cells and the control group that received DPBS (Fig. 2c). By day 28 (Fig. 2d), anti-SRBC Th cells were able to once again provide helper function in infected mice. This same trend continued with even greater effect on day 35 (Fig. 2e, $10^5$ versus $10^2$ cells and no Th cells). Likewise, when antigen-specific Th cells were injected into mice on various days of infection and spleen cells from these mice were assayed on day 56 of infection (data not shown), enhanced immune responses were obtained on all days except those days of infection when the most intense suppressive mechanisms are active, namely, 3 to 4 weeks postinfection.

It was of interest to determine the ability of T. cruzi-specific Th-cell lines added in vitro to spleen cells from infected animals to overcome suppressed immune responses. Spleen cells from infected mice were isolated and immunized in vitro with 30 μl of SRBC or $10^5$ TNP-T. cruzi in Mishell-Dutton cultures. The cells were then harvested from Mishell-Dutton cultures and subsequently used in the Jerne plaque assay with SRBC or SRBC conjugated to TNP, respectively, as the indicator systems for specific antibody. Parasite-specific Th-cell lines added to infected spleen cells in doses ranging from $10^2$ to $10^5$ Th cells increased responsiveness to parasite antigens during the course of infection except at 3 to 4 weeks postinfection, when the most intense suppression was observed (Fig. 3). The addition of T. cruzi-specific Th cells in vitro did not overcome suppression to SRBC antigen to a statistically significant level,
which suggests that parasite-specific Th cells added in vitro induce immune responsiveness only in an antigen-specific manner.

The ability of T. cruzi-specific Th cells to induce immune responsiveness when injected into infected animals on various days of infection was also investigated. T. cruzi-infected animals received \(10^6\) T. cruzi-specific Th cells intraperitoneally on various days of infection. On day 56, spleen cells from animals in all these experimental groups were isolated and utilized in Mishell-Dutton cultures with TNP-T. cruzi as the antigen. TNP-T. cruzi in doses ranging from 10 to \(10^5\) Formalin-fixed organisms were added in triplicate to culture wells. TNP-conjugated SRBC were then used as the indicator system in the Jerne plaque assay to determine hapten-specific DPFC response to the immunizing agent. T. cruzi-specific Th cells injected into infected animals enhanced immune responsiveness on most days of infection except on day 28, when suppressive mechanisms were most intense (Fig. 4).

It would appear that a small number of Th cells (10^5) injected into mice would be insufficient to provide significant functional activity unless these cells proliferated in vivo. Therefore, we investigated the ability of SRBC-specific Th cells treated or not treated with mitomycin C to enhance DPFC responses when injected into infected animals on day 28. Antigen-specific Th cells injected into infected mice must be capable of proliferation to provide enhanced DPFC responsiveness (Fig. 5).

It was thought that primed Th cells, when added to cultures of spleen cells from infected mice, could be inducing resident T cells to contribute to the observed enhanced responses. This was tested by adding antigen-specific Th
FIG. 2. Ability of SRBC-specific T_h cells injected into infected mice during infection to overcome suppression during infection. Groups of C57BL/6 mice received DPBS (□) or 10^2 (▲) or 10^3 (○) anti-SRBC T_h cell lines on day 7 (a), 14 (b), 21 (c), 28 (d), or day 35 (e) of infection with spleen cells from these animals in Mishell-Dutton cultures on day 49 of infection. Spleen cells were harvested from Mishell-Dutton cultures, and the Jerne plaque assay was used to determine DPFC responses. Experiments were performed with three mice per group.

cells to infected spleen cells isolated on day 31 of infection. These spleen cells had been either depleted of resident T cells by treatment with anti-Thy 1+ antibody and complement or had not been depleted of T cells. The addition of antigen-specific T_h to T cell-depleted infected spleen cells did not support enhanced DPFC responsiveness when compared with that of non-T cell-depleted infected spleen cells (Fig. 6). Therefore, we concluded that the addition of primed T_h cells cannot increase the DPFC response of infected spleen cells without the contribution of resident T_h cells.

The ability of splenic macrophages from infected mice to modulate DPFC responses of normal spleen cells and help provided by antigen-specific T_h cells were also investigated (Fig. 7). Various numbers of spleen cells, ranging from 1.56 × 10^5 to 5 × 10^6, from animals infected for 36 days were allowed to adhere to the bottom of 24-well plates for 3 h at 37°C. Spleen cells obtained from mice on day 36 of infection contained 16.5% nonspecific esterase-positive cells (8). The wells of the 24-well plates were washed gently after incubation, the supernatant fluid and nonadherent cells were removed, and 5 × 10^6 normal spleen cells plus an antigen (SRBC) were added to the culture wells. Anti-SRBC T_h cells, ranging from 10^2 to 10^6, were added in triplicate to wells of each culture plate containing the various dilutions of infected spleen cells. The normal spleen cells, antigen-specific T_h cells, plastic-adherent macrophages from infected animals, and antigen were maintained at 37°C for 5 days, at which time the number of DPFC was determined. In the absence of splenic macrophages from infected mice,
normal spleen cells showed an increase in responsiveness with increasing doses of antigen-specific Th cells. However, with increasing numbers of plastic-adherent cells from infected mice there were decreasing numbers of resultant DPFC even when normal spleen cells were coincubated with large numbers of antigen-specific Th cells (Fig. 7).

**DISCUSSION**

Reduction of cellular and humoral immune responses during T. cruzi infection results in profound suppression that continues into the postacute stage of the disease. The mechanism that mediates this suppression is unknown, but the Th-cell population appears to be the target for suppression (1, 11, 15). Existing evidence suggests that there is no depletion of T cells during infection (14), but rather that crucial functions of T cells are modified as a result of infection. It has been suggested that the inability of the Th-cell population to respond immunologically is related to a deficiency in production of IL-2 during infection (1, 11, 15). Harel-Bellan et al. (3) demonstrated that T cells from spleens of infected mice could not be stimulated by concanavalin A to produce or respond to IL-2. Tarleton and Kuhn (15) showed that infection with T. cruzi causes an early deficiency in the ability of spleen cells to produce IL-2 and that IL-2 production cannot be detected by 2 to 3 weeks of infection. Tarleton and Kuhn (15) and Reed et al. (11) showed that in vitro immune responses can be returned to nearly normal levels with the addition of exogenous IL-2. Choromanski and Kuhn (1) demonstrated that immune responses to SRBC and parasite-specific responses could be enhanced with in vivo administration of IL-2. In recent investigations, Tarleton (13) suggested that regulation of IL-2 production is mediated by suppressor cells that have the Thy 1+ Lyt 2- phenotype; he theorized that T-suppres-

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**FIG. 3.** Spleen cells from infected animals overcame suppressed anti-T. cruzi responses on most days of infection when tested by the addition of T. cruzi-specific Th cells in vitro. Doses of T. cruzi-specific Th cells, ranging from 10^2 to 10^5 in 10-fold serial dilutions, were added to spleen cells from infected animals on day 7 (a), 14 (b), 21 (c), 28 (d), 35 (e), or 42 (f) of infection. Spleen cells from infected animals received primary immunization in Mishell-Dutton cultures for 5 days with SRBC (●) or TNP-T. cruzi (□) as the antigen, and the Jerne plaque assay was used to determine DPFC responses.
FIG. 4. Addition in vivo of *T. cruzi*-specific T<sub>H</sub> cells to infected animals overcame suppressed immune responses; 10<sup>5</sup> *T. cruzi*-specific T<sub>H</sub> cells were injected into infected animals on various days of infection, and spleen cells were isolated from these animals on day 56. Spleen cells were cultured in Mishell-Dutton cultures with TNP-*T. cruzi* (TNP-TC) diluted from 10 to 10<sup>5</sup> and then used in the Jerne plaque assay to determine DPFC response. *T. cruzi*-specific T<sub>H</sub> were injected into animals on day 7 (a), 14 (b), 21 (c), 28 (d), 35 (e), 42 (f), or 49 (g).

Suppressor cells may have a large role in regulation of IL-2 production. Tarleton postulated that macrophages are inducers of lymphocyte suppressor effector cells, which thereby have a direct role in inhibiting IL-2 production (13). Therefore, induction of macrophages or suppressor T lymphocytes by parasites could provide mechanisms that reduce the ability of the T<sub>H</sub>-cell population to function in immune responses.

Inhibition of immune responsiveness in *T. cruzi*-infected mice seems to result in a suboptimal environment for elimination of the parasite from the host. Macrophages provide intense suppressive mechanisms that can be circumvented with appropriate experimental manipulation (1, 11, 15), such as the addition of SRBC-specific and parasite-specific T<sub>H</sub> cells in vivo and in vitro. The ability of primed antigen-specific T<sub>H</sub> cells to enhance immune responses appears to depend on the day of infection. During times of infection when suppressive mechanisms are most intense, i.e., 3 to 4 weeks postinfection, the addition of SRBC-specific T<sub>H</sub> cells or *T. cruzi*-specific T<sub>H</sub> cells to infected spleen cells or injection into infected animals cannot enhance antigenspecific responses, even when cells are added in excessive quantities. Thus, this suggests that the mechanism(s) of suppression that exists in vivo at this period of infection affects competent immune cells placed in the suppressive environment; i.e., primed antigen-specific T<sub>H</sub> cells are ren-
dered ineffective, and further immunologic mechanisms must be enhanced during this intense phase of infection to overcome suppression.

The present study shows that the addition of primed T_h cells can cause an augmentation of DPFC responses of spleen cells from infected mice. However, the addition of antigen-specific T_h cells can only enhance antibody responses to antigen when resident T cells are present in the spleen cell population. Comparison on day 31 of infected spleen cells that had been T cell depleted or not depleted of T cells suggests that the antigen-specific T_h cells can overcome the immunosuppression in T. cruzi infections by activating the resident T_h cells in some manner.

It seems clear that 10^5 primed T_h cells injected into infected animals is insufficient to provide effective help unless their numbers are expanded in vivo. This is supported by the observation that cells treated with mitomycin C and injected into infected animals were unable to enhance DPFC responses, presumably because of their inability to proliferate. In addition, the inability of primed T_h cells to enhance DPFC responses on day 21 may be due to prevention of this cell population from proliferating and exerting any effect on the resident T-cell population or to suppressive mechanisms in infected mice that do not allow primed T_h cells to express their helper activity. Although the ability of primed T_h cells to proliferate when injected into infected animals has not been tested directly, it is possible that inhibition of proliferation of T_h cells is a potent means of depressing immune responses.

The induction of suppression in experimental T. cruzi infections is mediated, at least in part, by suppressor macrophages (2). The data presented in this report show that increasing numbers of macrophages from infected spleen cells can abrogate the positive contribution of T_h cells in enhancing DPFC responses. On day 28 of infection, when macrophages are at their highest concentration in the spleen (14), the contribution of antigen-specific T_h cells is the least effective. At this time, suppressive effects of adherent cells are such that even 10^6 antigen-specific T_h cells added in vitro to 5 × 10^6 infected spleen cells in Mishell-Dutton cultures cannot overcome the suppression of antibody responses to SRBC. Thus, the variability in enhancement of antibody responses by the addition of antigen-specific T_h cells seems to depend on the quantity of suppressor cells present in the infected spleen cell population.

Inasmuch as the immune system of mice infected with T. cruzi is profoundly suppressed, any manipulation of the immune system that results in enhanced responses could have profound effects on parasite-specific immunity. Enhancement of the immune responses through injection of primed T_h cells provides a method with which to study immune regulation of infected animals. The ability of primed T_h cells to enhance immune responses on certain days of infection but not on other days of infection suggests that suppressive mechanisms that mediate down-regulation of the immune system are complex. At the most intense period of suppression, when injection of primed T_h cells does not
enhance responses, additional factors seem to be needed to up-regulate the immune system.

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LITERATURE CITED