INFECTION AND IMMUNITY, Apr. 1992, p. 1489–1498
0019-9567/92/041489-10$02.00/0

Vol. 60, No. 4

Effect of Antigen-Specific T Helper Cells or Interleukin-2 on Suppressive Ability of Macrophage Subsets Detected in Spleens of *Trypanosoma cruzi*-Infected Mice as Determined by Limiting Dilution-Partition Analysis

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Received 6 August 1991/Accepted 14 January 1992

***Trypanosoma cruzi*, a protozoan parasite and the causative agent of Chagas' disease, induces a state of lymphocyte hyporesponsiveness to both mitogenic and antigenic stimuli in mice during the acute phase of infection. Addition of spleen cells from *T. cruzi*-infected mice (SCinf) to microcultures of spleen cells from noninfected mice (SCn) suppresses the responsiveness of such cultures to antigenic challenge and to mitogenic stimulation. We analyzed the regulatory cell populations in SCinf by limiting dilution-partition analysis and found a complex regulatory circuit in *T. cruzi*-infected mice consisting of two suppressive macrophage subsets and an enhancing T-cell population. This T-cell population was able to abrogate or escape the suppressive ability of one suppressor macrophage subset, yet was suppressed by the other macrophage subset. To further study the cellular interactions of this regulatory circuit and analyze the suppressive abilities of the two suppressor macrophage subsets, we examined the effect of adding either primed T helper cells of known specificity or interleukin-2 to the limiting dilution-partition analysis microcultures. The results of these experiments suggest that one suppressor macrophage subset, which is abundant and, therefore, detected with low doses of SCinf, is able to suppress both mitogen- and primary antigen-specific responses but is unable to inhibit cells once they are already activated or primed. The other macrophage subset, which is presumably a less abundant or less active population (since high doses of SCinf are required to detect it), is able to suppress the response of activated or primed T cells by the inhibition of interleukin-2 production.**

Mice infected with the protozoan parasite *Trypanosoma cruzi* develop an experimental form of Chagas' disease. As a result of the infection, mice develop and exhibit a state of nonresponsiveness to immunological challenge with neoantigens, such as heterologous erythrocytes (8–10, 12, 22, 24), as well as suppressed parasite-specific responses (34). Furthermore, lymphocytes isolated from infected mice exhibit depressed proliferative responses following mitogenic stimulation with either T-cell or B-cell mitogens (11, 17, 25, 30).

However, the observed suppression is enigmatic because infected mice retain the ability in some cases to respond to antigens encountered prior to infection (13), immune responses to the parasite can be detected during the course of infection (32), and parasite-specific T helper cells are generated (1). Furthermore, the suppressive mechanism(s) induced by *T. cruzi* infection is not absolute, as evidenced by the results of Choromanski and Kuhn (5) which showed that repeated antigenic challenge, if timed at the correct interval, can overcome the suppressive state in an antigen-specific manner. Several cytokines and, particularly, interleukin-2 (IL-2) are also depressed in *T. cruzi*-infected mice (6, 14, 27, 28, 33); however, the administration of exogenous IL-2 can restore responsiveness both in vivo and in vitro (6, 33).

Analysis of regulatory cell populations which develop in the spleens of *T. cruzi*-infected mice by limiting dilution-partition analysis revealed the presence of at least three subsets of regulatory cell activity (2, 3). Two of the regulatory cell populations are macrophages which mediate suppressive events and were detected with the addition of either low doses or high doses of spleen cells from *T. cruzi*-infected mice (SCinf) to Mishell-Dutton microcultures of spleen cells from noninfected mice (SCn). The third regulatory subset was a T cell which was detectable upon the addition of intermediate doses of SCinf to the microcultures. This T-cell population had a positive or enhancing effect on the microcultures and could apparently counter the suppressive ability of one of the suppressor macrophage subpopulations, yet any beneficial effect of this regulatory T cell was abrogated by the second suppressor macrophage subpopulation.

The mechanism by which the T-cell population was able to abrogate the suppression mediated by the one subset of suppressive macrophages was unclear. Two possibilities were that (i) contained within the T cell population was a contrasuppressor T cell able to downregulate the one suppressor macrophage population or that (ii) the T cells represented a polyclonally stimulated population able to provide antigen-nonspecific help and that there was a differential ability between the two macrophage populations to suppress activated T cells. Because defects in T helper cell function have been reported in *T. cruzi*-infected mice (27, 28), the interaction of the two suppressor macrophage subsets with primed T helper cells of known antigenic specificity was examined in the present study. Furthermore, given the importance of the T-cell-derived lymphokine, IL-2, in promoting immune responses and the defects in IL-2 production observed in *T. cruzi*-infected mice, the effect of the suppressor macrophage subsets on the production of IL-2 and the ability of exogenously provided IL-2 to alleviate the suppression of each suppressor subset were also investigated.

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MATERIALS AND METHODS

Animals. Female C57BL/6 and C3HeB/FeJ mice (Jackson Laboratory, Bar Harbor, Maine) were 16 to 24 weeks old when used. Mice were housed five to a cage in a temperature-controlled room and were provided food and water ad libitum. The mice used in this study were maintained in accordance with National Institutes of Health guidelines.

Infections. Mice were infected with 10^3 blood-form trypomastigotes of the Brazil strain of *T. cruzi* contained in 0.1 to 0.2 ml of Dulbecco's phosphate-buffered saline (PBS) via intraperitoneal injection. Blood-form trypomastigotes were maintained by serial passage in C3HeB/FeJ mice as described previously (18).

Preparation of spleen cells. Mice anesthetized with ether were killed by cervical dislocation, and the spleens were removed. The spleens were teased apart in Dulbecco's PBS and further dissociated into single-cell suspensions by serial passage through 21-, 23-, and 26-gauge syringe needles. Erythrocytes (RBCs) were removed by hypotonic shock with cold distilled H_2O; this was followed by restoration of isotonicity with 10× Dulbecco's PBS. Cells were washed twice, counted, and adjusted to the desired concentration.

Cells used either as filler cells or as regulatory cells in limiting dilution-type microcultures were resuspended at 5×10^7 cells per ml and were treated with 0.05 ml of mitomycin C (0.5 mg/ml) per ml of cell suspension for 35 min at 37°C in a humidified environment containing 5% CO_2. The cells were then washed three times with medium and counted, and the concentration was adjusted as specified.

Preparation of anti-SRBC-specific T helper cell lines. C57BL/6 mice were immunized with three intraperitoneal injections of 0.2 ml of 10% sheep RBC (SRBC) at weekly intervals. Four days following the last immunization, the spleens were removed and the spleen cells were prepared as described above. T-cell-enriched spleen cells were prepared by collection of the nonadherent cells after passage over a nylon wool column as described by Julius et al. (16). The nonadherent cells were adjusted to 10^7 cells per ml and depleted of Lyt 2^+ cells by treatment with a 1/20 dilution of anti-Lyt 2 antibody (Accurate Chemical and Scientific Corp., Westbury, N.Y.) at 4°C for 1 h and then resuspension in a 1/10 dilution of Low-Tox rabbit complement (Accurate Chemical and Scientific Corp.) for 1 h at 37°C. The cells were washed and adjusted to 5×10^7 cells per ml in modified Click's medium containing 15% fetal bovine serum (FBS) and 20 U of human recombinant IL-2 (Hoffmann-La Roche Inc., Nutley, N.J.) per ml. A 1-ml sample of the T-cell-enriched spleen cell suspension was plated per well in Linbro 24-well tissue culture plates (Flow Laboratories, McLean, Va.) containing mitomycin C-treated feeder spleen cells which had previously been incubated for 24 h with 10% SRBC ghosts. T cells were maintained on an alternating 4-day cycle of transfer to new medium or transfer to new feeder cell layers.

Prior to use in the limiting dilution-partition analysis microcultures, the helper activity of the various T helper cell lines was evaluated by the ability of 1×10^5 T helper cells to increase the plaque-forming response of Mishell-Dutton cultures containing 5×10^6 spleen cells stimulated with SRBC.

In experiments in which T cells were depleted from spleen cell preparations, the cells were treated with anti-Thy 1.2 diluted 1/10 with cytotoxicity medium and using Low-Tox complement (Cedarlane Laboratories, Hornby, Ontario, Canada).

Preparation of IL-2. IL-2 (lot MC-1) was prepared from culture supernatants of the constitutively IL-2-producing Gibbon tumor T-cell line, MLA-144 (15, 23). MLA-144 cells were cultured in RPMI 1640 medium containing 10% FBS at a starting concentration of 5×10^5 cells per ml for 3 days. The culture supernatants from several cultures were pooled and centrifuged to remove any MLA-144 cells, filter sterilized by passage through 0.22-μm-pore-size Acrodisc filters (Gelman Sciences, Inc., Ann Arbor, Mich.), placed in aliquots, and stored frozen at −70°C until use. The IL-2 content of this supernatant fluid was determined to be 62.5 U/ml by comparison to a reference standard (human IL-2 from the Jurkat cell line, 500 U/ml) from the Biological Response Modifiers Program (Biological Resources Branch, NCI-FCRF, Frederick, Md.), using the CTLL-2 assay. Activity (units per milliliter) in the MLA-144 culture supernatant was estimated by the weighted probit parallel line method of analysis (31).

Limiting dilution analysis microcultures. Limiting dilution microcultures were performed as described in detail earlier (2, 19). Prior to dispensing the cells into the microwells of Terasaki trays (Robbins Scientific Corp., Mountain View, Calif., or Dynatech Laboratories, Inc., Alexandria, Va.), mitomycin C-treated filler and regulatory cell suspensions, both at 3.75×10^6 cells per ml, were mixed in various ratios to give dilutions of the regulatory cells. For Mishell-Dutton-type microcultures, the mixtures of filler and regulatory cells were then added to responder cells at a concentration of 15×10^6 cells per ml in a ratio of 2 parts fillers plus regulators to 1 part responder cells immunized with 30 μl of 1% SRBC per ml of cell suspension. Each Mishell-Dutton microculture received 20 μl of the cell mixture, resulting in each culture containing 50,000 fillers and regulators and 100,000 responder cells. Trays were incubated for 6 days at 37°C in a humidified environment containing 5% CO_2. At the end of the culture period, the fraction of responding and nonresponding microcultures was determined by testing the microculture supernatant fluids of each individual microculture for the presence or absence of detectable SRBC-specific antibody by using the micro-enzyme-linked immunosorbent assay (microELISA) described below.

For mitogen-stimulated microcultures, the mixtures of filler plus regulatory cells were added to responder cells at a concentration of 7.5×10^6 cells per ml in the ratio of 2 parts filler plus regulatory cells to 1 part responder cells. This resulted in each microculture receiving 25,000 responder cells plus 50,000 filler and regulatory cells. Phytohemagglutinin-P (PHA-P) (1 μl; 100 μg/ml; Sigma Chemical Co., St. Louis, Mo.) was added to each microculture, and the trays were incubated for 72 h at 37°C in a humidified environment containing 5% CO_2. Twelve hours prior to termination of the cultures, 1 μl of [^{3}H]thymidine (6.7 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.) containing 0.1 μCi was added to each culture. Cultures were harvested by resuspending the cells in each well by agitation with a Titertek 6 channel Terasaki pipettor (Flow Laboratories) and then were transferred to glass filter pads (type A/E; Gelman Sciences), prescored and cut to fit the Terasaki trays. Each well was washed two times with 10 μl of PBS, and the well's contents were transferred to the filter pad after each wash. After the contents of all wells in a tray were transferred to the filter pad, the pad was placed on a Büchner funnel and washed by suction two times with 10 ml of PBS, once with 5% trichloroacetic acid, and then once with absolute methanol. Filters were allowed to dry, cut to separate the individual wells, and placed into scintillation vials with 2 ml of ScintiVerse II fluid (Fisher Scientific Co., Fairlawn, N.J.).

Radioactivities of the samples were counted with a Beckman LS7500 scintillation counter.

The mitogen-stimulated microcultures were scored as follows. The mean counts per minute of [^{3}H]thymidine incorporated by cultures receiving only responder and filler (no regulatory) cells were determined. The proliferative response of individual microcultures was considered suppressed if the amount of [^{3}H]thymidine incorporated by the microculture was less than the [^{3}H]thymidine incorporation 2 standard deviations below the mean of microcultures receiving responder and filler cells only (no regulatory cells). Because the emphasis was the examination of suppressed responses, the results are presented as the fraction of nonsuppressed (i.e., responding microcultures [±95% confidence intervals]) instead of the more commonly expressed fraction of nonresponding microcultures (20). The amount of [^{3}H]thymidine incorporated by each microculture is plotted against the dose of regulatory cells present in the microculture.

Determination of IL-2 content in microcultures by induced proliferation of CTLL-2 cells. (i) Maintenance of CTLL-2 cells. CTLL-2 cells were cultured at a starting concentration of 10^5 cells per ml in RPMI 1640 medium containing 10% FBS to which was added IL-2-containing MLA-144 supernatant (MC-1) to a final concentration of 15%. CTLL-2 cell cultures were passaged every 2.5 to 3 days, at which time the concentration of CTLL-2 cells had usually reached 5×10^5 to 6×10^5 cells per ml.

(ii) Assay of IL-2 content. CTLL-2 cells were washed twice ($400 \times g$ for 10 min) in RPMI 1640 medium containing 5% FBS. After the second wash, the cells were resuspended in 10 ml of medium and incubated for 20 min at 37°C in a humidified atmosphere containing 5% CO_2 to allow release of any residual cell-bound IL-2 obtained from the propagation culture medium (21). The CTLL-2 cells were washed two more times as above and resuspended in modified Click's medium (RPMI 1640 base [4]) containing 10% FBS. Cells were counted and adjusted to concentration as required.

Culture supernatant (10 μl) from each limiting dilution microculture was transferred to a Terasaki tray well, and 10 μl of CTLL-2 cells, at a concentration of 2.5×10^5 cells per ml, was added to each well. Following incubation for 20 h at 37°C in a humidified atmosphere containing 5% CO_2, the CTLL-2 cultures were given 1 μl of [^{3}H]thymidine (0.1 μCi) and cultured for an additional 6 h. Cultures were harvested as described above for mitogen-stimulated cultures.

Determination of anti-SRBC antibody in microculture supernatants. Culture supernatant (5 μl) was removed from each individual microculture and tested for the presence of antibody against SRBC by use of a microELISA spot test. The preparation of antigen-coated plates, wash procedure, and the conditions of the assay have been described in detail elsewhere (2). Briefly, the individual culture supernatants were transferred to SRBC-coated Terasaki tray wells which previously had been fixed and blocked overnight. The trays were then incubated for 1 h at room temperature and washed, and 10 μl of biotinylated anti-mouse immunoglobulin G (IgG), IgA, and IgM (Cappel Worthington, Malvern, Pa.) diluted 1/5,000 in 1% milk in PBS was added. After incubation for 1 h at room temperature, the trays were washed and 10 μl of avidin-urease (Allelix, Inc., Mississauga, Ontario, Canada) diluted 1/500 in 1% bovine serum albumin in PBS was added. Following incubation at room temperature for 1 h, the plates were washed, and 10 μl of substrate solution consisting of urea, bromocresol purple indicator, and EDTA (7) was added to each well. Trays were incubated for 2 h at 37°C to allow development of the reactions and then scored for the number of positive and negative reaction wells per tray.

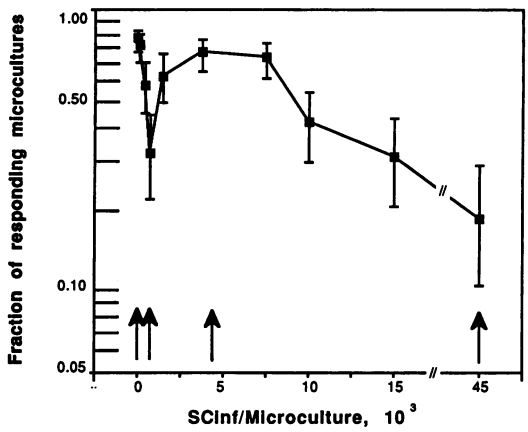

FIG. 1. Regulation of anti-SRBC responses in Mishell-Dutton cultures of SCn by SCinf. The fraction of responding Mishell-Dutton microcultures (±95% confidence interval) was determined by the microELISA detection of SRBC-specific antibody in individual microcultures (n = 72 microcultures per SCinf dose). Arrows indicate the regions of the response profile at which the effect of SRBC-specific T helper cell addition was tested (see Fig. 2).

Hemolytic plaque assay. Direct plaque-forming cell (PFC) responses were measured by using the Cunningham slide technique (7). The microcultures in each tray were resuspended by agitation with a Titertek 6-channel Terasaki pipettor. After resuspension of all the cultures within a tray, the resuspended cells were collected and all identical cultures on the tray were pooled. Cells were pelleted by centrifugation, washed once with Hanks balanced salt solution plus 0.1% gelatin, and resuspended in plaque-developing solution (4). Appropriate dilutions were made in developing solution, and 30 μl of the suspension was added to triplicate wells of Air-Lock PFC slides (Spiral Scientific, Cincinnati, Ohio). Slides were incubated for 1 h at 37°C in a humidified 5% CO_2 environment and then scored for plaques.

RESULTS

Effect of antigen-specific T helper cell lines on suppressive ability of SCinf. Titration of the number of mitomycin C-treated SCinf into Mishell-Dutton microcultures stimulated with SRBC and then the analysis of the microculture supernatants for the presence of specific antibody gave a characteristic pattern for the regulation of the responses. Plotting the fraction of microcultures producing SRBC-specific antibody for a large number of replicate cultures at each regulatory cell dose demonstrated the occurrence of two dose zones of suppressed responses with an intervening zone where responsiveness was restored to the level of microcultures receiving no SCinf (Fig. 1). Previous experiments established that the two dose zones of suppression were mediated by macrophages, whereas the restoration of responsiveness was due to the presence of a T-cell population (3). To further examine the interaction of T cells with the suppressor macrophage subsets, we added SRBC-specific T helper cells to a replicate series of microcultures at four points in the dose curve. These points, indicated by the

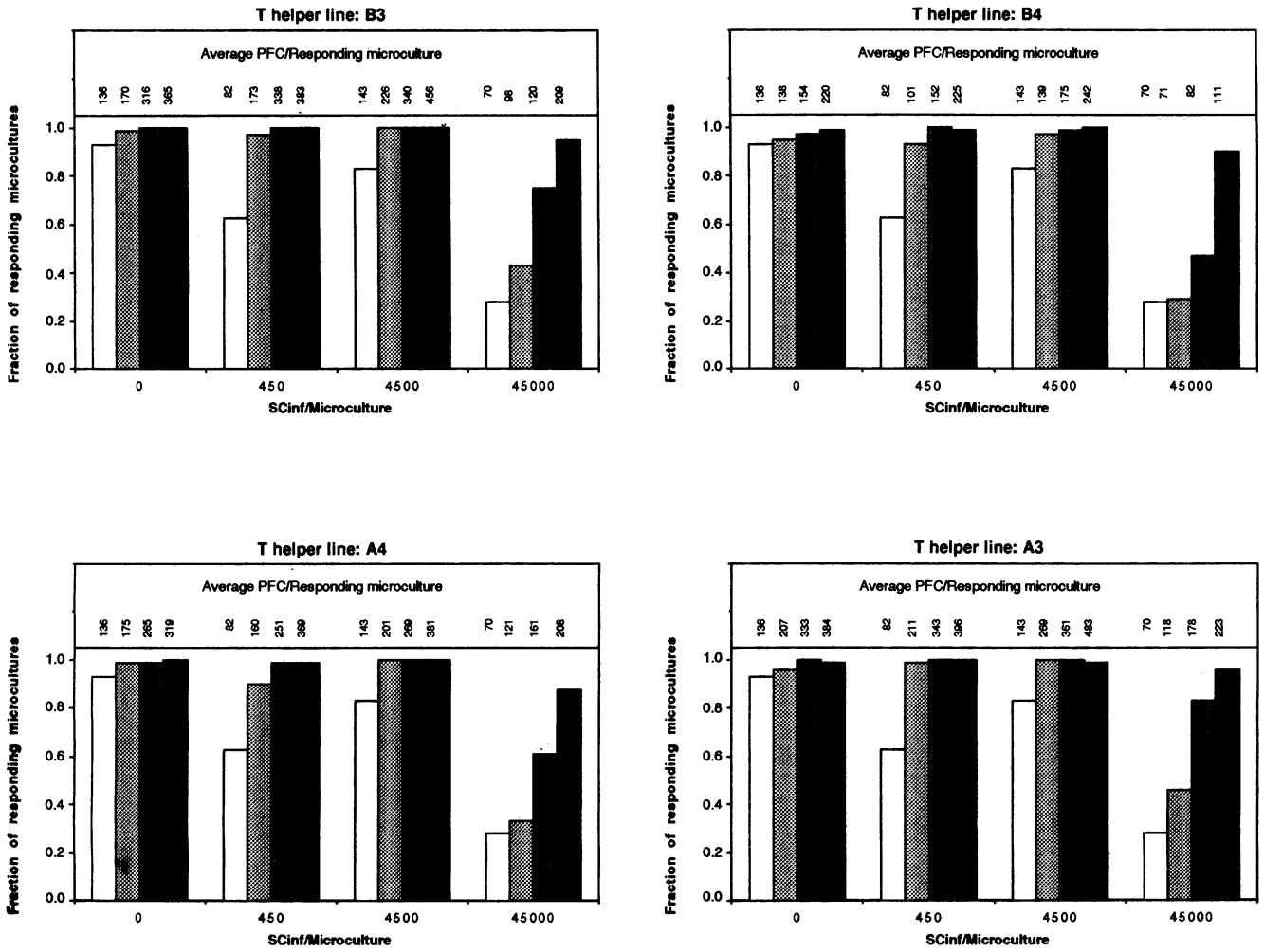

FIG. 2. Effect of SRBC-specific T helper cell addition on the response of limiting dilution-partition microcultures. SRBC-specific T helper cells (0 [open bars], 1 [lightly stippled bars], 10 [medium-stippled bars], or 100 [darkly stippled bars] T helper cells per microculture) were added to microcultures (n = 72 microcultures per SCinf dose) containing the indicated numbers of SCinf as regulatory cells. The fraction of responding microcultures was determined by the microELISA detection of SRBC-specific antibody in the individual microcultures, and the average number of PFC per microculture was calculated as described in Materials and Methods.

arrows in Fig. 1, correspond to (i) a point of normal responsiveness (no SCinf added) and to points in (ii) the low-dose zone of suppression, (iii) the intermediate-dose zone of restored responses, and (iv) the high-dose zone of suppression. Four T helper cell lines were tested which were characterized as high (A3)-, medium (B3)-, or low (A4 and B4)-activity helpers by their relative abilities to enhance the antigen-specific response of Mishell-Dutton cultures (data not shown). Figure 2 shows the effect of adding these helper cells to the microcultures on the suppressive ability of the titrated SCinf cells. Addition of even a single primed T helper cell to the microcultures was able to relieve the suppression of the suppressor macrophage subpopulation present in low doses of SCinf (450 SCinf per microculture). This was evident in that both the fraction of responding microcultures was elevated to normal levels and the PFC response per responding microculture was comparable to that of the control microcultures receiving helper cells but no SCinf. The suppression induced by high doses of SCinf (45,000 SCinf per microculture) was more resistant to the effect of the primed T helper cells, and the T helper cells were only partially effective. Although primed T helper cells were able to counter the suppression, this effect was highly dependent on the number of T helper cells present, and in some cases, the fraction of responding microcultures returned to levels comparable with the normal response only after the addition of as many as 100 antigen-specific T helper cells to the microcultures. However, although all the microcultures could be classified as responsive by the production of specific antibody, the cultures were clearly suppressed because the PFC response per responding microculture was only 50 to 65% of the PFC response of microcultures receiving an equivalent number of T helper cells but no SCinf.

Addition of the SRBC-specific T helper cell lines to the microcultures which had received an intermediate dose of SCinf (4,500 SCinf per microculture) had only a minor effect on the fraction of responding microcultures because the majority of the microcultures were already responsive (Fig. 2). However, if the average PFC response was examined, there was some evidence of synergy between specific T helper cells added to the microcultures and the T cells

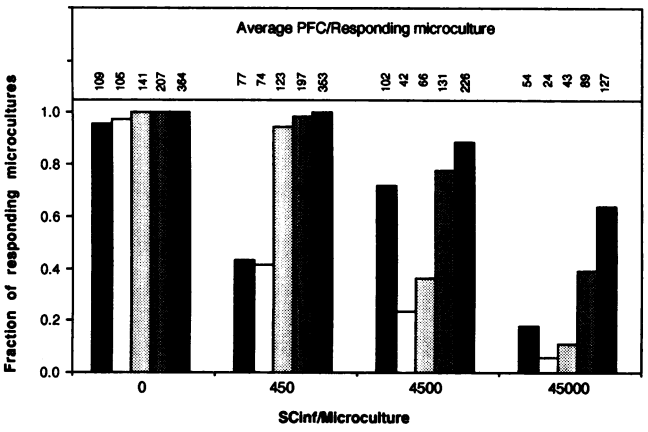

FIG. 3. Effect of adding the SRBC-specific T helper cell line A4 on the regulation of Mishell-Dutton microcultures by SCinf depleted of endogenous T cells. Mishell-Dutton microcultures containing the indicated number of SCinf (solid bars) or SCinf depleted of T cells with anti-Thy 1.2 and complement received the indicated number of T helper cells per microculture (open bars, 0 T helper cells; lightly stippled bars, 1 T helper cell; medium-stippled bars, 10 T helper cells; darkly stippled bars, 100 T helper cells). The fraction of responding microcultures (n = 72 microcultures per SCinf dose per T helper cell dose) was determined by the microELISA detection of SRBC-specific antibody in the microcultures, and the average number of PFC per microculture was calculated as described in Materials and Methods.

present in the SCinf. There was an increase in the number of PFC for the comparison of responses between microcultures containing intermediate doses of SCinf which were not supplemented with the antigen-specific T helper cells and those microcultures receiving increasing numbers of the helper cells. For example, compare the response of 143 average PFC per responding microculture in the absence of antigen-specific T helper cells versus the response of 361 PFC when 10 antigen-specific T helper cells (line A3) were added to microcultures containing 4,500 SCinf. Also, the PFC response for microcultures receiving the same dose of antigen-specific T helper cells showed an increase in the number of PFC in those microcultures containing an intermediate dose of SCinf compared with the response when no SCinf were present. For microcultures supplemented with 100 T helper cells (line A3), the average PFC per responding microculture increased from 384 in the absence of SCinf to 483 in the presence of 4,500 SCinf.

Effect of SRBC-specific T helper cells on suppressive ability of T-cell-depleted SCinf in Mishell-Dutton cultures. Because there appeared to be a synergistic interaction between the resident T cells of SCinf and the SRBC-specific T helper cells, it was conceivable that the ability of the antigen-specific T helper cells to partially alleviate the suppression in the high-dose zone was dependent on T cells in the SCinf. Therefore, the ability of antigen-specific T cells to affect the responsiveness of Mishell-Dutton microcultures receiving T-cell-depleted SCinf as regulatory cells was examined (Fig. 3). Depletion of T cells from the SCinf did not affect the low-dose zone of suppression but did eliminate enhancement of responsiveness with intermediate doses of SCinf. Instead, the high-dose zone of suppression was slightly increased. Addition of antigen-specific T cells to the microcultures containing 450 SCinf was able to restore the responsiveness of the microcultures, even with the addition of a single antigen-specific T helper cell. T helper cells remained capable of partially alleviating the suppression caused by high doses of T-cell-depleted SCinf. There was an increase in both the fraction of microcultures responding and the average number of PFC per responding microculture.

Production of IL-2 in microcultures receiving SCinf as regulatory cells. SCn were stimulated with PHA in the presence of various doses of SCinf. As shown in Fig. 4, the response curve for the microcultures has two dose zones at which the number of cultures responding was suppressed, with an intervening zone of nonsuppressed responses. This pattern was also evident from [^{3}H]thymidine incorporation by the individual microcultures receiving the various doses of SCinf.

When 10 μl of each individual microculture supernatant was removed from the PHA-stimulated microcultures and tested for IL-2 activity, the results showed that there was little effect on the production of IL-2 in the cultures which received low doses of SCinf (Fig. 5). However, the produc-

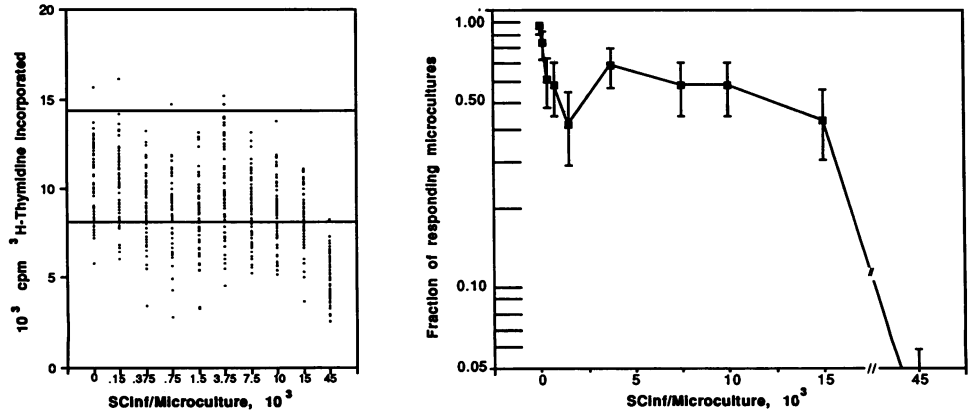

FIG. 4. Limiting dilution-partition analysis of SCinf regulatory activity affecting the proliferative response of SCn stimulated with PHA. Left panel: Each datum point represents the PHA-induced proliferation of an individual microculture of SCn containing the indicated number of SCinf (n = 60 microcultures per SCinf dose). The solid horizontal lines indicate the [^{3}H]thymidine incorporation level 2 standard deviations above and below the mean response of microcultures receiving filler cells only (no SCinf). Right panel: Fraction of responding microcultures following the addition of various doses of SCinf to the microcultures. Error bars indicate 95% confidence intervals. Microcultures were considered suppressed by the criteria described in Materials and Methods.

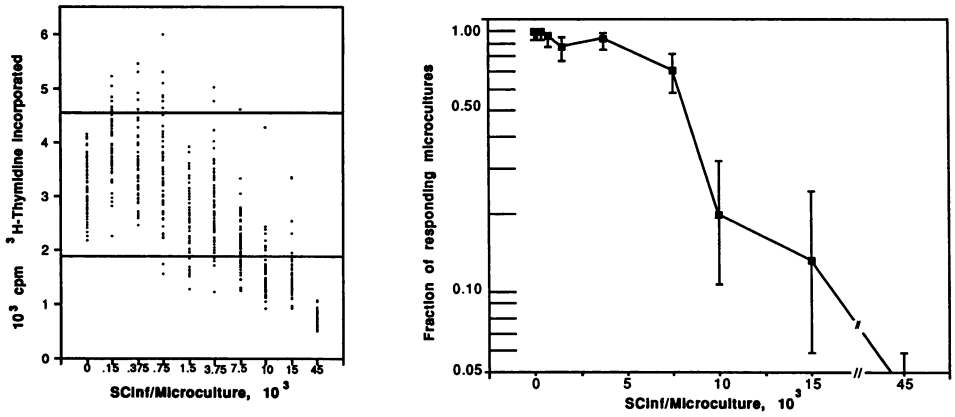

FIG. 5. Production of IL-2 in PHA-stimulated microcultures containing various numbers of SCinf. Individual microculture supernatants were tested for the presence of IL-2 by the CTLL-2 proliferation assay. Left panel: Each datum point represents the IL-2-induced proliferation of CTLL-2 cells by an individual microculture supernatant obtained from the experiment shown in Fig. 4 (n = 60 microcultures per SCinf dose). Right panel: Fraction of responding microcultures in the CTLL-2 assay. CTLL-2 microcultures were considered nonresponsive if the counts per minute of [^{3}H]thymidine incorporated were more than 2 standard deviations below the mean response of microcultures receiving filler cells only (no SCinf).

tion of IL-2 was inhibited by the presence of high doses of SCinf in the microcultures. This was demonstrable by both [^{3}H]thymidine incorporation and the resultant fraction of responding microcultures. Microculture supernatants from Mishell-Dutton cultures of SCn challenged with SRBCs and cocultured with various doses of SCinf gave similar results (data not shown). However, the level of IL-2 measured in the Mishell-Dutton cultures was lower throughout the dose curve of SCinf.

Effect of supplementing the microcultures with IL-2 on the suppressive ability of SCinf. The proliferative response of the microcultures supplemented with IL-2 (Fig. 6) was approximately doubled throughout the dose range of SCinf, resulting in a higher response range than for those cultures which were not treated with IL-2 (Fig. 4). Despite the increased baseline proliferative response, low doses of SCinf were still able to suppress the proliferative response, and IL-2 was unable to alleviate the suppression. However, addition of IL-2 to the microcultures which had received high doses of SCinf did abrogate the suppression and restored the responsiveness of the microcultures. Although [^{3}H]thymidine incorporation by the microcultures was still slightly depressed compared with the proliferative response of microcultures which did not receive any SCinf, the response of the majority of microcultures receiving high doses of SCinf was increased by the addition of IL-2 to within the normal response range. (Similar experiments showed that supplementing Mishell-Dutton microcultures with IL-2 had the same effect on the regulation of the microculture responses by SCinf [data not shown].)

Effect of preincubating IL-2-containing medium with various doses of SCinf on the ability of the supernatants to support the proliferation of CTLL-2 cells. Microcultures consisting of various numbers of mitomycin C-treated SCinf, ranging from 0 to 45,000 SCinf per microculture, and filler cells (mitomycin C-treated SCn) at a constant cell density of 50,000 total cells per microculture were incubated with 1 U of IL-2 per microculture for 12 h. Following this incubation, 10 μl of

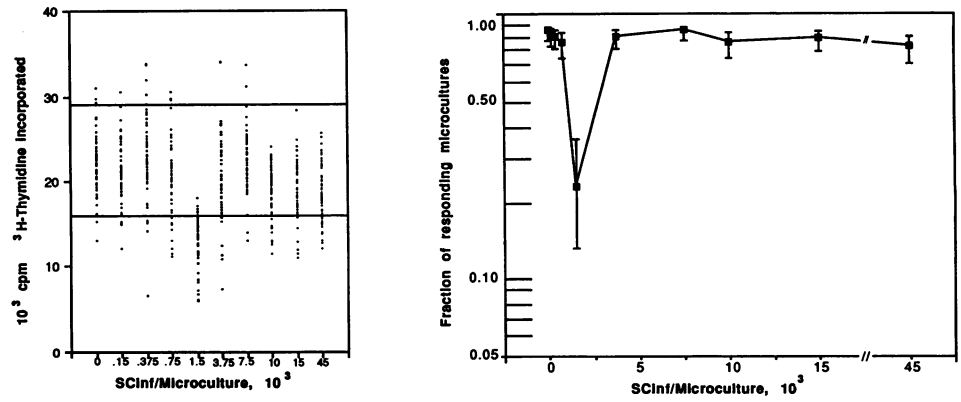

FIG. 6. Effect of IL-2 addition on the responsiveness of PHA-stimulated microcultures of SCn containing various numbers of SCinf. Left panel: Each point represents the PHA-induced proliferation of an individual microculture of SCn containing the indicated number of SCinf (n = 60 microcultures per SCinf dose) in the presence of 1 U of IL-2 per microculture. The solid horizontal lines indicate the [^{3}H]thymidine incorporation level 2 standard deviations above and below the mean response of microcultures receiving filler cells only (no SCinf). Right panel: Fraction of responding microcultures in the presence of IL-2 following the addition of various doses of SCinf to the microcultures. Error bars indicate 95% confidence intervals. Microcultures were considered suppressed by the criteria described in Materials and Methods.

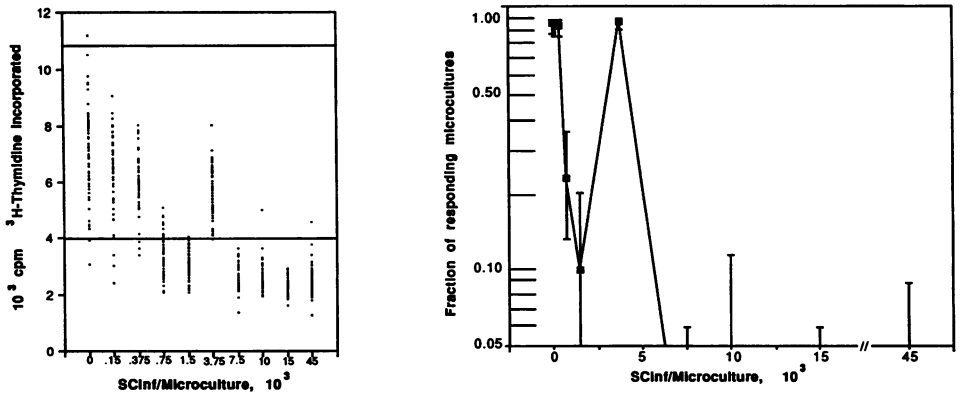

FIG. 7. Effect of incubating IL-2-containing supernatants with various doses of SCinf on their ability to support the growth of CTLL-2 cells. IL-2-containing medium was incubated with microcultures of the indicated doses of SCinf overnight, and a portion of the supernatant from each microculture was tested for residual IL-2 activity by its ability to support the proliferation of CTLL-2 cells. Left panel: Each point represents the IL-2-induced proliferation of CTLL-2 cells by an individual culture supernatant (n = 60 microcultures per SCinf dose). Right panel: Fraction of responding microcultures in the CTLL-2 assay. CTLL-2 microcultures were considered inhibited if the counts per minute of [^{3}H]thymidine incorporated were more than 2 standard deviations below the mean response of supernatants incubated with filler cells only (no SCinf).

each microculture supernatant was individually tested for residual IL-2 activity by its ability to support the proliferation of CTLL-2 cells. As shown in Fig. 7, the ability of the microculture supernatant fluids to support the proliferation of CTLL-2 cells declines substantially beginning with those culture supernatants preincubated with a low dose of SCinf (0.75×10^3 SCinf per microculture). A slight increase in the proliferative response occurred when the CTLL-2 cells were cultured with supernatants which had been preincubated with an intermediate dose of SCinf. However, this increased proliferative response of the CTLL-2 cells did not reach the magnitude of proliferation of many of the control CTLL-2 microcultures (no SCinf). Despite the proliferative increase in the middle region of the dose curve, further increase in the dose of SCinf with which the IL-2-containing medium was preincubated caused the residual IL-2 activity in the culture supernatants to decrease.

Proliferative response of CTLL-2 cells to IL-2 when cocultured with various doses of SCinf. Microcultures of CTLL-2 cells were incubated with IL-2-containing medium (1 U per microculture) for 2 h prior to the addition of various doses of SCinf to the microcultures. Addition of SCinf to the microcultures caused the proliferative response of the CTLL-2 cells to decrease as the SCinf dose increased (Fig. 8). There was but a single dose zone of suppressive (inhibitory) activity which was reflected in the steady decline in proliferation beginning with the addition of low doses of SCinf.

DISCUSSION

We have previously demonstrated that the restoration of responsiveness in limiting dilution-partition analysis microcultures used to analyze the ability of SCinf to suppress either mitogen-stimulated or Mishell-Dutton microcultures was dependent on the presence of a T-cell population contained within the SCinf (3). Apparently, this T-cell population reached sufficient concentration with the addition of intermediate doses of SCinf to the microcultures to counter-

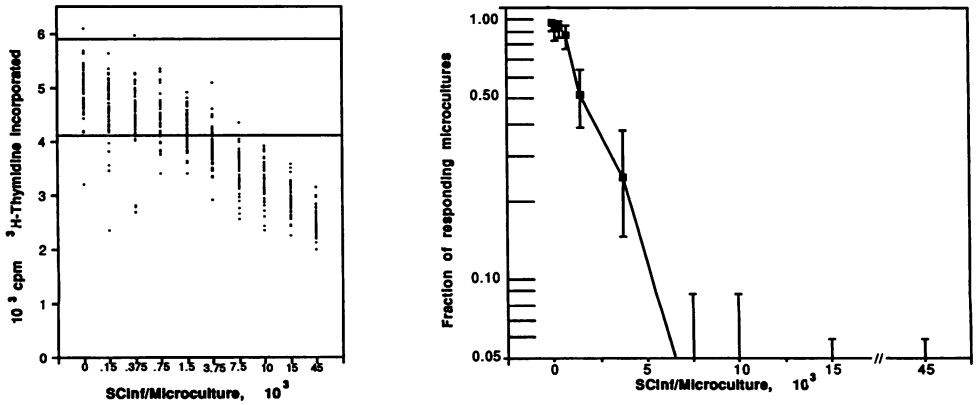

FIG. 8. Effect of SCinf on the IL-2-stimulated proliferation of CTLL-2 cells. Microcultures containing 2,500 CTLL-2 cells per culture were incubated with the indicated number of SCinf in the presence of IL-2. CTLL-2 proliferation was considered inhibited in individual microcultures if the amount of [^{3}H]thymidine incorporated was more than 2 standard deviations below the mean response of CTLL-2 cells incubated with filler cells only (no SCinf). Left panel: Proliferative response of individual microcultures (n = 60 microcultures per SCinf dose). Right panel: Fraction of responding microcultures.

act or abrogate the suppression mediated by macrophages detected with low doses of SCinf. However, this T-cell subset's ability to alleviate the suppression was somehow circumvented by the addition of high doses of SCinf which contained a second macrophage subset that was responsible for the expression of the suppression.

The T-cell population could have counteracted the low-dose zone of suppression by acting as contrasuppressors causing the downregulation of the suppressive ability of the macrophage population. Alternatively, the polyclonal stimulation of splenic T cells in *T. cruzi*-infected mice could lead to the development of a polyclonally activated population of T cells which might be aiding the microculture responses by providing nonspecific helper activity, perhaps by the production of lymphokines. Cunningham and Kuhn (11) have previously suggested that SCinf are polyclonally activated based on their increased ability, compared with normal T cells, to collaborate with B cells in generating plaque cell responses to SRBC and trinitrophenyl groups.

Cunningham and Kuhn (11) observed the ability of purified T cells from *T. cruzi*-infected mice to increase the proliferative response of SCn to lipopolysaccharide. This would be consistent with the ability of polyclonally stimulated T cells to provide nonspecific help through the possible secretion of multiple lymphokine activities, some of which were directly affecting B-cell responses. Furthermore, the ability of antigen-specific T helper cells to abrogate the low-dose zone of suppression in the absence of resident T cells in SCinf would argue against the necessity of a contrasuppressor to downregulate the suppressive macrophage subset detected with low doses of SCinf. Therefore, it was conceivable that the two dose zones of macrophage-dependent suppression in the limiting dilution-partition analysis microcultures reflect the presence of two different suppressive macrophage subsets, of which one subset is effective at inhibiting the initiation of proliferative responses and primary antigen-specific responses of Mishell-Dutton cultures. However, if T cells which were already activated or stimulated were present, they could aid the microcultures in overcoming the suppression induced by this macrophage subset. The second suppressor macrophage subset would then, presumably, function by having the ability to suppress or downregulate already primed or activated T cells.

To examine these possibilities, we tested the ability of T helper cell lines of known antigen specificity to counteract the suppression induced by either macrophage subset. The addition of very low doses of antigen-specific T helper cells, even a single T helper cell, to the microcultures was sufficient to counteract the suppression induced by low doses of SCinf. The limiting dilution titration of antigen-specific T helper cells into microcultures of T-cell-depleted spleen cells demonstrated the ability of a single helper cell to allow microcultures to generate detectable antibody responses in limiting dilution-type microcultures (25). The addition of the antigen-specific T helper cells revealed a more complex interaction with the second suppressive macrophage subset. The presence of antigen-specific T helper cells was clearly beneficial because the addition of the higher doses of the specific T helper cells to the cultures allowed the majority of microcultures, even in the presence of high doses of SCinf, to be responsive based on the production of antigen-specific antibody. However, the PFC response generated by the microcultures was clearly still suppressed. The degree to which PFC are suppressed seems to be dependent on the dose of antigen-specific T helper cells which is present. This suggests that the ability of the second macrophage subset to suppress T helper cell populations is not absolute and is not an all-or-none phenomenon but instead may relate to the relative dose ratio of T helper cells and suppressor macrophages in the microcultures. If the ratio between activated antigen-specific T helper cells and one subset of suppressive macrophages is important to the responsiveness of *T. cruzi*-infected mice, then strategies able to shift this ratio in favor of more antigen-specific T helper cells could be beneficial to the immunoresponsiveness of infected animals.

Choromanski and Kuhn (5) have demonstrated that the repeated injection of antigen into *T. cruzi*-infected mice, if administered at a correctly timed interval between challenges, can break the immune suppression in an antigen-specific manner. The present study suggests that, with a single antigenic challenge, the induction of antigen-specific T helper cells remains below the threshold needed to elicit a detectable response by the first suppressor macrophage subset. Any T helper cells which were able to avoid this first suppressive mechanism and become activated would then be inhibited by the second suppressor macrophage subset. Repeated administration of antigen would need to be properly timed so that it is not too closely spaced to allow the maximum expansion of the T helper cells by the first antigenic challenge under the dampening conditions. This would be necessary to generate as large an antigen-specific T helper cell pool as possible so that the second antigen dose can expand this pool to sufficient numbers to compete with the suppression of the second suppressor macrophage subset. If too long an interval occurred between the repeated administrations of antigen, then the second suppressor macrophage subset would downregulate the activated T helpers induced by the first antigen dose and the T helper cell pool size could not be further expanded by subsequent antigen stimulation above the threshold required for a detectable response.

Addition of antigen-specific T helper cells into the Mishell-Dutton microcultures in the presence of the SCinf-endogenous T-cell population showed that there was a synergistic interaction between the two T-cell populations. The SCinf-endogenous T cells, presumably polyclonally stimulated or activated, could provide the added boost to further enhance the effect of antigen-specific T helper cells in partially alleviating the high dose of suppression by the secretion of IL-2. Previous studies by a number of investigators (6, 14, 26–29, 33) have demonstrated defects in cytokine production in *T. cruzi*-infected mice and that addition of IL-2, particularly, in vivo or in vitro enhances responsiveness of SCinf.

Because it was possible that the beneficial effect of the SCinf-endogenous T cells could have been through the elaboration of IL-2 and that the suppressive macrophage population detected with high doses of SCinf was in some way able to inhibit or downregulate IL-2 production or responsiveness by T cells, the production of IL-2 and the ability of IL-2 to restore responses in both PHA-stimulated and antigen-stimulated microcultures was examined. Production of IL-2 appeared to be inhibited by high doses of SCinf but not by low doses of SCinf, and supplementing the microcultures with exogenous IL-2 readily reversed the suppression in the high-dose zone of suppression but failed to alleviate the suppression by low doses of SCinf. The ability of T helper cells to abrogate the low-dose zone of suppression and the inability of supplemental IL-2 to do so indicates that an actual cellular element may be required for abrogation of the low-dose zone of suppression. It is also possible that other cytokines are important in affecting

suppressor macrophages in the low-dose zone of suppression.

The ability of SCinf to bind IL-2 does not appear to be inhibited because preincubation of medium containing IL-2 with increasing doses of SCinf effectively depletes the IL-2 from the medium, except at one point at which intermediate doses of SCinf are utilized for the adsorption. This one point may reflect the equilibrium between the adsorption of IL-2 by the SCinf and the production of IL-2 by SCinf-endogenous T cells present in intermediate doses of SCinf. Increasing the SCinf dose further introduces the second suppressive macrophage, which shuts down IL-2 production and shifts the equilibrium back in favor of depletion. One could argue that the SCinf were secreting an inhibitor rather than binding the IL-2, but this would be inconsistent with the ability of exogenously provided IL-2 to restore responsiveness to the microcultures.

It also appears that SCinf have an increased ability to bind IL-2 compared with SCn because the SCinf were mixed with SCn as filler cells to keep the total cell number constant throughout the SCinf dose curve. Incubation of the medium containing IL-2 with increasing doses of SCinf removed the IL-2 activity compared with the ability of SCn alone (no SCinf per microculture). The increased binding ability of SCinf compared with that of SCn lends further support to the idea that T cells in SCinf could be polyclonally activated or stimulated because an increase in the number of IL-2 receptors occurs on activated T cells.

In summary, the two zones of suppression detected by the limiting dilution-partition analysis of SCinf reflect the presence of two suppressive macrophage populations with differing abilities to suppress activated cells. The first subset, which is abundant and therefore detected with low doses of SCinf, is able to suppress both mitogenic and primary antigen-specific responses but is apparently unable to inhibit cells once they are already activated or primed. The second macrophage subset, which is presumably a less abundant or less active population because high doses of SCinf are required to detect it, is able to suppress the response of activated or primed T cells by the inhibition of IL-2 production. The two suppressive mechanisms working in concert would provide an effective two-pronged regulatory mechanism by first damping the induction of responses below a needed threshold and then inhibiting any activated cells that escaped the suppression of the first mechanism.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI-13128 from the National Institute of Allergy and Infectious Diseases.

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