IMMUNOREGULATION OF CUTANEOUS LEISHMANIASIS

T Cell Lines that Transfer Protective Immunity or Exacerbation Belong to Different T Helper Subsets and Respond to Distinct Parasite Antigens

BY PHILLIP SCOTT, PATRICIA NATOVITZ, ROBERT L. COFFMAN,* EDWARD PEARCE, AND ALAN SHER

From the Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892; and the *DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California 94304

Immunity in leishmaniasis, a chronic protozoal infection of man, is mediated by the stimulation of T cells to produce lymphokines, such as IFN-γ, that activate macrophages to kill the obligate intracellular Leishmania parasites (1). We recently demonstrated that immunization with a soluble extract of the parasite, termed soluble leishmanial antigen (SLA), protects BALB/c mice against a normally fatal challenge with Leishmania major, and that this immunity is associated with the induction of cell-mediated immunity (2). In an effort to identify the immunogens within SLA, we separated the preparation into distinct fractions by anion-exchange chromatography, and assayed the ability of each fraction to be recognized by T cells from SLA-immunized mice, as well as to induce protection. While two fractions, 1 and 9, stimulated T lymphocytes, only fraction 9 was capable of inducing protection against challenge infection (3). This observation led us to postulate that recognition of leishmanial antigens by T cells is not the sole criterion that defines a protective leishmanial immunogen. One possible explanation for our results is that the T cells recognizing the protective and nonprotective fractions differ in their immunologic activity. This hypothesis is explored in the present paper.

Several recent studies have shown that helper T cell clones of the L3T4 phenotype can be divided into two subsets, designated Th1 and Th2, based upon the types of lymphokines produced following stimulation with antigens or mitogens (4–6). Cloned lines of the Th1 type produce IL-2 and IFN-γ, while Th2 cells produce IL-4 and IL-5, but fail to produce IL-2 or IFN-γ. To determine if differential induction of Th1 and Th2 cells might explain the results of our immunization studies, we established T cell lines responsive to protective and nonprotective leishmanial antigens. The properties of these cell lines were analyzed by determining: (a) their antigen specificity using conventional proliferation assays as well as T cell immuno-blotting techniques; (b) the lymphokine profiles of these cells after stimulation; and (c) their ability to adoptively transfer protection. Our results support the concept that protective and nonprotective T cells belong to different CD4+ T cell subsets.

Address correspondence to P. Scott, National Institutes of Health, Laboratory of Parasitic Diseases, NIAID, Bldg. 4, Rm. 126, Bethesda, MD 20892.

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Materials and Methods

Mice. Female BALB/c mice (5–8 wk old) were obtained from the Division of Research Sciences, National Institutes of Health, Bethesda, MD.

Parasites and Antigens. A clone of L. major (MRHO/SU/59/P strain) was used for these studies. SLA and fractions 1 and 9 were prepared as previously described (2, 3). Briefly, SLA was prepared from promastigotes by sonication, and the supernatant was collected after centrifugation at 100,000 g. SLA was fractionated by anion-exchange chromatography on a Mono Q column, and fractions 1 and 9, corresponding to the first and last peaks off the column, were collected, dialyzed against PBS, and stored at -70°C until use.

Establishment of Cell Lines. BALB/c mice were immunized intraperitoneally with either fraction 9 (10 µg) or fraction 1 (50 µg) accompanied by the bacterial adjuvant, Corynebacterium parvum (100 µg). 1 wk later mice received a boost of antigen alone. 2 wk later splenic lymphocytes from immunized mice were collected and stimulated with antigen in vitro following established protocols (7). The cells from fraction 1-immunized mice (designated line 1) were stimulated with fraction 1 (50 µg/ml), while the cells from fraction 9-immunized mice (designated line 9) were stimulated with whole SLA (50 µg/ml), since fraction 9 was not available in sufficient quantities for continuous in vitro passage. The cells were grown in DME with 4,500 mg/ml glucose (Advanced Biotechnologies Inc., Silver Spring, MD) containing 10% FCS, 2 mM glutamine, 100 U/ml of penicillin, 100 µg of streptomycin sulfate, 25 mM Hepes, and 5 × 10^{-5} 2-ME. In the initial stimulation, T cells (2 × 10^6/ml) were exposed to antigen for 12 d without IL-2 and subsequently rested and stimulated in weekly cycles with 5 × 10^6/ml irradiated (3,300 rad) syngeneic spleen cells. For stimulation, lines 1 and 9 received, in addition to antigen, highly purified human rIL-2 from Escherichia coli (8, 9) (35 U/ml; generously provided by Cetus Corp., Emeryville, CA).

Lymphocyte Proliferation. To measure the responses of T cells to leishmanial antigens, 10^4 cells with 5 × 10^5 irradiated (3,300 rad) syngeneic spleen cells were cultured in 96-well flat-bottomed tissue culture plates for 4–6 d with varying concentrations of antigen. Antigen responsiveness was then measured by the colorimetric MTT proliferation assay (10).

Adoptive Transfers. T cell lines were harvested after rest and 5 × 10^6 cells were injected intravenously into BALB/c mice that had been irradiated with 200 rad. Mice were challenged in the footpad with 10^5 stationary L. major promastigotes. The increase in footpad thickness was measured during the course of infection by using a dial micrometer (L. S. Starrett Co., Athol, MA) and swelling in the infected footpad was compared with the contralateral uninfected footpad. No difference in lesion development was found in nonirradiated mice and animals receiving 200 rad irradiation (data not shown). Normal T cells were purified by depletion of B cells by two rounds of panning over affinity-purified rabbit anti-mouse Ig as described (11). In some experiments unfractionated splenic cell preparations (10^9) were used as a source of normal T cells. No differences in the course of infection were observed between animals receiving T cells and unfractionated cells.

Bioassays. IFN-γ was measured in an ELISA following the procedure described by Curry et al. (12). Briefly, IFN-γ in culture supernatants was trapped by immobilized Hb170 rat mAb recognizing mouse IFN-γ, and detected by a polyclonal monospecific rabbit anti–mouse IFN-γ (raised according to the methods of Curry et al. [12]) followed by a peroxidase-conjugated goat anti–rabbit Ig antisera (Bio-Rad Laboratories, Richmond, CA). Quantitation was by comparison with a known amount of murine rIFN-γ (generously provided by Genentech, Inc., South San Francisco, CA). Plates were then washed and developed with a peroxidase substrate (ABTS, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) and read on an ELISA reader.

T cell growth factors were measured by their ability to support proliferation of the mouse HT2 mouse T cell line in the presence of 11B1 anti-IL-4 antibody (13) or S4B6 anti-IL-2 antibody (4) using the colorimetric MTT assay as previously described (4). Test supernatants were harvested from T cell lines (10^5/ml) after a 24-h stimulation with Con A (5 µg/ml).

IL-5 was measured in an IL-5-specific ELISA assay using two mAbs (14).

T Cell Immunoblotting. T cell immunoblotting was done as previously described (15). Briefly, SDS-PAGE was performed under reducing conditions with 500 µg of leishmanial antigen (fractions 1 or 9) applied to a single well trough. After electrophoresis and transfer of the
proteins to nitrocellulose (0.1-μm pore size), 6-mm discs were punched out of the blot into 96-well plates. T cells (10^4) and irradiated spleen cells (5 x 10^5) were added, and proliferation was measured by [H]thymidine uptake from day 4-5.

**Analysis of Cell Surface Phenotype.** The surface antigens of the T cell lines were characterized by indirect immunofluorescence after reaction with anti-L3T4 (GK1.5) and anti-Ly-2 (Becton Dickinson & Co., Sunnyvale, CA).

**Results**

**Antigenic Responsiveness and Cell Surface Phenotype of Leishmanial T Cell Lines.** Spleen cells from BALB/c mice immunized with either fraction 1 or fraction 9 were harvested and stimulated in vitro with fraction 1 (line 1) or SLA (line 9). SLA was used for stimulation of line 9 since fraction 9 was not available in sufficient quantities for continuous in vitro passage. As described in the Materials and Methods, the cells were stimulated with antigen and IL-2 and rested in cycles lasting 4-10 d, following established protocols. After 6-10 stimulation and rest cycles, the antigen specificities of the lines were determined by measuring cell proliferation. Line 1 responded to both SLA and fraction 1, but not to fraction 9, while line 9 responded to SLA and fraction 9, but not to fraction 1 (Fig. 1, A and B). Interestingly, a second line that we attempted to establish against fraction 9 (line 9.2) responded poorly to fraction 9, but did respond to fraction 1 and SLA (Fig. 1 C). Analysis of the surface phenotype of the cell lines demonstrated that all 3 lines were L3T4+ Ly-2−.

**Analysis of the Antigens Recognized by Line 1 and Line 9 by T Cell Immunoblotting.** The cell lines were established with FPLC fractions containing multiple proteins. To identify the relative number and molecular weights of antigens recognized by these T cells we used the technique of T cell immunoblotting. In spite of the fact that lines 1 and 9 had been maintained in culture for >3 mo, they both exhibited a heterogeneous response with regard to antigen specificity (Fig. 2). These results indicate that no single clone predominated after prolonged in vitro culture. Line 1 recognized a large number of antigens contained in fraction 1, the majority of which had a mol wt <68 x 10^3. There was a similar heterogeneity in the response of line 9 to fraction 9 antigens, although there appeared to be fewer proteins that induced proliferation. The major antigens recognized by Line 9 were of approximate mol wt 10-12 x 10^3, 23-35 x 10^3, and 50-68 x 10^3.

**Analysis of the Lymphokines Produced by T Cell Lines.** Supernatants from all the cell lines stimulated with Con A produced lymphokines capable of stimulating growth of the mouse HT2 T cell line. Inhibition with mAbs directed against either IL-2 or IL-4 identified the T cell growth factors produced by lines 1 and 9.2 as IL-4, and those produced by line 9 as IL-2 (Table I). When these T cell supernatants were analyzed for the presence of IL-5, only lines 1 and 9.2 produced detectable levels of IL-5 (Table I). T cell supernatants from both Con A and antigen-stimulated cells were also tested for the presence of IFN-γ. Line 9 produced significant levels of IFN-γ in response to Con A, SLA, or fraction 9, while lines 1 and 9.2 produced only minimal amounts of IFN-γ (Table II).

**In Vivo Biologic Activity of Leishmanial T Cell Lines.** To assess the protective effects of the T cell lines, cells were harvested from culture after rest and intravenously injected into BALB/c mice. Recipients and controls were immediately challenged in the footpad with 10^9 L. major promastigotes and the course of infection was followed.
Figure 1. Proliferative response of cell lines to leishmanial antigens. T cells from line 1 (A), line 9 (B), and line 9.2 (C) were stimulated with 0.1, 1, 10, or 25 μg/ml of SLA (●), fraction 1 (◆), or fraction 9 (■) in the presence of APC. After 4 d in culture, cell responsiveness to antigens was determined using the MTT cell proliferation assay as described in Materials and Methods. For each data point the background response has been subtracted. One representative experiment out of three for each cell line is presented.

Figure 2. T cell immunoblots of (A) line 1 to fraction 1 and (B) line 9 to fraction 9. T cells were incubated with portions of nitrocellulose corresponding to different molecular weight antigens within each fraction in the presence of APC as described in Materials and Methods. Proliferation was measured by incorporation of [3H]thymidine at 4 d, and the results presented represent the mean cpm of quadruplicate wells with the background cpm subtracted.
Mice receiving normal T cells did not exhibit a course of infection significantly different from control animals, and in all cases mice developed large lesions with ulceration and eventual loss of the infected footpad (Fig. 3A). In contrast, mice that received line 9 were significantly protected against infection. The protection observed was equivalent to, if not better than, that obtained by active immunization (3). On the other hand, mice that received cells of line 1 demonstrated accelerated lesion development (Fig. 3A). In addition, cells of line 9.2 were also found to exacerbate lesion development (Fig. 3B). These results were confirmed in five experiments summarized in Fig. 4.

Discussion

BALB/c mice infected with L. major develop nonhealing cutaneous lesions at the site of parasite inoculation, metastatic lesions at both cutaneous and visceral sites, and they eventually die from the infection (1). However, a variety of immunologic manipulations, such as irradiation with 550 rad (16), anti-L3T4 treatment (17), and immunization (2, 3, 18, 19), protect BALB/c mice against fatal infection, and in some cases lead to complete healing. At present, it is generally thought that alterations in the expression of different CD4+ T cell subsets determine these changes in susceptibility to infection (20). The experiments described here were aimed at determining the nature of the T cells contributing to either susceptibility or resistance by establishing T cell lines reactive against protective and non-protective soluble leishmanial antigens.

Table I

| Line   | IL-2  | IL-4  | IL-5   |
|--------|-------|-------|--------|
|        | U/ml  | U/ml  | ng/ml  |
| 1      | <5    | 3807  | 17.3   |
| 9      | 175   | <5    | <0.06  |
| 9.2    | <5    | 5558  | 88.4   |

Supernatants were collected 1 d after stimulation of 10^6 T cells with 5 μg/ml Con A, and were tested in the presence or absence of antibody to IL-2 or IL-4 as described in Materials and Methods. IL-5 was measured in an ELISA assay.

Table II

| Stimulation | IFN-γ secretion |
|-------------|-----------------|
|             | Line 1 | Line 9 | Line 9.2 |
| Con A       | 0.8    | 614    | 1.4      |
| SLA         | 0.2    | 315    | <0.01    |
| Fr.1        | 0.2    | 40     | <0.01    |
| Fr.9        | 0.6    | 228    | <0.01    |

Supernatants from Con A-stimulated cells were prepared as described in Table I. Supernatants from antigen-stimulated cultures were collected 3 d after stimulation of 10^6 T cells with 25 μg/ml antigen. The amount of IFN-γ was determined using an ELISA described in Materials and Methods.
We previously found that BALB/c mice can be protected against lethal infection with *L. major* by immunization with SLA, and that two fractions derived from SLA stimulated T cells (fractions 1 and 9), while only one (fraction 9) stimulated protective immunity (3). Similarly, we now report that cell lines reactive against fraction 1 are unable to induce protection, while T cells recognizing fraction 9 antigens transfer protective immunity equivalent to that obtained by active immunization. This is the first description of a T cell line transferring protection in cutaneous leishmaniasis, and serves to further demonstrate the paramount importance of cell-mediated immunity in controlling leishmanial infections. More significant, however, is the observation that protective and nonprotective T cell lines differ in the types of lymphokines produced after mitogen and antigen stimulation. Line 9 produces IL-2 and IFN-γ, while lines 1 and 9.2 produce IL-4 and IL-5. The lymphokine profile
for line 9 corresponds to that described for the Th1 subset, whereas lines 1 and 9.2 have the properties of the Th2 subset. The initial classification of CD4 cells into Th1 and Th2 types was accomplished by examining the profile of lymphokines produced by murine T cell clones in vitro (4). Our results indicate that even T cell lines that exhibit significant heterogeneity with regard to antigen specificity may be primarily composed of one Th cell subset, and in addition, that the in vitro differences observed in these Th cell subsets are important in determining how T cells influence the outcome of an infection.

Previous attempts to establish protective Leishmania-specific T cell lines have been unsuccessful, even though protection by adoptive transfer of fresh immune T cells has been demonstrated (1, 21, 22). One explanation for these results is that after in vitro culture with crude antigen preparations, T cells recognizing nonprotective antigens and/or of the Th2 phenotype may predominate. On the other hand, the success of our adoptive transfers may be related to establishing line 9 with a defined protective antigen preparation. A more important issue, however, is why previously established cell lines (22), as well as lines 1 and 9.2, not only fail to protect mice, but actually exacerbate the infection. One possibility is that lymphokines preferentially produced by these cells down-modulate the response of macrophages to IFN-γ and/or enhance parasite growth within macrophages. In this regard, we recently found that supernatants derived from mitogen-stimulated line 1 T cells inhibit the ability of macrophages to respond to IFN-γ for macrophage killing of Leishmania (manuscript in preparation). IL-4 or IL-5, which are only produced by the Th2 cell lines, would be candidates for such modulation. In addition, other lymphokines that are synthesized in larger amounts by our Th2 cell lines might contribute to the enhanced lesion development. For example, in preliminary experiments we have found that line 1 produces significantly more colony-stimulating factor (granulocyte/macrophage CSF and/or IL-3) after stimulation than line 9, and both of these factors have been reported to enhance lesion development (20). We are currently investigating the in vitro and in vivo effects of these lymphokines upon leishmanial infection.

Both of the soluble fractions recognized by these cell lines are composed of many proteins, and, using the T cell immunoblotting technique, we found that the cell lines exhibited heterogeneity with regard to antigen specificity. Nevertheless, in the present studies recognition of fraction 1 proteins appeared to be primarily associated with stimulation of Th2 cells. This correlation was strengthened when we found that a second cell line (line 9.2), meant to be established against fraction 9, preferentially recognized fraction 1 antigens and had a Th2 lymphokine profile. Although the reason for the development of anti-fraction 1 reactivity by line 9.2 is, as yet, unclear, it might be due to the presence of a crossreactive antigen found in both fractions 1 and 9. These results may appear to contradict our earlier observations that fresh T cells from SLA-immunized mice produced IFN-γ when stimulated with fraction 1 (3). However, recent studies indicate that newly sensitized CD4 T cells may have the capacity to produce lymphokines associated with both Th1 and Th2 cells, and that chronic stimulation, certainly in vitro and possibly in vivo, leads to the commitment of these cells to one of the two types of Th cells (23). Thus, on the basis of our observations we suggest that stimulation of T cells with fraction 1 may preferentially lead to the development of Th2 type cells. On the other hand,
recognition of fraction 9 antigens was a characteristic of the Th1 cell line. We similarly suggest that stimulation with this parasite fraction may preferentially lead to the development of Th1 cells. Thus, while it is difficult to generalize from the analysis of three cell lines, it is possible that fraction 1 antigens preferentially stimulate Th2 cells, while fraction 9 proteins stimulate Th1 cells, an observation that suggests that certain parasite antigens may preferentially stimulate one class of Th cell. We are now analyzing a larger number of cell lines and clones with defined proteins to investigate this hypothesis.

The data presented here indicate that the effector T cells responsible for protection in leishmaniasis are of the Th1 subset, which is not surprising since protection in this disease is mediated by macrophage-activating factors, such as IFN-γ. Thus, we would argue that immunization leads to preferential induction of Th1 cells, while during a normal infection in BALB/c mice Th2 cells are primarily stimulated, leading to nonhealing infections. Similarly, we would suggest that irradiation or in vivo treatment with anti-L3T4 mAb shifts the balance of Th subsets towards Th1 cells. This hypothesis is supported by recent observations of Locksley and coworkers (20), who found that lymphokines associated with Th2 cells predominated in draining lymph nodes of L. major-infected BALB/c mice, while lymphokines associated with Th1 cells were prevalent in a genetically resistant strain, as well as in BALB/c mice treated with anti-L3T4 mAb. At present, the factors contributing to the preferential induction of Th2 cells in Leishmania-infected BALB/c mice are unknown. Nevertheless, it is clear that the identification of two T cell subsets contributing to either protection or exacerbation in leishmaniasis offers a framework with which to interpret previous observations in the field and design future studies.

Summary

BALB/c mice can be protected against a normally fatal Leishmania major infection by immunization with a partially purified, soluble subfraction of the parasite (fraction 9). In this study, we demonstrate that a T cell line established against fraction 9, designated line 9, transfers protection equivalent to that obtained by active immunization. In contrast, T cell lines (lines 1 and 9.2) responsive to a nonprotective soluble fraction (fraction 1) not only failed to protect BALB/c mice against L. major, but exacerbated the infection. Most importantly, in addition to differing in their antigen specificity, protective and exacerbative T cell lines could be distinguished on the basis of the lymphokines produced, a characteristic previously used to separate murine Th cells into two subsets, designated Th1 and Th2. We found that the protective cell line, line 9, displayed the Th1 property of secreting IL-2 and IFN-γ, while the exacerbating lines secreted IL-4 and IL-5, a characteristic of Th2 cells. Our results demonstrate that Th1 and Th2 cells may have dramatically different effects on the outcome of an infection, and suggest that susceptibility and resistance in experimental leishmaniasis may depend upon a balance between the Th subsets induced.

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