Learning from lesions: Patterns of tissue inflammation in leprosy

(cell-mediated immunity/T-lymphocyte subsets/precursor-frequency analysis/immunopathology/suppressor cells)

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ABSTRACT The clinical forms of leprosy constitute a spectrum that correlates closely with the degree of cell-mediated immunity. Patients with tuberculoid leprosy develop strong cell-mediated responses and have only a few, localized lesions, whereas patients with multibacillary lepromatous leprosy are specifically unresponsive to antigens of *Myobacterium leprae*. T cells of the CD4+ subset predominate in tuberculoid lesions, whereas the CD8+ cells predominate in lepromatous lesions. Monoclonal antibodies that distinguish subpopulations of CD4+ and CD8+ cells were used to analyze the distribution of T cells infiltrating lesions across the disease spectrum. In lepromatous lesions, T cells of T-suppressor phenotype (9.3−) were the predominant CD8+ cells and suppressor/inducer cells (2H4+, Leu-8+) represented half of the CD4+ subset. In tuberculoid lesions, helper T cells (CD4+4B4+) outnumbered suppressor/inducer T cells by 14:1, compared with a ratio of 1:2:1 in peripheral blood. Analysis of the precursor frequency of antigen-reactive T cells permitted us to estimate that there was a 100-fold enrichment of T cells able to proliferate in response to *M. leprae* antigens in tuberculoid lesions (2/100), when compared with blood from the same patients. The methods used here to characterize the T-lymphocyte subsets and frequency of antigen-reactive T cells in leprosy may be useful in analyzing immunological reactions occurring in lesions of other inflammatory and autoimmune diseases.

There are at least two compelling reasons to study leprosy. The disease itself, affecting 10–15 million people worldwide, poses a significant health and economic burden on third-world countries. Second, because leprosy is a spectral disease in which pathology and immunology are inextricably related, it provides a unique critical model for investigating immunoregulatory mechanisms in humans.

Leprosy is not a single critical entity but rather comprises a spectrum of clinical manifestations that correlate remarkably well with immunological responses to the organism (1). Patients with tuberculoid leprosy have a few localized lesions with rare organisms and a strong cell-mediated immune response directed against *Myobacterium leprae* antigens that ultimately kills and clears the bacilli, although often with concomitant injury to nerves. In contrast, lepromatous leprosy patients have numerous skin lesions containing extraordinarily high numbers of acid-fast bacilli and show specific immunological unresponsiveness to antigens of *M. leprae* in vivo and in vitro.

The selective mechanisms of accumulation of lymphocytes, their antigen specificity, and immunological functions in inflammatory lesions of human infectious and autoimmune diseases remain largely unknown. Since the focal point of the immune response to *M. leprae* is the tissue granuloma, consisting of a collection of lymphocytes and macrophages, some of these questions can be approached directly through the study of cells infiltrating the lesions across the spectrum of leprosy.

Immunohistological studies using monoclonal antibodies to characterize T-lymphocyte subsets in granulomas revealed striking differences in the various types of leprosy (2, 3). Tuberculoid (TT and BT) lesions contain a predominance of T cells of the CD4+ helper/inducer subset, whereas lepromatous lesions contain mainly T cells of the CD8+ suppressor/cytotoxic subset in proportions quite distinct from the normal peripheral blood CD4+/CD8+ ratio (4). The CD4+ subset has recently been further divided into immunoregulatory subpopulations by a series of monoclonal antibodies (5, 6, 11, 17, 18). The 2H4−4B4+ subset has been shown to proliferate in response to soluble antigens and to enable B cells to produce immunoglobulin and is thought to comprise helper T cells. The suppressor/inducer subpopulation of CD4+ cells has been reported to express the 2H4−4B4− (5) and/or Leu-8+ (17, 18) surface markers. In addition, the CD8+ subset has been divided into reciprocal immunoregulatory subsets with the suppressor T-cell subset being CD8+9.3−CD11+ and the cytotoxic T-cell subset being CD8+9.3+CD11−. Because CD4+ and CD8+ cells are present in varying proportions in lesions across the spectrum of leprosy, it was of interest to explore differences in the immunoregulatory T-cell populations in cellular reactions of leprosy lesions.

Although phenotyping of lymphocytes in leprosy lesions has provided important information about the nature of cells present in the inflammatory sites, the delineation of the immune function of these cells is of potentially greater interest. We recently developed methods for isolating lymphocytes directly from lesions (4, 8). In the present study, CD4+ T cells were isolated from lesions across the spectrum of leprosy and characterized with respect to their surface markers and antigen reactivity. Using a limiting-dilution analysis to estimate the precursor frequency of antigen-reactive T cells, we were able to demonstrate the enrichment of antigen-specific CD4+ T cells in specific inflammatory lesions relative to peripheral blood.

MATERIALS AND METHODS

Patients. For immunostaining studies, 18 untreated patients with leprosy, 8 tuberculoid (TT/ BT or BT) and 10 lepromatous (BL or LL), were classified according to the criteria of Ridley and Jopling (1). Seven untreated tubercul-
loid and 7 untreated lepromatous patients were studied for reactivity of cells extracted from lesions.

Tissues. A portion of an ellipsoid skin biopsy specimen was quick-frozen for immunostaining as described (9), and the remainder was transported to the laboratory for extraction of lymphocytes (4, 8).

Monoclonal Antibodies. Primary mouse monoclonal antibodies were used at concentrations predetermined by titrations on cells from reactive tonsils. Anti-2H4 (Coulter) was used at a 1:50 dilution, anti-B4 (Coulter) at 1:400, anti-Leu-8 (Becton Dickinson) at 1:100, and anti-9.3 (John A. Hansen, Seattle) at 1:300, but the anti-CD11 marker Leu-15 (anti- CR3, Becton Dickinson) did not provide adequate tissue staining. Each specimen was additionally stained with anti-CD3, which recognizes a pan-T-cell marker (Leu-4, 1:200); anti-CD4, which identifies the helper/inducer T-cell subset (Leu-3a, 1:25); and anti-CD8, which identifies the suppressor/cytotoxic T-cell subset (Leu-2a, 1:50).

Immunoperoxidase Staining. Indirect single immunoperoxidase staining and double immunostaining techniques have been reported in detail (9). Double staining was performed on all specimens pairing anti-2H4 or anti-Leu-8 with anti-Leu-3a and anti-9.3 with anti-Leu-2a. (Anti-4B4 was not satisfactory for the double staining method.) The immunoperoxidase reaction yielded a red product from 3-amin-9-ethylcarbazole in the presence of hydrogen peroxide. The glucose oxidase system (Vecstastain kit, Vector Laboratories, Burlingame, CA) produced a blue color with the substrate nitro blue tetrazolium.

Quantitation of Stained Cells. Percent single- or double-staining cells was determined as previously described (9). Double-stained cells were identified when both red and blue colors could be seen on the same cell or when a purple color was present.

The percent of Leu-3a+ cells staining with anti-2H4+ or anti-Leu-8 could be calculated as the number of double-stained cells counted divided by the total number of Leu-3a+ (single- or double-staining) cells counted. This ratio was multiplied by the percent Leu-3a+ to give the percentage that were CD4+2H4+. The CD4+2H4− phenotype was calculated as the percent Leu-3a− minus percent CD4−2H4−. CD8− subpopulations were similarly calculated using double staining with anti-9.3.

Peripheral Blood Cells. PBMCs were separated by Ficoll-Hypaque centrifugation from 10 tuberculous and 8 lepromatous patients. Cells were stained simultaneously with fluorescein-isothiocyanate-conjugated anti-T4 (Coulter) and phycoerythrin-labeled anti-2H4 (Coulter) or anti-4B4 and analyzed by flow cytometry.

Extraction of Lymphocytes from Biopsy Specimens. Lymphocytes were extracted from skin specimens as previously reported in detail (4) and then labeled with fluorescein isothiocyanate-conjugated monoclonal antibodies anti-CD4 (T4, Coulter) and anti-CD8 (T8, Coulter) and phycoerythrin-conjugated 2H4 and 4B4. Cell populations were then analyzed and sorted to >99% purity by flow cytometry.

Lines from Tissue-Extracted Lymphocytes. CD4+ cells were expanded and maintained with interleukin 2 (IL-2; Electro-Nucleonics, Oak Ridge, TN) by seeding 1000 cells per well in the presence of lethally irradiated (3000 rads; 1 rad = 0.01 Gy) autologous or HL-A-DR-matched peripheral blood mononuclear cells (PBMCs) as feeders (8). Some lines were established in the presence of IL-2 alone (to expand cells activated in situ). Other lines were established using M. leprae (Dharmandra lepromin prepared by M. Abe, Tokyo) or Mycobacterium tuberculosis H37Ra (10 μg/ml; Difco) plus IL-2 to stimulate antigen-specific cells.

Proliferative Response of T-Cell Lines. The ability of cell lines to proliferate in response to M. leprae and M. tuberculosis was measured by [3H]thymidine incorporation as previously described (8). Controls consisted of medium alone for background, tetanus toxoid as an irrelevant antigen, and 10% (vol/vol) IL-2 as a positive control to demonstrate cell viability.

Precursor Frequency Analysis. T lymphocytes were derived from five tuberculoid lesions and the peripheral blood of four tuberculoid patients and five lepromin skin test-positive contacts of leprosy patients. In three tuberculoid patients, limiting-dilution analysis was simultaneously performed on T lymphocytes from peripheral blood and skin lesion biopsy.

T lymphocytes were isolated by sedimentation of sheep erythrocyte rosettes. Replicate cultures (sets of 24) were established in round-bottom microtiter wells. Various numbers of T lymphocytes were cocultured with antigen-presenting cells (10⁶ irradiated erythrocyte-rosette-negative PBMCs). Identical sets were established with either M. leprae or medium (control). For tissue-derived cells, replicates were established containing 0.3, 1, 5, 10, 20, and 50 T lymphocytes per well. PBMC replicates were established containing 500, 1000, 2500, 5000, 7500, and 10,000 T lymphocytes per well. Cultures were incubated at 37°C in the presence of 7% CO₂. On day 6, 10 μl of IL-2 was added to each well and on day 9, 50% of the medium in each well was removed and replaced with fresh medium containing 10% human type AB serum and 5% IL-2. On day 12, cultures were incubated with [3H]thymidine (1 μCi per well) and harvested 6 hr later for measurement of incorporated radioactivity by liquid scintillation counting.

For each set of 24 replicates, antigen-containing cultures were scored as positive if the proliferative response (measured in counts per minute) was greater than the mean plus 3 standard deviations of control cultures without antigen. The percent negative cultures was used to determine the precursor frequency according to the Poisson distribution and the χ² minimization (10).

RESULTS

Patterns of T-Cell Subsets in Lesions. It was initially important to characterize the percentages and location of T-lymphocyte subsets in the lesions of the patients studied here in order to establish that the distribution was consistent with previously reported findings (3). T cells (CD3+) comprised 61% of total cells counted in tuberculosis lesions and 40% of cells in lepromatous lesions. The CD4+/CD8− ratio in tuberculoid granulomas was 1.7, compared with a ratio of 0.6 in lepromatous granulomas. CD4+ cells were distributed throughout the tuberculoid granuloma, with CD8− cells confined to the mantle surrounding it. In lepromatous leprosy lesions, both CD4+ and CD8− subsets were admixed throughout the biopsy specimen.

Anti-2H4 (or anti-Leu-8, data not shown) monoclonal antibodies stained lymphocytes that comprised ~1% of all cells in both tuberculoid and lepromatous lesions. In lepromatous granulomas, 2H4+ cells were distributed throughout (data not shown), whereas in tuberculoid granulomas, 2H4+ cells were invariably restricted to the mantle surrounding the granuloma (Fig. 1a). Anti-4B4 monoclonal antibodies stained lymphocytes and macrophages throughout both types of granulomas, staining more than 60% of lymphocytes in tuberculoid granulomas (Fig. 1b) and fewer than 40% in lepromatous granulomas (data not shown).

Because 2H4 and Leu-8 are expressed on both CD4+ and CD8+ populations, it was important to perform double staining to determine the proportion of CD4+ cells expressing these markers in lesions. Similarly, we wished to estimate the percentage of cells that were CD4+4B4+. Approximately 5% (3% in tuberculoid and 6% in lepromatous) of the cells present in lesions were CD4+2H4+. More signifi-
cantly, CD4+2H4- cells comprised 36% of cells in tuberculosis granulomas but only 7% of cells in lepromatous granulomas. Furthermore, CD4+ cells found in the center of the tuberculosis granuloma and associated with macrophages were essentially all 2H4-. The putative suppressor/inducer subset of CD4+ cells, identified as CD4+2H4+ or CD4+Leu-8+, was localized primarily in the mantle zone surrounding the granuloma, the same region, incidentally, in which the CD8+ cells are found. The percentage of CD4+ cells staining with anti-2H4 was identical to that staining with anti-Leu-8 in all sections examined.

Several experimental observations in our leprosy patients support the claim that the CD4+4B4+ subset approximates the CD4+2H4- subset: (i) measurement of the CD4+4B4+/CD4+2H4+ ratio by sorting of cells extracted from two tuberculosis and two lepromatous biopsy specimens gave results identical to the CD4+2H4-/CD4+2H4+ ratio measured by immunoperoxidase in the tissue sections from the same biopsy specimens; (ii) of the CD4+2H4- cells sorted from the peripheral blood of leprosy patients, more than 90% were found to be positive for 4B4; and (iii) the cells morphologically identifiable as lymphocytes located in the core of the tuberculosis granuloma that were CD4+2H4- were also positive for 4B4.

Our analysis indicates that the ratio of CD4+4B4+ (helper/inducer) cells to CD4+2H4+ (suppressor/inducer) cells was 14:1 in tuberculosis lesions and 1.1:1 in lepromatous lesions. In contrast, the ratio of CD4+4B4+ cells to CD4+2H4+ cells in peripheral blood was 1.2:1 in tuberculous patients and 1.9:1 in lepromatous patients (Fig. 2).

The distribution of CD8+ subpopulations was investigated in leprosy lesions by using the monoclonal antibody anti-9.3 (7). Cells positive for the 9.3 antigen were present throughout all types of granulomas and comprised 16% of the cells in lepromatous lesions and 38% of the cells in tuberculosis lesions. In lepromatous lesions, the CD8+9.3- subset, reported to be a T-suppressor subset, predominated; in tuberculosis lesions, the CD8+9.3+ subset, reported to comprise cytotoxic T cells, was the predominant CD8+ subpopulation (Fig. 2).

IL-2-Dependent T-Cell Lines from Leprosy Lesions. To ascertain whether cells of the helper phenotype present in the lesions were antigen-specific and activated in situ, CD4+ or CD4+4B4+ cells were extracted from lesions and established as T-cell lines in vitro by culture in the presence of IL-2 alone. Our reasoning was that by expanding all T cells activated in situ to express the IL-2 receptor (Tac), rather than selecting initially with antigen, we could establish whether a significant percentage of the Tac+ cells was specifically responsive to M. leprae antigens. All the T-cell lines from tuberculous leprosy lesions established in this manner were found to be CD3+CD4+CD8-DR+ Tac+2H4-4B4+. Seven such lines were found to be antigen-specific and proliferated in response to M. leprae and, to a variable extent, to M. tuberculosis but not in response to tetanus toxoid (Fig. 3). From lesions of lepromatous patients, seven CD4+4B4+ lines were produced that were IL-2-dependent but unresponsive to mycobacterial antigens. Six additional lines derived from lepromatous lesions in the presence of M. leprae plus IL-2 were similarly unresponsive to M. leprae. However, two of the lines derived from lepromatous lesions in the presence of M. tuberculosis did...
proliferate in response to *M. leprae* but were unresponsive to *M. leprae*. Overall, it would appear that a proportion of the CD4+ cells in tuberculoid lesions are indeed activated by *M. leprae* antigens in situ and can be expanded by IL-2 alone while retaining specificity for mycobacterial antigens. In contrast, CD4+ cells derived from lepromatous lesions do not proliferate in response to *M. leprae*, and this specific unresponsiveness could not be overcome by the addition of IL-2, *in vitro* culture in the absence of CD8+ suppressor cells, or stimulation by cross-reactive mycobacteria.

**Determination of Precursor Frequency of *M. leprae*-Reactive Lymphocytes.** Although differences in the percentage of the CD4+ subsets of patients with tuberculoid leprosy were found in the blood versus lesions, we wished to know if there was a difference in the percentage of lymphocytes responsive to *M. leprae*. This was investigated by performing limiting-dilution analysis of precursor frequencies of *M. leprae*-reactive cells (Fig. 4). We observed that in tuberculoid lesions, ~2% of T cells proliferated in response to *M. leprae* compared with ~0.02% in the peripheral blood of tuberculoid patients and patient contacts. There is thus as much as a 100-fold greater concentration of *M. leprae*-reactive T-lymphocytes in the lesion as compared to the peripheral blood.

**DISCUSSION**

We have attempted to combine immunohistologic techniques to determine the phenotype of lymphocyte populations infiltrating lesions of leprosy together with functional studies of T cells in order to investigate the pathogenesis of tissue inflammation in leprosy. In tuberculoid leprosy, patients develop high levels of cell-mediated immunity *in vivo* and *in vitro* and have sharply defined lesions containing characteristically few acid-fast bacilli. The tuberculoid granuloma is organized into distinct immunological microenvironments. Immunohistologic studies revealed that the core of the granuloma is composed of well-differentiated macrophages microanatomically apposed with T cells of a helper (CD4+4B4+2H4-) phenotype (6). When T-cell lines were established from these cells, they proliferated in response to *M. leprae* and produced interferon γ, characteristic of T helper cells in cell-mediated immunity.

The outer mantle of the tuberculoid granuloma contains CD8+ cells of both cytotoxic (9.3+) and suppressor (9.3-) phenotypes. In addition there are CD4+ lymphocytes bearing the 2H4 and Leu-8 markers. Since most individuals infected with *M. leprae* develop no signs of clinical disease, it is not surprising to find cells of the suppressor/inducer and suppressor phenotypes in lesions of tuberculoid patients, who have not fully resisted the pathogen. Since this type of lesion is generally self-limited, the predominant T helper and cytotoxic cells may limit the granuloma, albeit often at the cost of some nerve and tissue damage.

The lepromatous granuloma consists of a preponderance of CD8+ cells with a CD4+/CD8+ ratio of 0.6:1 in tissue lesions as compared to 2:1 found in peripheral blood (4). Macrophages laden with bacilli are closely associated with both CD4+ and CD8+ subsets in the lesions. We established CD8+ lines and clones from lepromatous lesions and showed that these cells function *in vitro* as antigen-specific T suppressor cells and are restricted by class II histocompatibility antigens (4, 8). Similar findings of peripheral blood T-suppressor cells in leprosy patients have been reported by Ottenhoff *et al.* (12) and Kikuchi *et al.* (13). In the present study, it is clear that half of the CD4+ cells in the lepromatous lesions are 2H4+ and Leu-8+, consistent with a suppressor/inducer function. Furthermore, the predominant CD8+ cell was 9.3- (that is, of the suppressor T-cell subset). Yet CD4+4B4+ lines and clones from lepromatous lesions failed to respond to *M. leprae*, even when supplemented with IL-2 or with antigen plus IL-2. These data suggest that CD8+ suppressor cells may contribute, over a prolonged period of time in this chronic disease, to the functional depletion of *M. leprae*-reactive CD4+ lymphocytes from the immunologic repertoire of lepromatous patients. One must be cautious, however, in imputing functional significance to T-cell subsets defined solely by reactivity with monoclonal antibodies to 4B4, 2H4, Leu-8, and 9.3 surface antigens.

**Fig. 4.** Limiting-dilution analysis of the frequency of antigen-responsive T-lymphocyte precursors in the lesions and blood of tuberculoid leprosy patients. (Left) Standard plot of experiment demonstrating the *M. leprae*-responsive precursor frequency measured simultaneously in T lymphocytes derived from the lesion (●) and blood (○) of a tuberculoid leprosy patient. (Right) Summary of limiting-dilution data demonstrates that 2% of T lymphocytes in tuberculoid lesions respond to *M. leprae*, whereas 0.02% of cells in the blood of tuberculoid patients and patient contacts are responsive.
since it is not fully clear at present whether they identify lineage-specific, differentiation, or functional markers.

Convit et al. (14) devised an immunotherapeutic strategy for leprosy by using a combined vaccine of live Mycobacterium bovis BCG (bacillus Calmette–Guérin) and killed M. leprae to induce cell-mediated immune responses in 65–85% of previously unresponsive lepromatous (BL and LL) patients. It is not clear what cells are mediating the immune protection. It will be of great interest to follow the dynamics of accumulation of subpopulations of CD4⁺ and CD8⁺ cells in patients undergoing immunotherapy, in order to gain insight into the T-cell subsets required for overcoming unresponsiveness and activating macrophages to eliminate intracellular pathogens.

One of the functional problems in immunology is understanding the mechanisms of localization of specifically sensitized lymphocytes at sites of infection and inflammation. Early studies in experimental animals showed that the vast majority of cells infiltrating delayed-type hypersensitivity lesions were nonsensitized, consisting primarily of the cells of the mononuclear phagocyte lineage. While endothelial cells can be induced to express class II antigens and can activate sensitized T cells in vitro, there is little evidence that lymphocytes are locally activated by specific antigen presented by vascular endothelium to enter the local tissue. From a wide variety of studies of lymphocyte trafficking (15), it would appear that activated T lymphocytes and possibly memory T cells have the ability to home selectively to inflammatory sites. Entry into the tissues may require prior antigen activation, but tissue entry appears to be independent of antigen recognition at the site. This current stochastic model suggests that initial localization of T cells to tissue sites would be independent of antigen. If this were the exclusive factor determining cell representation in lesions, the percentage of specifically sensitized cells at inflammatory sites would be expected to be quite low.

The fundamental observation of the present work is that while the CD4⁺/CD8⁺ ratio is 2:1 in both peripheral blood and lesions of tuberculoid patients, the tissue population does not represent a random filtrate from blood. Indeed, the ratio of helper (CD4⁺4B4⁺) cells to suppressor/inducer (CD4⁻2H4⁺) cells was 1.2:1 in blood but 14:1 in lesions. While limiting dilution analysis has been used to assess the precursor frequency in animals and in human peripheral blood (16), this technique provides a new approach to the measurement of the frequency of antigen-reactive precursor T cells directly in inflammatory lesions of human disease. We determined that 2% of the T lymphocytes in tuberculoid leprosy skin lesions could proliferate in response to M. leprae, but when measured simultaneously, only 0.02% of peripheral blood lymphocytes were responsive to the same antigen. It is thus apparent that there is not only a 10-fold enrichment in the CD4⁺4B4⁺ helper subset in tuberculoid leprosy lesions but as much as a 100-fold enrichment in antigen-reactive T lymphocytes in these same lesions relative to peripheral blood. It is of interest that very similar findings were obtained by precursor-frequency analysis of T cells from lesions of patients with cutaneous leishmaniasis (19). If homing to these sites is not antigen-specific, then the selective accumulation can be accounted for either by prolonged retention in the lesions containing antigen or by selective expansion of M. leprae-reactive T cells in the antigen-containing lesions. If the 2% of cells expressing IL-2 receptors (9) represent the antigen-reactive cells, then it is possible that the marked enrichment for M. leprae-reactive cells is largely related to in situ proliferation.

In summary, we can learn much from lesions. We have used a variety of immunologic techniques to measure phenotype, function, and precursor frequency of T lymphocytes to further our understanding of the mechanism(s) of tissue inflammation in leprosy. We hope that application of these approaches to the study of other chronic infectious and autoimmune diseases will provide insight into the immunopathogenesis of these disorders in humans.

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