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In Situ Identification of Activated Ta1+ T Lymphocytes in Human Leprosy Skin Lesions[1]

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Leprosy is a chronic granulomatous disease caused by infection with *Mycobacterium leprae.* The immunological responses of patients to this bacterial infection form a spectrum and correlate with the various clinical categories ([14]). Patients with tuberculoid leprosy have few localized lesions with rare organisms and a strong cell-mediated immune response directed against *M. leprae* antigens. In contrast, lepromatous leprosy patients have widespread disease with numerous bacilli, and are specifically unresponsive to the antigens of *M. leprae.* The nature of this unresponsiveness is an area of intensive immunologic investigation.

Immunohistological studies using monoclonal antibodies to characterize T-lymphocyte subsets in skin lesions of the various types of leprosy also correlated with the clinical categories and have given valuable information ([5, 8, 9, 12, 15, 16]). For example, the helper/inducer subset predominates in tuberculoid lesions, but the suppressor/cytotoxic predominates in lepromatous lesions. Such studies give information as to cell phenotype but cannot address the important questions concerning the antigenic specificity and functional properties of the lymphocytes observed in tissues or identify the mechanism of lymphocyte accumulation.

In an attempt to increase the power of *in situ* phenotypic studies, considerable attention has been given to putative activation antigens. That is to say, antigens observed to be expressed upon "turned on" lymphocytes *in vitro* are sought *in situ.* To date, the candidate activation antigens have not correlated well with the host's immunity as directed against *M. leprae.* For example, the T cells in both lepromatous and tuberculoid tissues expressed HLA-DR with equal frequency ([5, 9, 12, 15, 16]). Interleukin-2 (IL-2) positive cells, thought to be producing IL-2, a cytokine required for T-cell proliferation and a potent stimulator of interferon-gamma production, are more numerous in tuberculoid lesions compared with lepromatous lesions ([5, 7]). However, patients with lepromatous leprosy who also had erythema nodosum leprosum and who are also unresponsive to *M. leprae* have similar percentages of IL-2-containing cells in lesions compared with tuberculoid leprosy patients ([11]). The IL-2 receptor, which is expressed on T lymphocytes in response to presented antigen, was equally prevalent in tuberculoid and lepromatous tissues ([7]). Thus, three potential activation antigens did not correlate well with the host's observed cell-mediated responses to *M. leprae.*

Recently, a new T-lymphocyte surface antigen, Ta1, has been demonstrated to be unique to IL-2-dependent, activated T lymphocytes ([2]). Ta1 is a 105 kD molecule, distinct from the IL-2 receptor. Although this antigen is only weakly expressed on small numbers of resting peripheral T lymphocytes, it is strongly expressed on large numbers of activated T lymphocytes ([2, 4]). This antibody has been shown to be a marker for T cells that have undergone antigen-triggering *in vivo,* and the Ta1+ subset appears to include T memory cells ([3]). When lymphocytes were stimulated with antigen or mitogen, IL-2 receptors are the first to appear on the cell surface, followed by the Ta1 molecule, and then HLA-DR ([2]). Seeking a better activation antigen for *in situ* studies, we investigated the distribution and num-

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bers of Ta1 positive T lymphocytes in the skin lesions of patients with leprosy.

MATERIALS AND METHODS

Patients. Thirty-eight patients with leprosy, 8 tuberculoid (TT/BT or BT), 10 lepromatous (polar or subpolar LL), 10 lepromatous with erythema nodosum leprosum (ENL), and 10 patients with reversal reaction were classified according to the criteria of Ridley ([14]). Diagnoses of reactional states were made according to criteria previously published ([6, 13]). Five positive lepromin skin tests from TT/BT or BT patients were also included in this study according to a previous protocol ([1]).

Tissues. Punch or ellipsoidal skin biopsy specimens were embedded in OCT medium (Ames Co., Elkhart, Indiana, U.S.A.), and snap-frozen in liquid nitrogen. The tissues were then stored at −70°C until sectioning. Cryostat sections were cut to 5 μm thickness and air dried overnight. Before immunostaining, tissue sections were fixed in reagent-grade acetone at room temperature for 10 min, air dried, and then hydrated in modified phosphate-buffered saline (PBS).

Monoclonal antibodies. Primary mouse monoclonal antibodies were used at concentrations predetermined by checkerboard titrations. The activated T-lymphocyte marker anti-Ta1 (Coulter Electronics, Inc., Hialeah, Florida, U.S.A) was used at 1:50. Each specimen was also stained with a panel of monoclonal antibodies, including pan-T cells (Leu4, 1:400; Becton, Dickinson & Co., Rutherford, New Jersey, U.S.A.), helper/inducer T cells (Leu3a, 1:100), suppressor/cytotoxic T cells (Leu2a, 1:100), and anti-HLA-DR antibody (1:200; Becton, Dickinson). Controls consisted of omission of the primary antibody or the use of irrelevant antibodies on sections for the same period of incubation.

Single immunoperoxidase staining. After acetone fixation and PBS hydration, slides were incubated sequentially with monoclonal antibodies and peroxidase-conjugated goat anti-mouse IgG (1:20; Tago Inc., Burlingame, California, U.S.A.) for 15 min each. Five-minute PBS washes were performed between all incubations. The sections were then incubated with aminoethyl carbazol (AEC) in the presence of hydrogen peroxide for 8 min. Slides were then washed in tap water for 5 min, counter-stained with Mayer's hematoxylin for 1 min, washed, and mounted with glycerin-gelatin.

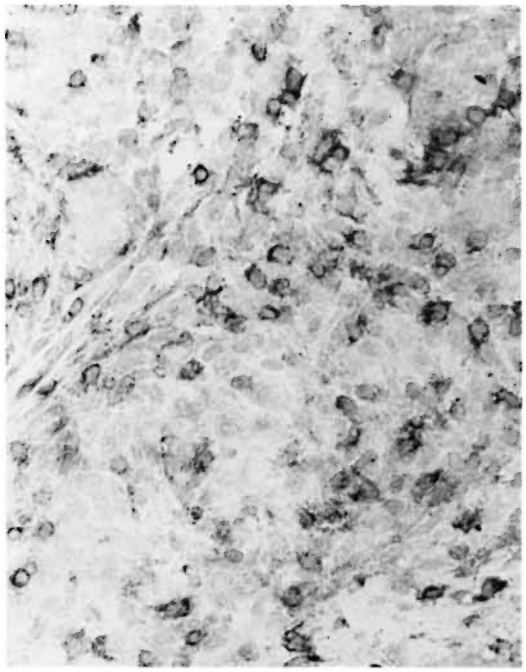

FIG. 1. Ta1 positive T lymphocytes in leprosy skin lesions. Shown is a tuberculoid leprosy lesion. Both large and small lymphocytes stain positively for Ta1 and are distributed throughout the granuloma (anti-Ta1, immunoperoxidase counterstained with hematoxylin, ×250).

Double-staining method. Double-staining was performed on four lepromatous and four tuberculoid specimens pairing anti-Ta1 with Leu4, Leu3a, and Leu2a. Frozen sections were single-stained as described above. After AEC incubation of the sections, slides were washed with PBS and then incubated with secondary monoclonal antibody for 30 min. Subsequently, the slides were incubated with biotinylated horse anti-mouse IgG (1:50; Vector Labs., Burlingame, California, U.S.A.) for 30 min. This was followed with avidin-biotin-glucose oxidase complex (Vectastain kit, Vector) incubation for 30 min. The blue color was developed with a glucose oxidase substrate (Vector) at 37°C (Immunostain plate; Ortho, Raritan, New Jersey, U.S.A.) for 20 min. The slides were then washed with tap water for 1 min and mounted with glycerin-gelatin. Con-

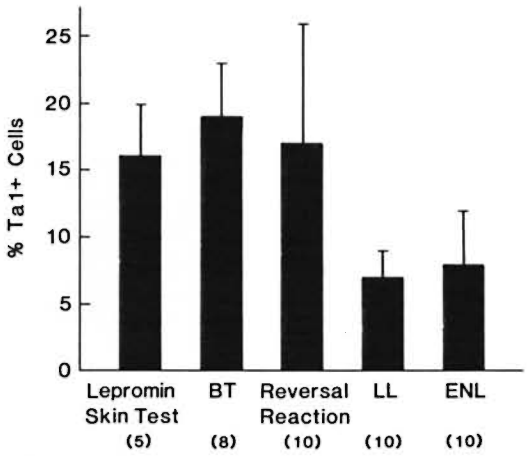

FIG. 2. Percentage of Ta1 positive cells in different types of leprosy skin lesions. Shown are the mean ± standard deviation for lepromin skin test, tuberculoid (BT), reversal reaction, lepromatous (LL), and erythema nodosum leprosum (ENL). Numbers of lesions studied are shown in parentheses.

trols were performed to demonstrate that the second labeling system did not cross-react with antibodies already added in the first reaction.

Quantification of stained cells. Because Leu4, Leu3a, Leu2a, and Ta1 positive cells were numerous, the percent positive cells were directly estimated by two independent observers and averaged. Double-stained positive cells were counted when both red and blue colors could be seen on the same cell or a purple color could be identified compared to the red or blue colored single-stained cells in the same section. The percent of double-stained cells was calculated as double-stained cells divided by total numbers of the single-stained plus double-stained cells.

Peripheral blood staining. Peripheral blood mononuclear cells were separated by Ficoll-Hypaque centrifugation from 10 normal controls, 10 tuberculoid, 10 lepromatous, and 10 ENL patients. Cells were stained with phycoerythrin-labeled anti-Ta1 (Coulter) and analyzed with a FACS IV. Controls consisted of omission of the antibody and antibody staining of a B-cell line (negative control) and a T-cell clone (positive control).

RESULTS

Using conventional hematoxylin and eosin (H&E) staining, the lesions of tuberculoid leprosy and the positive lepromin skin test were characterized as epithelioid granulomas; lepromatous leprosy and ENL lesions showed undifferentiated mononuclear phagocyte infiltration. A variable degree of epithelioid change was present in reversal reaction lesions; more in those two classified as borderline tuberculoid, and less in the eight classified as borderline lepromatous. Staining with Leu4, Leu3a, and Leu2a showed localization and percentages of positive cells similar to other patient groups previously reported ([8, 9]).

Ta1 antigen was expressed on the cell surface of both small and large nucleated cells in tuberculoid tissues, lepromin skin tests, and reversal reaction skin lesions. In contrast, most of the Ta1 antigen was expressed on small nucleated cells in lepromatous and ENL tissues. In all tissues, Ta1 positive cells were distributed throughout the granuloma (Fig. 1). The percentage of Ta1 positive cells in lepromatous specimens (7% ± 2%) is significantly less than the tuberculoid specimens (19% ± 4%), lepromin skin tests (16% ± 4%), and reversal reaction skin lesions (17% ± 4%). In contrast, there was no significant difference in Ta1 positive cells in ENL tissues (8% ± 4%) compared to lepromatous tissues (Fig. 2).

Further characterization of Ta1 positive cells was performed by double-immunostaining with Leu3a and Leu2a in tuberculoid and lepromatous specimens. More than 90% of the Ta1 positive cells double-stained with Leu3a in the tuberculoid specimens and less than 10% of the Ta1 positive cells double-stained with Leu2a. In lepromatous tissues, approximately 80% of Ta1 positive cells double-stained with Leu3a and 20% of the Ta1 positive cells double-stained with Leu2a.

There was no significant difference in the percentage of Ta1 positive cells in the peripheral blood in controls versus tuberculoid, lepromatous, or ENL patients, indicating that percentages in lesions were independent of those in the blood.

DISCUSSION

The current study showed the prevalence of Ta1 positive T lymphocytes in leprosy lesions to correlate well with the clinical states of the patients. Ta1 positive lymphocytes were more frequent in tuberculoid

leprosy skin lesions than in lepromatous lesions. Unlike the staining pattern with anti-IL-2, patients with ENL had similar percentages of Ta1 positive lymphocytes compared with lepromatous leprosy. Positive lepromin skin tests and reversal reactions of leprosy, known examples of delayed-type hypersensitivity reactions, had similar numbers of positive Ta1 cells compared with tuberculoid leprosy. Thus, Ta1, as judged by correlation with clinical and immunological categories of leprosy, is a better *in situ* marker for activated T lymphocytes than is HLA-DR, Tac, or IL-2 positivity.

Because, in the process of antigen or mitogen stimulation of T lymphocytes, Ta1 expression appears between that of Tac and HLA-DR, it is of interest that the frequency of Ta1 positive cells differs in tuberculoid and lepromatous lesions, but that of Tac- and HLA-DR-bearing cells do not. While an exact explanation for these differences is not available, it is likely that not all T cells expressing HLA-DR are antigen triggered, Ta1 positive. Because both represent responses to antigen, the discrepancy between Ta1 positive and Tac positive cell frequencies is more difficult. Possible explanations include the underestimation of Tac-bearing cells with the conditions used or receptor modulations occurring *in vivo* which are not found *in vitro*.

The presence of anti-Ta1 staining on small T-helper/inducer lymphocytes in lepromatous and ENL lesions compared with the large cells in tuberculoid lesions suggests that these cells may be resting or memory T cells ([2, 3]). The presence of Ta1 in lepromatous lesions on small lymphocytes but not lymphoblasts indicates a possible defect in the proliferation of antigen-reactive T-helper/inducer cells. To be sure, there is no data concerning the antigenic specificity of the Ta1 positive cells in these lesions. However, the frequency of Ta1 positivity (and the absence of lymphoblasts) was similar in lymphocyte-poor lepromatous and lymphocyte-rich ENL tissues. Thus, one must consider the possibility that these Ta1 positive cells might contain a *M. leprae*-specific population. Because *M. leprae*-transformable T lymphocytes have not been convincingly demonstrated in lepromatous patients, this is admittedly speculative but it is consistent with our suppressor cell data ([10]). Specifically, we have been able to clone from lepromatous lesions suppressor/cytotoxic (CD8+) T cells which can suppress HLA-DR-matched, helper/inducer (CD4+), *M. leprae*-responsive clones derived from tuberculoid lesions. The mechanisms of this suppression is the subject of current investigation. That a *M. leprae*-specific, Ta1 positive cell within lepromatous lesions is the target of the suppressor cell is one consideration. Thus, the diminished number of Ta1 positive helper/inducer cells in lepromatous and ENL lesions indicates that the unresponsiveness of lepromatous patients may be related to failure of activation and/or proliferation of antigen-reactive T-helper cells.

SUMMARY

Using a monoclonal antibody, anti-Ta1, that identifies antigen-activated T lymphocytes *in vitro*, we sought to identify activated T lymphocytes in leprosy skin lesions. Greater numbers of Ta1 positive T lymphocytes were observed in tuberculoid leprosy, lepromin skin tests, and reversal reactions as compared with lepromatous leprosy or erythema nodosum leprosum (ENL) ($p < 0.001$). With a double-staining technique, we found that the majority of these activated T lymphocytes were of the helper/inducer phenotype. No differences of Ta1 positive lymphocytes were observed in the peripheral blood. The defective cell-mediated immune response in lepromatous and ENL patients correlates with, and may be related to the failure of T-helper/inducer activation or proliferation in the presence of *Mycobacterium leprae*.

RESUMEN

Utilizando un anticuerpo monoclonal, anti-Ta1, que identifica a los linfocitos T activados *in vitro*, por antígeno, se trató de identificar a estos linfocitos en lesiones de piel de pacientes con lepra. Se observaron mayores números de linfocitos T Ta1+ en los pacientes con lepra tuberculoide, en las intradermoreacciones a la lepromina y en las reacciones reversas, que en los pacientes con lepra lepromatosa o eritema nodoso leproso (ENL $p < 0.001$). Con una técnica de doble tinción, encontramos que la mayoría de estos linfocitos T activados fueron del fenotipo cooperador/inductor. No se observaron diferencias de linfocitos Ta1+ en la sangre periférica de los grupos estudiados. La defectuosa respuesta inmune celular en los pacientes con lepra lepromatosa y ENL, correlacionó con (y podría estar

relacionada a) la falla en la activación de los linfocitos T cooperadores/inductores o en su capacidad de proliferación en respuesta al *Mycobacterium leprae*.

RÉSUMÉ

On a cherché à identifier dans les lésions cutanées de la lèpre les lymphocytes T activés, en ayant recours à une technique d'anticorps monoclonaux anti-Ta1, qui identifie *in vitro* les lymphocytes T activés par des antigènes. Les plus grands nombres de lymphocytes T-Ta1+ ont été observés dans la lèpre tuberculoide, dans les épreuves cutanées à la lépromine, et dans les réactions réverses, par comparaison avec les chiffres notés dans la lèpre lépromateuse ou dans l'érythème noueux lépreux (ENL) ($p < 0{,}001$). Avec une technique de double coloration, on a observé que la majorité de ces lymphocytes T activés appartenait au type adjuvant/inducteur (helper/inducer). Aucune différence dans les lymphocytes Ta1+ n'a été relevée dans le sang périphérique. La déficience dans la réponse d'immunité à médiation cellularie chez les malades lépromateux et chez ceux atteints d'ENL correspond à l'absence d'une activation des cellules T adjuvantes/inductrices et à leur prolifération en présence de *Mycobacterium leprae*; elle pourrait être reliée à ces phénomènes.

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