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LEUKOCYTE SUBSETS IN THE GRANULOMATOUS RESPONSE PRODUCED AFTER INOCULATION WITH *MYCOBACTERIUM LEPRAE*-BCG IN LEPROMATOUS PATIENTS

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Abstract. Leukocyte subsets present in the granulomatous response produced after the inoculation of a mixture of *Mycobacterium leprae* and BCG in lepromatous leprosy patients were characterized in situ using monoclonal antibodies and an immunoperoxidase technique. The granuloma produced after *M. leprae*-BCG inoculation showed a distribution pattern similar to tuberculoid granulomas. T lymphocytes bearing the CD8 phenotype (T cytotoxic suppressor) were sequestered to the periphery of the epithelioid tubercles and T helper-induced CD4+ lymphocytes were distributed throughout the infiltrate. Langerhans cells CD1+ were increased in the epidermis, and in dermis they were localized mainly in the mantle surrounding the granuloma. Most of the dermal infiltrate produced after the inoculation of *M. leprae*-BCG expresses the HLA-DR antigen. Similarly, most keratinocytes were also positive to this MHC antigen. The granulomatous response to BCG was similar to the inoculation of a mixture of *M. leprae*-BCG, however acid-fast bacilla were still present. The inoculation of *M. leprae* produced a macrophage granuloma with no clearing of the bacilla which resembles the lepromatous leprosy granuloma.

The clinical pathological spectrum of leprosy in its diverse forms is the expression of the immunological response of the human host to infection by *Mycobacterium leprae*: the two forms at either end of that spectrum are lepromatous leprosy (LL), in which patients are anergic to *M. leprae* antigens, and tuberculoid leprosy (TL), in which patients have a cell-mediated immune response to the bacillus.[1–3]

Convit et al. in 1972[4] developed a skin test to determine patients' degree of competency in clearing *M. leprae*. The test consists of intradermal inoculation of 0.1 ml of 640×10^6 heat-killed bacilli/ml. Histological analysis 1 month later revealed that LL patients do not eliminate the bacilli whereas TL patients do. These authors also demonstrated that if another mycobacterium antigen is inoculated, such as BCG (*M. bovis* Bacille Calmette-Guerin) or *M. lepramurium*, the lepromatous patients produced an epithelioid granuloma and eliminated the bacilli. These results demonstrate the specific inability of the lepromatous granuloma to remove *M. leprae*.

The local in vivo activation of lepromatous macrophages and consequent clearing of the bacilli can be obtained after intradermal inoculation of a mixture of *M. leprae* and BCG.[4]

Immunological changes such as reactions to lepromin, the test for bacillary clearance, and in vitro lymphocyte transformation become positive in indeterminate and in Mitsuda-negative contacts after the inoculation of *M. leprae*-BCG.[5] In this study, leukocyte subsets in the granulomatous response (GR) produced after the inoculation of *M. leprae*-BCG in LL patients are characterized using monoclonal antibodies and immunoperoxidase techniques.[6–11]

MATERIALS AND METHODS

Patients

The cases (n = 8) were classified as LL according to the criteria of Ridley and Jopling.[1] Patients were inoculated with 6×10^8 bacilli of *M. leprae* purified from tissue of experimentally infected armadillos by the Draper protocol[12] and killed in the autoclave (121°C, 15 min). Live BCG in variable concentrations (0.01–0.2 mg) was injected depending on the reaction to 2 units of PPD.[4]

Inoculations were done intradermally in the volar surface of the forearm. Lepromatous pa-

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tients were inoculated with *M. leprae*-BCG (n = 3), BCG (n = 2), or *M. leprae* (n = 3). Biopsies (6 mm in diameter) from the reactions were sampled after 30 days.

For immunoperoxidase studies, the skin biopsies were embedded in OCT compound, snap-frozen in liquid nitrogen, and stored at −20°C until examination.

Monoclonal antibodies

The well characterized monoclonal antibodies used were diluted in a modified phosphate buffered saline (PBS), pH 7.2.[13] These antibodies recognized mononuclear cell markers for the following subpopulations:[14] T suppressor/cytotoxic (CD8 = Leu-2; diluted 1:100); Pan T (CD3 = Leu-4; 1:100); Langerhans cells (CD1 = Leu-6; 1:100); macrophages (Leu-M3; 1:50) purchased from Becton Dickinson, Inc.; T helper (CD4 = OKT4; 1:50) purchased from Ortho Diagnostics, Inc.; HLA-DR (Ia) (I_3; 1:400) purchased from Coulter Clone Inc. Anti-interleukin-2 receptor (Tac = CD25; 1:1,500) was kindly provided by T. A. Waldman (National Cancer Institute).

Immunoperoxidase staining procedure

Frozen sections (5 μm) were cut with a cryostat and air dried overnight before the immunostaining procedure. Some sections were also stained with hematoxylin and eosin (H&E) and Fite-Faraco. Immunostaining was performed using the avidin-biotin (ABC) immunoperoxidase technique,[15] the sections being treated as follows: fixation in fresh acetone, 10 min; PBS, 5 min; primary monoclonal antibody (optimal dilution), 15 min; PBS, 5 min; biotinylated horse-anti mouse IgG (Vector Labs Inc., Burlingame, California) 1:30 in PBS (50 μg/ml), 15 min; PBS, 5 min; ABC (Vectastain kit, Vector Labs) 1:100, 15 min; PBS, 5 min; developing for 10 min with 90 μM H_2O_2 and 3-amino-9-ethyl-carbazole (final concentration 0.88 mM) which was dissolved in 50 mM N,N-dimethylformamide in 0.1 M acetate buffer, pH 5.2; rinse in water; counterstaining with Mayer's hematoxylin and mounting in glycerin-gelatin.

Mononuclear cell quantification

Cells were counted using a light microscope with millimetered scale (Carl Zeiss, Germany), calibrated to determine the number of cells/mm²

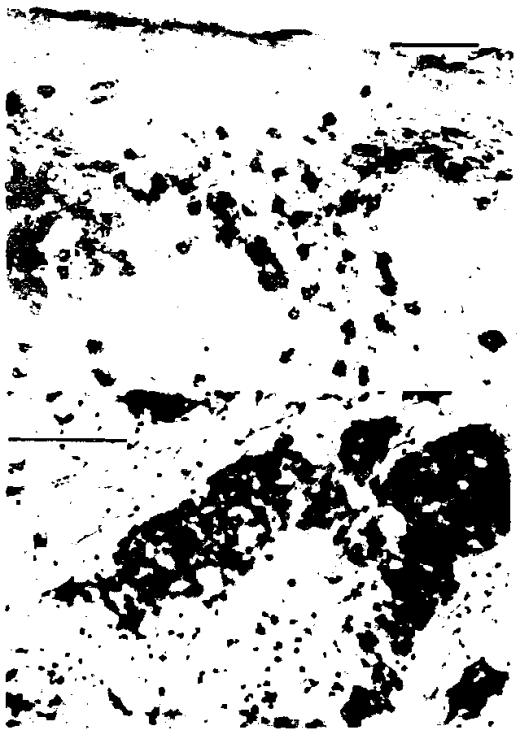

FIGURE 1. **Top.** T cytotoxic/suppressor cells in the granulomatous response to inoculation of *M. leprae*-BCG in a lepromatous leprosy patient. Note that the CD8+ T cells are arranged towards the periphery of the dermal granulomas and some are also present in the epidermis. Bar = 2.5 μm. **Bottom.** CD8− T cells in a tuberculoid granuloma after inoculation of BCG in a lepromatous leprosy patient. Bar = 10 μm.

in dermal infiltrates or epidermis. Only cells showing red-brownish immunostaining and a visible nucleus were counted as positive. Percentages of each phenotype were calculated. There are approximately 3,600 cells/mm² of infiltrate according to a previous count of the nucleated cells in an H&E stained section.

Statistical analysis

Cell counts were expressed as mean ± SD per mm² of dermal infiltrates or epidermis.

RESULTS

Histological examination demonstrated the presence of a GR. The GR produced after the inoculation of *M. leprae*-BCG was characterized

FIGURE 2. Langerhans cells (CD1+) distributed around the granulomas produced after inoculation of *M. leprae*-BCG in a lepromatous leprosy patient. Bar = 2.5 μm.

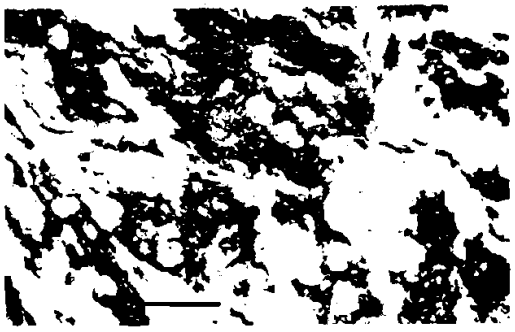

FIGURE 3. Abundant macrophages Leu-M3+ in the granulomatous response to *M. leprae* in a lepromatous leprosy patient. Bar = 2.5 μm.

by a well defined and circumscribed epithelioid granuloma in the dermis. These granulomas were mainly composed of epithelioid cells and no bacilli. The T cells bearing the cytotoxic suppressor CD8 phenotype were localized preferentially in the periphery of the granuloma (Fig. 1). In addition, a few of these T CD8+ cells were also observed in the epidermis. The T helper inducer lymphocytes (CD4+) were distributed throughout the granuloma. The same lymphocyte distribution was observed after inoculation with BCG alone (Fig. 1). However, most acid-fast bacilli were still present in the patient's granuloma after BCG inoculation. Only the BCG were phagocytosed by macrophages in this GR.

When the inoculation consisted solely of heat-killed *M. leprae*, the GR was composed of an admixture of leukocytes and bacilli-laden macrophages. Both T immunophenotypes CD8− and CD4+ were diffusely distributed throughout the granuloma. Epidermal Langerhans cells (CD1+) were increased over normal values[19] in the three reaction sites (Table 1). These cells were also observed surrounding the dermal granulomas formed after the inoculation of *M. leprae*-BCG (Fig. 2) and BCG.

HLA-DR− cells were almost universally present in the three types of GR analyzed. Most keratinocytes also expressed this antigen.

Lymphocytes expressing the interleukin-2 receptor (Tac) were also present in appropriate numbers in the GR to *M. leprae*-BCG (mean 748 ± 83 cells/mm²) and BCG (1,145 cells mm²)

TABLE 1

Mononuclear cell densities in the lesions of lepromatous leprosy patients after inoculation of M. leprae, M. leprae-*BCG, or BCG*

Phenotype	Granulomatous response*		
	M. leprae	*M. leprae*-BCG	BCG
CD8	1,325 ± 127 (36.8 ± 3.50)	1,023 ± 134 (28.41 ± 3.72)	940 ± 241 (26.11 ± 6.69)
CD4	1,011 ± 580 (28.08 ± 16.11)	1,083 ± 144 (30.08 ± 4.00)	705 ± 298 (19.58 ± 8.77)
CD3	1,076 ± 196 (29.88 ± 5.44)	1,015 ± 127 (28.19 ± 3.52)	935 ± 94 (25.97 ± 2.61)
Tac	359 ± 176 (9.97 ± 4.88)	748 ± 83 (20.77 ± 2.30)	1,145 (31.80)
Leu-M3	2,110 ± 338 (58.60 ± 9.39)	673 ± 182 (18.19 ± 5.05)	624 ± 24 (17.33 ± 0.66)
CD1 epidermis	656 ± 152	796 ± 351	991 ± 12
CD4/CD8	0.84 ± 0.47	1.05 ± 0.14	0.73 ± 0.12

* Expressed in cells/mm² ± SD (% of the designated cells ± SD).

but their numbers diminished in the GR to *M. leprae* (mean 359 ± 176 cells/mm²).

The inoculation of *M. leprae* produced an undifferentiated granuloma mainly composed of macrophages Leu-M3+ (mean 2,110 ± 338 cells/mm², 58.6%) (Fig. 3). This granuloma was full of bacilli localized in globi.

DISCUSSION

The immunohistological findings confirm Convit's observations[4] that the injection of heat-killed *M. leprae*-BCG in nonreactor lepromatous patients induced an immune granuloma with elimination of both mycobacteria. Convit's findings provided the experimental basis for the use of the mixture of two mycobacteria, one providing the necessary specific antigens and the other triggering macrophage digestion, in studies of immunotherapy and immunoprophylaxis in leprosy.[5–17]

The particular arrangement of the leukocyte immunophenotypes observed in the dermal infiltrates of the GR to a mixture of *M. leprae* and BCG was very similar to that described for tuberculin and Mitsuda reactions.[5,18,19] This granulomatous response was characterized by a very active epidermis featuring Langerhans cell hyperplasia, infiltrating T lymphocytes, and HLA-DR+ keratinocytes. Similarly, the location of T cytotoxic/suppressor CD8+ cells in the lymphocytic mantle surrounding the epithelioid tubercle, after inoculation of BCG or the mixture, appears to be the consequence of immune granuloma formation.

The percentages in dermis of leukocytes bearing the CD8, CD4, CD3, CD1, and HLA-DR immunophenotypes were very similar in the three tests. However, marked differences were observed in the GR to *M. leprae* which showed a decrease in the numbers of T cells positive for the interleukin-2 receptor and an increase in the numbers of Leu-M3− macrophages. The low density of Tac+ cells in the GR coincides with a previous report[20] which suggests that the anergic response of LL patients is associated with a defective expression of interleukin-2 receptors by T cells.

In LL lesions IL-2+ T cells are scarce,[21] epidermal immunoactivity is diminished, dermal CD1+ Langerhans cells are rare, and T cytotoxic/suppressor cells predominate.[8] The modifications observed in this study after inoculation with *M. leprae*-BCG are in part consistent with a delayed type hypersensitivity immunopathogenesis.

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