

## *In vitro* Studies on Dermal Granulomas of Human Leprosy: Characterization of Cells Using Monoclonal Antibodies<sup>1</sup>

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Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae*. The lesions in tuberculoid leprosy are characterized by an epithelioid cell granuloma with abundant lymphocytes forming dense collections around the epithelioid cells. On the other hand, lepromatous leprosy is characterized by a granuloma composed of sheets of macrophages loaded with *M. leprae* along with plasma cells and a few lymphocytes diffusely distributed in the granuloma. Most of the studies made to understand the immunological mechanisms in leprosy have been mainly carried out using *in vitro* tests on peripheral blood-derived lymphocytes and monocytes (7). Monoclonal antibodies against cell-surface antigens have been used to delineate the immunopathological mechanisms involved in the granulomas of leprosy (4, 5, 9, 10). A better understanding of the immunological mechanisms involved in granuloma formation could be made by isolating the cells from these granulomas.

Recently, we have standardized a method for obtaining single cell suspensions from leprosy granulomas. The suspensions contained lymphocytes and "large cells." The numbers of lymphocytes were higher in the suspensions of tuberculoid granulomas in comparison to those in suspensions of lepromatous granulomas. A higher percentage of lymphocytes from the tuberculoid granulomas formed rosettes with sheep erythrocytes and also showed the presence of esterase dots in the cytoplasm. However, they did not form rosettes with antibody

and complement-coated erythrocytes (EAC). Most of the "large cells" from both of the granulomas were esterase positive, exhibited peroxidase activity, and did not carry receptors for the C3 component of complement (6).

In this study, monoclonal antibodies against T-cell subsets and Ia-like antigens have been used for further characterization of the cells in the suspensions from leprosy granulomas.

### MATERIALS AND METHODS

RPMI 1640 (GIBCO Laboratories, U.K.) was used containing 100 µg/ml of streptomycin and 100 U of penicillin (both from Indian Drugs and Pharmaceuticals Ltd.) per ml. Collagenase type I (from *Clostridium histolyticum*) was obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

**Monoclonal antibodies.** The following monoclonal antibodies were obtained from Ortho Pharmaceutical Corporation, U.S.A.: OKT11 (a pan T-cell marker), OKT8 (suppressor/cytotoxic T marker), OKIa (recognizing activated T cells, macrophages, and B cells). Leu3a (recognizing inducer/helper T cells) was obtained from Becton Dickinson Monoclonal Center, U.S.A. Fluorescein (FITC) conjugated sheep anti-mouse Ig F(ab)<sub>2</sub> was obtained from New England Nuclear, Boston, Massachusetts, U.S.A.). Fluorescein-conjugated rabbit anti-human IgM (recognizing B cells) was from Dakopatts A/S, Denmark.

**Skin biopsies.** Skin biopsies (15 × 5 mm in size) were taken from 21 untreated leprosy patients attending the outpatient clinic of the Central JALMA Institute for Leprosy, Agra, India. Each biopsy was bisected on removal; one half was fixed in Formol-Zenker's fluid and processed for histology

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THE TABLE. Characteristics of the cells in the suspensions from leprosy granulomas.

	Percentage positive cells <sup>a</sup>	
	Tuberculoid leprosy	Lepromatous leprosy
	Epithelioid cell granuloma (N = 11)	Macrophage granuloma (N = 10)
<b>Lymphocytes</b>		
No. viable cells/biopsy	0.90 ( $\pm 0.14$ ) $\times 10^6$	0.30 ( $\pm 0.08$ ) $\times 10^6$
OKT11	69.9 $\pm$ 3.65	<2
Leu3a	61.5 $\pm$ 4.12	<2
OKT8	26.7 $\pm$ 3.70	<2
OKIa	72.7 $\pm$ 7.5	—
B cells	<2	<2
Ratio Leu3a+ : OKT8+ cells	2.79 $\pm$ 0.61	—
<b>Macrophages</b>		
No. viable cells/biopsy	0.39 ( $\pm 0.05$ ) $\times 10^6$	0.37 ( $\pm 0.09$ ) $\times 10^6$
Ia	90–100	90–100

<sup>a</sup> Values are mean  $\pm$  S.E.M. of percentage of positive-staining cells unless otherwise noted.

by paraffin-embedded blocks; the other half was used for obtaining cell suspensions. The biopsies were graded histologically according to the criteria of Ridley and Jopling (<sup>8</sup>). Patients classified as BT and TT were grouped as tuberculoid, and those classified as BL and LL were grouped as lepromatous cases.

**Preparation of single cell suspensions from the granulomas.** Pilot experiments were done by incubating the biopsies a) with different concentrations of collagenase and b) for different intervals of time. From these experiments, the optimal conditions for the isolation of cells from the biopsies were standardized.

The biopsies were collected in RPMI 1640 containing antibiotics, and cleaned free of fat and connective tissues. They were then sliced into small pieces, suspended in 1 ml of RPMI 1640, and incubated with 1 mg of collagenase for 4 hr at 37°C, with intermittent shaking every 1 hr. The supernatants were collected and centrifuged at 200  $\times$  g for 10 min. The pellet was then washed once with medium and resuspended. The number of viable lymphocytes and viable "large cells" were quantitated using the trypan blue exclusion (0.2% w/v phosphate buffered saline) test in a hemocytometer.

**Immunofluorescence.** A smear of the cell suspension (25  $\mu$ l) was made and fixed in acetone-chloroform (1:1) for 20 min. The smears were layered with 25  $\mu$ l of a 1:20 dilution of the monoclonal antibodies at

room temperature for 45 min. Smears layered with phosphate buffered saline (PBS) served as controls. Subsequently, the smears were washed in 0.85% w/v saline for 10 min. They were then incubated with 25  $\mu$ l of a 1:80 dilution of fluorescein (FITC)-conjugated sheep anti-mouse Ig F(ab)<sub>2</sub> for 30 min, and washed in 0.85% saline for 15 min. The smears were mounted in 90% glycerol-PBS and viewed by epi-illumination using an HBO 50 mercury lamp and a Leitz inverted microscope. B cells were defined by direct immunofluorescent staining with FITC-conjugated rabbit anti-human IgM.

Smears of Ficoll-Hypaque-purified mononuclear cells from the peripheral blood of normal individuals and skin lesions from leprosy patients were used as controls to test the efficacy of the monoclonal antibodies.

The nature of the cells in suspension showing positive staining was based on morphological assessment in a) smears stained by immunofluorescence and b) parallel smears stained with Geimsa stain. Quantitation of the positive cells was made and expressed as a percentage. A minimum of 100–200 cells were counted (particularly in the suspensions of tuberculoid granulomas), depending on the concentration of the cells.

## RESULTS

Skin biopsies from 21 untreated leprosy patients were studied—11 tuberculoid (BT/

TT) and 10 lepromatous (BL/LL) leprosy cases confirmed histologically.

Preliminary experiments were carried out on the cryostat sections of leprosy lesions to assess the optimal dilution of antibodies required for staining. The antibodies were also used by indirect immunofluorescence on peripheral blood mononuclear cells. OKT11, Leu3a, and OKT8 antibodies stained 70–80%, 40–50%, 15–20% of peripheral blood mononuclear cells, respectively.

**Characteristics of cells in the suspensions from the granulomas.** Single cell suspensions prepared from the dermal granulomas showed the presence of lymphocytes and macrophages. The numbers of lymphocytes were significantly higher in the granulomas of tuberculoid lesions in comparison to those in the granulomas of lepromatous lesions. The viability of the cells in the suspensions was 70–80%.

**Lymphocytes.** The Table shows that 70% of the lymphocytes in tuberculoid granulomas expressed OKT11 and Ia-like antigens. At the same time, 61% of the lymphocytes were Leu3a+, while only 27% expressed OKT8 antigens. The ratio of Leu3a+:OKT8+ cells in the tuberculoid lesions was  $2.79 \pm 0.61$ . In contrast, the lepromatous granulomas contained few positive lymphocytes (<2%) expressing OKT11 and Leu3a or OKT8 antigens. Occasional B cells (<2%) were noticed in both types of lesions.

Thus, it would appear that the predominant lymphocytes in the leprosy lesion are activated T lymphocytes expressing Ia-like antigens. Leu3a+ (helper/inducer) cells appear to be in higher proportions in the tuberculoid lesions.

**Macrophages.** Macrophages from the leprosy granulomas had a very granular appearance by light microscopy. It is interesting that most of the macrophages from both types of granulomas expressed Ia-like antigens.

## DISCUSSION

There are two main advantages of characterization of cells in single cell suspensions over *in situ* studies for understanding the immunopathological mechanisms involved in the granuloma formation: a) It is

easier to characterize and quantitate lymphocytes and macrophages in the granulomas, and b) it can be used as an experimental system for functional studies of the lymphocytes and macrophages infiltrating the granulomas.

We have recently shown that it is possible to prepare single cell suspensions from the dermal granulomas. The single cell suspension from a tuberculoid granuloma contains a significantly higher number of lymphocytes in comparison to a single cell suspension from a lepromatous granuloma. A high percentage of lymphocytes from the tuberculoid granuloma formed rosettes with sheep erythrocytes. In addition, these cells exhibited esterase dots in the cytoplasm which is known to be a marker of T cells. Most macrophages from both granulomas were esterase positive and exhibited peroxidase activity (6).

In the present study, we have used monoclonal antibodies for further characterization of these cells in suspension. A high percentage of lymphocytes in suspensions from tuberculoid granulomas expressed OKT11 and Ia-like antigens. The proportion of Leu3a+ cells was greater in comparison to OKT8+ cells. The ratio of Leu3a+:OKT8+ cells was also higher in these granulomas. By contrast, lepromatous granulomas contained only a few positive lymphocytes expressing OKT11 or Leu3a or OKT8 antigens. The ratio of Leu3a+:OKT8+ cells cannot be enumerated accurately in these granulomas because the patients were untreated and, as such, the lymphocytes in the granulomas were very low in number. These results show a good correlation with the *in situ* characteristics of cells infiltrating the leprosy lesions as reported by us (5) and by other workers (4,10). A similar ratio of Leu3a+:OKT8+ cells has been recorded in other conditions where epithelioid cell granulomas are prevalent (3,4).

Most of the macrophages from both types of granulomas were granular in appearance and expressed Ia-like antigens. Recent studies suggest that the antigen-presenting capacity of macrophages is related to the expression of Ia-like antigens on their surface (11). It is interesting that the macrophages from the suspensions of lepromatous granulomas express Ia-like antigens and

may, therefore, possess the ability to present antigen. Incidentally, a high proportion of macrophages from lepromatous granulomas were found to be adherent to a plastic surface (6). Adherent cells have been shown to be involved in antigen presentation (2). An analogous finding of similar Ia expression has been noted in macrophages isolated from vigorous and immunomodulated schistosome granulomas in mice (1). This, therefore, raises the question of why there is a paucity of T lymphocytes or a disturbance in lymphocyte traffic in the lepromatous granulomas. Further studies are aimed at delineating the mechanisms of lymphocyte traffic in these granulomas.

It appears from the present study that it is possible to obtain the constituent cells of the granulomas in different types of leprosy as single cell suspensions and to characterize them. This should help in further understanding the immunological mechanisms of granuloma formation in leprosy.

#### SUMMARY

Single cell suspensions from granulomas of leprosy cases were prepared to enable an *in vitro* study on the characteristics of infiltrating cells. In all, biopsies from 21 untreated cases of tuberculoid leprosy and lepromatous leprosy were analyzed. The granulomas were found to contain lymphocytes and macrophages. The numbers of lymphocytes were higher in the suspensions of tuberculoid granulomas in comparison to lepromatous granulomas. A high percentage of lymphocytes from tuberculoid granulomas expressed OKT11 and Ia-like antigens, thereby indicating the presence of activated T cells. The proportion of Leu3a+ cells was greater in comparison to OKT8+ cells in these granulomas. In lepromatous granulomas, only a few positive lymphocytes expressing OKT11 or OKT8 antigens were observed. The ratio of Leu3a+:OKT8+ cells ( $2.79 \pm 0.61$ ) was higher in the tuberculoid granulomas than in the lepromatous granulomas. Most macrophages from both types of granulomas expressed Ia-like antigens.

#### RESUMEN

Se prepararon suspensiones de células aisladas a partir de granulomas de casos de lepra para estudiar *in*

*vitro* las características de las células infiltrantes. Se analizaron las biopsias de 21 casos de lepra tuberculoides o lepromatosa sin tratamiento. Se encontró que los granulomas contenían linfocitos y macrófagos. Los números de linfocitos fueron mayores en los granulomas tuberculoides que en los lepromatosos. Un alto porcentaje de los linfocitos de los granulomas tuberculoides expresaron antígenos OKT11 y antígenos Ia, indicativos del estado activado de los linfocitos T. La proporción de células Leu 3a+ fue mayor que la de células OKT8+ en estos granulomas. En los granulomas lepromatosos sólo se observaron unos cuantos linfocitos OKT11+ y OKT8+. La relación de células Leu3a+:OKT8+ ( $2.79 \pm 0.61$ ) fue mayor en los granulomas tuberculoides que en los lepromatosos. La mayoría de los macrófagos de ambos tipos de granulomas expresaron antígenos Ia.

#### RÉSUMÉ

On a préparé des suspensions de cellules isolées recueillies dans des granulomes de lèpre, en vue de mener une étude *in vitro* sur les caractéristiques des cellules d'infiltration. Au total, on a analysé les biopsies récoltées chez 21 malades atteints de lèpre tuberculoides ou lépromateuse, non traités. On a observé que les granulomes contenaient des lymphocytes et des macrophages. Le nombre de lymphocytes était plus élevé dans les suspensions de granulomes tuberculoides que dans les granulomes lépromateux. Une proportion élevée de lymphocytes obtenus à partir de granulomes tuberculoides ont exprimé des antigènes OKT11 et du type Ia. Ceci indique la présence de cellules T activées. Dans ces granulomes, la proportion de cellules Leu3a+ était plus élevée que celle de cellules OKT8+. Dans les granulomes lépromateux, par contre, un petit nombre seulement de lymphocytes positifs ont exprimé les antigènes OKT11 ou OKT8. Le rapport des cellules Leu3a+/OKT8+ était plus élevé dans les granulomes tuberculoides que dans les granulomes lépromateux ( $2.79 \pm 0.61$ ). La plupart des macrophages provenant de l'une ou l'autre espèce de granulomes exprimaient des antigènes du type Ia.

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