In vitro Studies on Dermal Granulomas of Human Leprosy—Cellular Characteristics[1]

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Leprosy is a chronic granulomatous disease caused by *Mycobacterium leprae*. This disease manifests itself as a spectrum with highly immune, paucibacillary, tuberculoid leprosy at one end and low-immune, multibacillary, lepromatous leprosy at the other. The lesions in tuberculoid leprosy are characterized by an epithelioid cell granuloma with abundant lymphocytes forming dense collections around the epithelioid cells. 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 investigations to understand the immunological mechanisms in leprosy have been mainly carried out by using *in vitro* tests on peripheral blood-derived lymphocytes and monocytes ([10]). Scant information is available on the nature of the infiltrating cells in the granulomas of leprosy lesions ([10, 11]). In the present study, an attempt has been made to prepare a single cell suspension from these granulomas and to study some of the properties of these cells *in vitro*.

MATERIALS AND METHODS

RPMI 1640 was obtained from GIBCO Laboratories, U.K.; penicillin/streptomycin from Indian Drugs and Pharmaceuticals Ltd., and α-napthyl acetate from Loba Chemie, India. 3-3′-Diaminobenzidine tetrahydrochloride and collagenase Type I (*Clostridium histolyticum*) was obtained from Sigma Chemical Company, U.S.A., and anti-sheep hemolysin from Span Diagnostics, Surat, India. To 100 ml of RPMI 1640, 10,000 μg/10,000 I.U. of streptomycin-penicillin were added.

Skin biopsies. Skin biopsies (15 × 5 mm in size) were taken from 44 untreated leprosy patients attending the outpatient clinic of Central JALMA Institute for Leprosy, Agra. Each biopsy was bisected on removal. One half was fixed in formal-Zenker's fluid and processed by conventional paraffin-embedded blocks. The other half was used for cellular studies. The biopsies were graded histologically on the criteria of Ridley and Jopling ([12]). The patients with BT or TT were grouped as tuberculoid and those with BL or LL were grouped as lepromatous cases. Only those results are reported where hematoxylin and eosin (H&E) and Ziehl-Neelsen stains of formalin-fixed tissues showed typical histopathological features.

Preparation of single cell suspension from the granulomas. Pilot experiments were done using a) different concentrations of collagenase, and b) incubating 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 cut 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 × *g* for 10 min. The pellet was washed once with medium and resuspended. The number of viable lymphocytes and viable "large cells" were quantitated, using the trypan blue exclusion (0.2% in phosphate buffered saline) test, in a hemocytometer.

Adherence. Depending on the yield, 0.5 × 16^6 viable "large cells" were adhered to a plastic Petri dish (35 × 10 mm) in serum-free medium for 2 hr at 37°C. The supernatants were removed, centrifuged at 200 × *g* for 10 min, and the number of "viable

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TABLE 1. *Histopathological analysis of leprosy lesions.*

	Tuberculoid (BT-TT) 22 patients	Lepromatous (BL-LL) 22 patients
Area occupied (% of dermis)	52.2 ± 5.0[a]	59.0 ± 5.2[a]
Lymphocytes	++	±
Epithelioid cells	++	−
Macrophages	−	++
Acid-fast bacilli	−	+++++

[a] Values are mean ± standard error of the mean.

large cells" were counted. Results were expressed as the percentage of "nonadherent viable large cells."

Antibody- and complement-coated erythrocytes (EAC). Sheep erythrocytes were washed three times in phosphate buffered saline (PBS) before use and a 5% suspension was prepared. Anti-sheep hemolysin (IgM) at 1:100 dilution in PBS (0.01 M phosphate, pH 7.4) was used. One ml of 5% sheep erythrocytes was incubated with 1 ml of antibody at 37°C in a water bath for 30 min. After incubation, the suspension was centrifuged at 400 × *g* for 5 min. The pellet was washed three times in PBS and finally resuspended in 1 ml of PBS. This suspension was incubated with 1 ml of fresh mouse serum (1:10 dilution in PBS) at 37°C for 45 min, in a water bath, and then centrifuged at 400 × *g* for 5 min. The pellet was washed three times in PBS, resuspended in 1 ml of PBS, and stored at 4°C until used. This preparation results in a 5% suspension of EAC.

Rosetting technique. *E.-rosettes:* 0.3×10^6 to 0.5×10^6 cells from the granuloma were incubated with 1% sheep erythrocyte suspension, in equal volumes, at 37°C for 10 min. They were then centrifuged at 100 × *g* for 5 min, and incubated further for 1 hr at 4°C. The cells were then gently mixed and the number of rosettes were counted. Cells surrounded by three or more red cells were considered a "rosette" and a minimum of 200 rosettes was counted in each sample.

EAC-rosettes: 0.3×10^6 to 0.5×10^6 cells from the granuloma were incubated with 1% EAC suspension, in equal volumes, at 37°C for 30 min. The number of rosettes were counted as described above.

Histochemical technique for enzyme activity. A smear of the cell suspension was made and fixed in methanol for 5 min. The peroxidase staining was carried out as described by Van Furth ([15]), and nonspecific esterase staining by the method of Horwitz, *et al.* ([5]). The smears were stained in duplicate. The peroxidase reaction was characterized by a brown color in the cytoplasm, and the presence of nonspecific esterase by a black-brown color in the cytoplasm. In addition, the smears of infiltrating cells were also stained by the Ziehl-Neelsen method to detect the presence of acid-fast bacilli in the "large cells."

Smears of Ficoll-Hypaque purified mononuclear cells from the peripheral blood of normal individuals were used as controls to test the efficacy of the various reagents.

Statistical test. The results were analyzed by Student's *t* test ([2]).

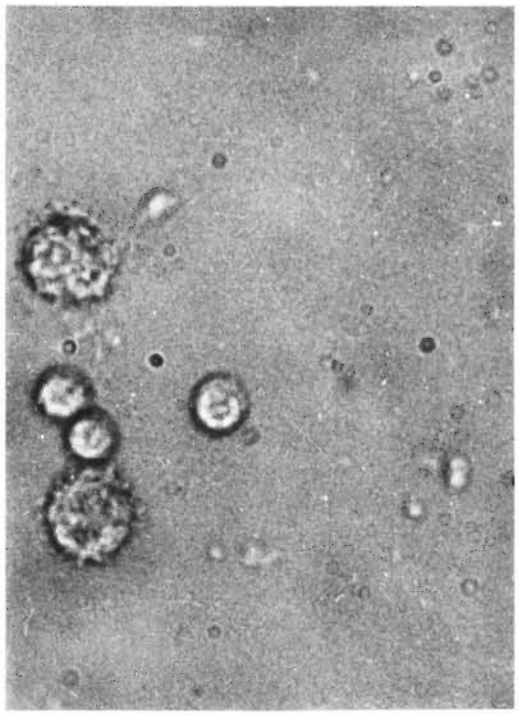

FIG. 1. Large cells from a granuloma. Note the granular appearance (×280).

RESULTS

Skin biopsies from 44 untreated leprosy patients were studied—22 tuberculoid (BT-TT) and 22 lepromatous (BL-LL). Only those results are included where the type of leprosy was confirmed by light-microscopic

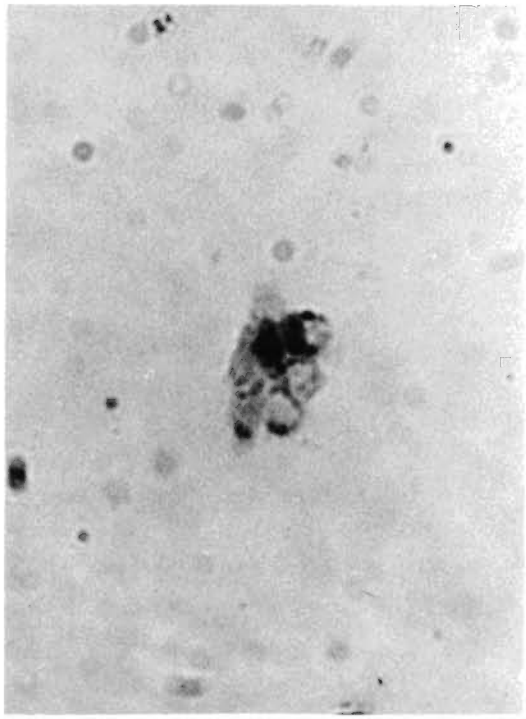

FIG. 2. Large cells from a granuloma showing the presence of esterase (×280).

examination of conventionally fixed tissues as per the criteria of Ridley and Jopling ([12]). The histopathological analysis of the granulomas is given in Table 1.

Infiltrating cells in the granulomas

Tables 2 and 3 summarize the results of the properties of the infiltrating cells isolated from the dermal granulomas of tuberculoid and lepromatous lesions.

Smear preparations of the single cell suspension from the dermal granulomas stained with Giemsa stain showed the presence of "large cells" and lymphocytes. The lymphocytes were significantly higher in the granulomas of the tuberculoid lesions in comparison with the lepromatous lesions ($p < 0.001$). The viability of these "large cells" and the lymphocytes was about 80%. These "large cells" showed a marked granular appearance by light microscopy in both types of granulomas (Fig. 1).

Lymphocytes. It is evident from Table 2 that 58% of the lymphocytes in the tuberculoid granulomas formed rosettes with sheep erythrocytes (E). In contrast, the lepromatous granulomas contained only a few E-rosette-forming lymphocytes. In addition, about 60% of the lymphocytes in the tuberculoid granulomas also showed the presence of esterase as dots in the cytoplasm. The lymphocytes did not form any rosettes with EAC in either of the granuloma types. The Ficoll-Hypaque purified peripheral blood mononuclear cells from the controls formed rosettes with EAC. The results suggest that the lymphocytes infiltrating the granulomas appear to be T lymphocytes.

Cells of mononuclear phagocyte series (MPS)

A high percentage of "large cells" from the tuberculoid granulomas was nonadherent to a plastic surface. In contrast, lepromatous granulomas contained a greater proportion of adherent "large cells" (Table 3).

About 80–90% of the "large cells" infiltrating the tuberculoid and lepromatous granulomas were esterase positive and exhibited peroxidase activity (Table 3, Fig. 2). Ziehl-Neelsen staining of the smears of the

TABLE 2. *Properties of lymphocytes in the granulomas.*

Properties	Tuberculoid patients		Lepromatous patients	
	Epithelioid cell granuloma	No. studied	Macrophage granuloma	No. studied
No. of viable lymphocytes per biopsy	$0.90 \times 10^6 \pm 0.14 \times 10^6$[a]	14	$0.30 \times 10^6 \pm 0.08 \times 10^6$[a,b]	15
Viability	80%	14	80%	15
E-rosettes (%)	57.6 ± 8.9[a]	5	1.2 ± 0.97[a]	5
EAC-rosettes	Not detectable	5	Not detectable	5
Nonspecific esterase (%)	Dots in the cytoplasm 60.4 ± 5.4[a]	5	Not detectable	5

[a] Values are mean ± standard error of the mean.
[b] $p < 0.001$, compared to number of viable lymphocytes from tuberculoid granulomas, Student's t test.

TABLE 3. *Properties of "large cells" in the granulomas.*

Properties	Tuberculoid patients		Lepromatous patients	
	Epithelioid cell granuloma	No. studied	Macrophage granuloma	No. studied
No. of viable "large cells" per biopsy	$0.39 \times 10^6 \pm 0.05 \times 10^6$[a]	14	$0.37 \times 10^6 \pm 0.09 \times 10^6$[a]	15
Viability	80%	14	80%	15
Appearance	Very granular	14	Very granular	15
Viable nonadherent "large cell" at 2 hr (%)	43.0 ± 4.4[a]	4	12.9 ± 2.7[b]	7
Nonspecific esterase	80–90%	5	80–90%	5
Peroxidase	80–90%	5	80–90%	5
Acid-fast bacilli	Not detectable	4	+	4
E-rosettes (%)	0	5	0	5
EAC-rosettes (%)	0	5	0	5

[a] Values are mean ± standard error of the mean.
[b] $p < 0.02$, compared to tuberculoid granulomas, Student's t test.

cell suspension showed the presence of acid-fast bacilli in the "large cells" of the lepromatous granulomas but not in the "large cells" of the tuberculoid granulomas. The "large cells" from both tuberculoid and lepromatous granulomas did not form any rosettes with sheep erythrocytes or EAC, suggesting that the "large cells" in the granulomas appear to be cells of the MPS.

DISCUSSION

The salient features of the present study are: a) Single cell suspensions prepared from tuberculoid and lepromatous granulomas contain lymphocytes and "large cells" (macrophages). The number of lymphocytes was significantly higher in the suspensions of tuberculoid granulomas in comparison to the suspensions of lepromatous granulomas. These "large cells" from both types of granulomas were granular in appearance by light microscopy. b) A high percentage of lymphocytes in the tuberculoid granulomas formed rosettes with sheep erythrocytes. In contrast, the lepromatous granulomas contained only a few E-rosette-forming lymphocytes. EAC-rosettes were not detectable in either type of granuloma. c) A high proportion of lymphocytes in the tuberculoid lesions showed the presence of esterase as dots in the cytoplasm. d) A high percentage of "large cells" from the tuberculoid granulomas was nonadherent to a plastic surface. In contrast, lepromatous granulomas contained a greater proportion of adherent "large cells." e) A high proportion of "large cells" from both the granulomas was esterase positive and exhibited peroxidase activity. However, these "large cells" did not carry C3 surface receptors. f) The "large cells" from the lepromatous granulomas showed the presence of *M. leprae* organisms, but the bacilli were not detectable in the "large cells" of the tuberculoid granulomas.

The present study was initiated to understand the properties of infiltrating cells in the dermal granulomas of leprosy. Preliminary experiments were carried out to determine the optimal concentrations of collagenase and the optimal time required for the isolation of cells. It was found that biopsies incubated with low concentrations of collagenase for a short time (30–45 min) gave quite a low yield of the isolated cells, and so a higher concentration of collagenase and longer incubation periods were required. A similar concentration of collagenase as described in the present study has been used in the isolation of cells from the dermal granulomas of mice infected with *Schistosoma mansoni* antigen ([1]). It is evident that single cell suspensions from tuberculoid granulomas contain significantly higher numbers of lymphocytes in comparison to the suspensions from lepromatous granulomas. A high percentage of lymphocytes from the tuberculoid granulomas formed rosettes with sheep erythrocytes. Further, these cells exhibited esterase dots in their cytoplasm which is known to be a marker of T cells ([5]). It was interesting that these lymphocytes did not form rosettes with EAC (Table 2). These findings suggest that

the lymphocytes derived from a tuberculoid granuloma appear to be T lymphocytes. Similar results have been reported on the *in situ* characteristics of infiltrating cells in the leprosy lesion by rosetting techniques [4] and by immunofluorescence with monoclonal antibodies [7, 9].

The suspensions from both the granulomas contain "large cells" in addition to lymphocytes. It is clear from the present study that a high percentage of these "large cells" from both the granulomas was esterase positive and exhibited peroxidase activity (Table 3). However, they did not form any rosettes with sheep erythrocytes. These features indicate that the "large cells" belong to cells of the MPS. A high proportion of "large cells" from the tuberculoid granulomas were nonadherent to a plastic surface, while the "large cells" from the lepromatous granulomas were adherent. Moreover, the "large cells" from the lepromatous granulomas showed the presence of *M. leprae* organisms, but this was not detectable in the "large cells" of tuberculoid granulomas, indicating that the "large cells" in the tuberculoid granulomas differ in some of their properties from similar cells of the lepromatous granulomas. One possible explanation for nonadherence of "large cells" to a plastic surface may be that the experiment was carried out in serum-free medium. As a control, we used peripheral blood mononuclear cells and allowed them to adhere for two hours in serum-free medium. These cells showed good adherence to the plastic surface.

It was interesting that the "large cells" from both types of granulomas did not possess C3 surface receptors. Some possible explanations for this could be: a) during the process of granuloma formation, these receptors may have been lost; b) the effect of lymphokines on these cells; c) the high concentration of collagenase used for the isolation of the cells. The last possibility is unlikely. As a control, we incubated a similar concentration of peripheral blood mononuclear cells (Ficoll-Hypaque purified) derived from adherent cells with the same concentration of collagenase for four hours. These cells displayed good C3 receptors, indicating that collagenase treatment does not alter the surface properties of cells. A similar phenomenon was observed in the murine system [1]. Lymphokines have been shown to affect the surface properties of macrophages [3]. Similar results have been reported on the loss of surface receptors in skin lesions of leprosy [13] and in experimental systems using mouse [6] and guinea pig [14] macrophages. Furthermore, all of these findings support the results of our previous studies on mycobacteria-induced granulomas in guinea pigs [8].

In summary, it can be seen from the present study that it is possible to obtain constituent cells of the granuloma in different types of leprosy as single cell suspensions. This should help in studying their properties in greater detail in order to understand the pathogenesis of the granulomas across the leprosy spectrum.

SUMMARY

Single-cell suspensions from the granulomas of leprosy cases were prepared for an *in vitro* study of the properties of the infiltrating cells. Biopsies from 44 untreated patients with tuberculoid and lepromatous leprosy were analyzed. The granulomas were found to contain lymphocytes and "large cells" (epithelioid cells and macrophages). The number of lymphocytes was significantly higher in the suspensions from the tuberculoid granulomas in comparison to the suspensions from the lepromatous granulomas. A high percentage of lymphocytes from the tuberculoid granulomas formed rosettes with sheep erythrocytes, and also showed the presence of esterase as dots in the cytoplasm. However, the lymphocytes did not form rosettes with EAC. Most of the "large cells" from both types of granulomas were esterase positive, exhibited peroxidase activity, and did not carry receptors for C3. A high percentage of "large cells" in the tuberculoid granulomas was nonadherent to a plastic surface, while the lepromatous granulomas contained a high proportion of adherent "large cells."

RESUMEN

Se prepararon suspensiones celulares a partir de los granulomas de casos de lepra para el estudio *in vitro* de las propiedades de las células infiltrantes. Se analizaron biopsias de 44 pacientes con lepra lepromatosa o tuberculoide. Se encontró que los granulomas contenían linfocitos y "células grandes" (células epitelioides y macrófagos). El número de linfocitos fue signi-

ficativamente mayor en las suspensiones de los granulomas tuberculoides que en las suspensiones de los granulomas lepromatosos. En los granulomas tuberculoides, un alto porcentaje de los linfocitos formaron rosetas con eritrocitos de carnero y mostraron la presencia de esterasa a manera de gránulos citoplásmicos únicos. Sin embargo, los linfocitos de estos granulomas no formaron rosetas EAC. La mayoría de las "células grandes" de ambos tipos de granulomas fueron esterasa positivas, exhibieron actividad de peroxidasa y no tuvieron receptores para C3. Mientras que los granulomas tuberculoides tuvieron un alto porcentaje de "células grandes" no adherentes, las "células grandes" adherentes fueron muy abundantes en los granulomas lepromatosos.

RÉSUMÉ

On a mis en suspension des cellules isolées provenant de granulomes de lèpre, en vue d'étudier *in vitro* les propriétés des cellules d'infiltration. On a également analysé les biopsies récoltées chez 44 malades non traités, atteints de lèpre tuberculoïde ou de lèpre lépromateuse. Ces études ont permis d'observer que les granulomes contenaient des lymphocytes et des "cellules de large dimension" (cellules épithélioïdes et macrophages). Le nombre de lymphocytes était significativement plus élevé dans les suspensions provenant de granulomes tuberculoïdes que dans celles obtenues à partir de granulomes de lèpre lépromateuse. Dans un pourcentage élevé de cas, les lymphocytes provenant de granulomes tuberculoïdes ont provoqué la formation de rosettes avec des érythrocytes de mouton; on a également pu mettre en évidence la présence d'estérase, sous forme de ponctuations dans le cytoplasme. Néanmoins, les lymphocytes n'ont pas provoqué de rosettes avec EAC. La plupart des "cellules de large dimension", dans l'un ou l'autre type de granulomes, étaient positives pour l'estérase; elles présentaient également une activité péroxydasique, et n'avaient pas de récepteur pour C3. Dans un pourcentage élevé des cas, les "cellules de large dimension" provenant de granulomes tuberculoïdes ne présentaient pas d'adhérence à des surfaces plastiques, alors que les granulomes lépromateux contenaient par contre une proportion élevée de "cellules de large dimension" adhérentes.

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