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The *In Situ* Characteristics of Mononuclear Cell Infiltrates in Dermal Lesions of Leprosy[1]

Sunil K. Gupta, Lalit K. Bhutani, and Indira Nath[2]

Investigations over the past two decades have focused attention on the complex immunological deficits which lead to the diverse clinicopathological features seen in human leprosy ([8, 25]). Infection with *Mycobacterium leprae* is manifested as an immunological spectrum ranging from the limited, high resistant, paucibacillary, tuberculoid leprosy to the disseminated, low resistant, multibacillary, lepromatous leprosy. Protective immunity as indicated by the absence of bacilli and localization of the lesion in tuberculoid patients has been shown to correlate with normal numbers of circulating T cells and integrity of general and antigen specific T cell functions ([8, 25]). Mainly lymphocytes and monocytes from the peripheral blood have been used for immunological studies, and scant information is available on the identity and functional properties of mononuclear cells in the tissue granulomas ([11, 20]). A single report by Ridley, Ridley and Turk ([20]) has described the presence of C3 and Fc receptors on the cells in dermal leprosy lesions. These authors were unable to positively identify T lymphocytes due to the failure of sheep erythrocytes to adhere to lymphocytes in the tissues.

Our laboratory has been studying the mechanisms involved in cell mediated immunity in human leprosy by means of circulating T cell functions *in vitro* ([14, 15, 16, 17, 23]). An understanding of the nature of the cells in the skin, a primary site of the disease, may be important in assessing the factors involved in local immunity and the elimination of intracellular pathogens. Leprosy lesions vary from epithelioid cell granulomas surrounded by dense infiltrations of lymphocytes in the tuberculoid end of the spectrum to foamy macrophage collections with intracellular bacilli and a conspicuous absence of lymphocytes in lepromatous leprosy ([19]). This disease forms an interesting, natural model for the study of the diverse varieties of granulomas elicited by the same pathogen.

In the present study, cryostat sections of dermal lesions of patients across the leprosy spectrum were layered with erythrocytes which had been conventionally treated to make the erythrocytes capable of identifying immunological surface markers on the mononuclear cells. The density of hemadsorption by such erythrocytes was graded. Sheep red blood cells (SRBC) were coated with AET (2-aminoethyl-isothiouronium bromide hydrobromide) to identify T cells ([10]); SRBC were treated with hemolysin and complement (EAC) to identify C3 receptors ([1]); and ox erythrocytes were coated with IgG anti-erythrocyte antibody (EA) to identify Fc receptors ([12]). Such indicator cells have been routinely used in rosette tests to identify lymphocytes and monocytes in cell suspensions ([1, 10, 12, 14]). In addition, the enzyme activity of the cells of the mononuclear phagocytic series (MPS) in the various granulomas was assessed by histochemical staining for nonspecific esterase ([26]).

MATERIALS AND METHODS

Patients and tissue material

Skin biopsies were obtained from 38 untreated patients across the leprosy spectrum. The patients were classified by the clinicopathological criteria of Ridley and Jopling ([19]), and consisted of 5 with polar tuberculoid leprosy (TT), 12 with borderline tuberculoid leprosy (BT), 5 with borderline leprosy (BB), 6 with borderline lepromatous leprosy (BL) and 10 with polar lepromatous leprosy (LL). Each skin bi-

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[2] S. K. Gupta, M.Sc., Department of Biochemistry, All India Institute of Medical Sciences, New Delhi-110029, India. Current address: Department of Pathology, Royal College of Surgeons of England, Lincoln's Inn, Fields, London WC2A 3PN, England; L. K. Bhutani, M.D., Department of Dermatology; Indira Nath, M.D., Department of Pathology, All India Institute of Medical Sciences, New Delhi-110029, India.

opsy was divided into two portions. One was collected fresh in cryopreservatives (Cryokwik, International Equipment Company, Needham Heights, Massachusetts, U.S.A.) and stored for one to two days at −20°C prior to testing with indicator cells for the *in situ* identification of mononuclear cells. The other was processed routinely by buffered formalin-fixed, paraffin embedded blocks and stained with hematoxylin eosin and Ziehl-Neelsen stains.

The skin biopsies from the 38 leprosy patients were graded on the Ridley-Jopling scale ([19]) by histological examination of the routinely processed, paraffin-embedded tissues. The *in situ* nature of the lymphocytes, epithelioid cells, and foamy macrophages in the dermal granulomas of the patients was mainly assessed by the adherence of sheep and ox erythrocytes suitably treated to identify T cells, and cells bearing C3 and Fc receptors. Only those cases which showed reproducibility of the results on serial sections were included. The localization of the indicator erythrocytes was further confirmed by examining serial sections stained by hematoxylin and eosin.

Nonspecific esterase

Cryostat sections were fixed in a buffered formalin-acetone mixture for 30 sec at 4°C, washed three times with distilled water and air dried. Esterase staining was done essentially by the method of Yam, *et al.* ([26]), using α-naphthyl acetate (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) as the substrate.

Preparation of indicator cells

Mononuclear cells in dermal lesions were identified by their ability to adhere to suitably treated sheep (SRBC) and ox erythrocytes (OE) for the identification of T cells and cells bearing receptors for C_3 and Fc. Reproducibility of localization of indicator cells on a minimum of three serial sections was used as a criterion for identification of the various cell types.

Indicator cells for the identification of T cells. AET-treated SRBC were prepared essentially by the method of Kaplan and Clarke ([10]). SRBC collected in Alsever's solution and stored at 4°C for one week or less were used. They were washed four times in 0.01 M phosphate-buffered saline (PBS) at pH 7.4. A 2% w/v solution of AET (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) was prepared in distilled water and its pH was adjusted to 9.0 with 5 N NaOH. Packed SRBC (0.4 ml) were mixed gently with 10 ml of 2% AET, and incubated at 37°C for 30 min with frequent shaking. AET-SRBC were subsequently washed four times with PBS, and resuspended to 5% v/v in RPMI 1640 supplemented with 25% heat-inactivated fetal calf serum (FCS) (GIBCO Laboratories, Grand Island, New York, U.S.A.), and stored at 4°C. These cells could be used within four days without further washing.

Indicator cells for the identification of Fc receptors. Ox erythrocytes coated with anti-ox RBC IgG antibody (EA) were used to identify Fc receptors ([12]). The antibody was produced in rabbits. The rabbits were immunized with an initial intramuscular injection of 10% ox RBC in complete Freund's adjuvant (GIBCO Laboratories, Grand Island, New York, U.S.A.), followed by weekly intravenous injections of 2% ox RBC for four to six weeks. After a test bleed to assess the titer of hemagglutinating antibody, the animals were bled to collect serum. IgG antibody was purified by ion exchange chromatography using a DEAE cellulose column. The purity of the antibody was assessed by immunoelectrophoresis using anti whole rabbit serum. The IgG antibody was used in the present study at a subagglutinating titer of 1:32.

EA were prepared as follows: Ox-RBC were collected in Alsever's solution and used up to one month at 4°C. They were washed three times with PBS, and resuspended to a 5% concentration. Equal volumes of a subagglutinating titer of IgG antibody and 5% ox-RBC were incubated for 1 hr at 37°C. The cells (EA) were washed three times with PBS and used for up to three days, when kept at 4°C. Prior to use the cells were washed once with PBS.

Indicator cells for the identification of C3 receptors

Essentially the method of Bianco and Nussenzweig was followed ([1]). SRBC were washed as above and resuspended to a 5% v/v concentration. Equal volumes of 5% SRBC and a 1:100 dilution (a subagglutinating titer) of rabbit anti-SRBC antibody

(GIBCO Laboratories, Grand Island, New York, U.S.A.) were incubated at 37°C for 30 min. The cells were washed three times with PBS and resuspended to the initial 5% concentration. An equal volume of 1:10 diluted fresh mouse serum was added to the antibody coated SRBC and incubated at 37°C for a further 45 min. The cells (EAC) were washed as above with PBS, stored at 4°C, and used within three days. The EAC were washed once prior to use.

Identification of mononuclear cells in tissue sections

Six μ cryostat sections were picked up on coverslips, air dried for 2 min, and washed in Hank's balanced salt solution (HBSS) (GIBCO Laboratories, Grand Island, New York, U.S.A.). All indicator cells were used at a 2% v/v concentration in HBSS and loaded in plastic plates with wells. The coverslips with cryostat sections were inverted over the wells in order to submerge the tissues in the indicator cell suspensions. Once the coverslips were fixed to all the wells, the plate was carefully inverted, so that the indicator cells would lie over the tissue sections. Plates containing EA and EAC suspensions were incubated at room temperature for 1 hr. Sections with AET-SRBC were left overnight at 4°C. At the end of the incubation, the plates were carefully turned over and left for 5 to 10 min. The coverslips were gently disengaged and washed several times in PBS. After microscopic examination to ensure the clearance of nonadhering erythrocytes, the tissues were fixed in 1% glutaraldehyde in PBS. All procedures for AET-SRBC treated sections were performed at 4°C.

In order to enhance the color contrast of the adherent indicator erythrocytes, the sections were treated with 1% benzedine in Tris-buffer, pH 7.4, for 30 min, and subsequently washed in PBS. All sections were stained with 1% toludine blue or hematoxylin and eosin. In addition, serial sections of every biopsy were stained for acid-fast bacilli by the Ziehl-Neelsen stain. Some sections were also stained for nonspecific esterase after treatment with the indicator cells.

The density of adherence of the indicator cells in the dermal granulomas was graded arbitrarily from 1+ to 6+. All observations were independently assessed by two workers.

RESULTS

Lymphocytes. The intensity of infiltration of granulomas by lymphocytes was most marked in the tuberculoid (TT, BT), and least in the lepromatous (LL) groups of patients. In borderline lepromatous (BL) biopsies, there was variability in the degree of lymphocytic infiltration. Two biopsies had dense collections of lymphocytes scattered in the dermis; whereas two others had few focal infiltrates only.

AET-SRBC ahered mainly to lymphocytes and occasionally to the basal layers of the epidermis. They were not seen in areas containing epithelioid cells and foamy macrophages. In general, it was noted that the intensity of lymphocytic infiltration in the skin correlated with the density of adherence of AET-SRBC. The maximal adherence of AET-SRBC was seen in polar tuberculoid leprosy where the erythrocytes were observed on the lymphocytes surrounding the periphery of the epithelioid cell granuloma (Figs. 1A, 1B and 2). In contrast, active polar lepromatous leprosy showed a uniform absence of adherence by AET-SRBC (Figs. 1H and 2). The skin sections of the borderline group of patients showed a graded decrease in the adherence of AET-SRBC which correlated with the numbers of lymphocytes in the lesions (Figs. 1C through 1G). Some skin biopsies of BB and BL showed two patterns of adherence. Some had small focal collections; whereas others had an increased density of AET-SRBC (Figs. 1D through 1G and 2).

Epithelioid cells and macrophages. EA and EAC adhered mainly to the cells of the mononuclear phagocyte series in the dermal lesions. Significant differences in the degree of adherence of the indicator red cells were not observed in the various types of leprosy. However, EAC adhered more densely than EA. This was noted in the serial sections of the same biopsy as well as in the leprosy groups as a whole (Fig. 2). A prominent feature in the larger circumscribed granulomas was the dense adherence of EAC and EA to the peripherally located epithelioid cells/macrophages. Little or no adherence was observed in the central areas. EA adherence was also uni-

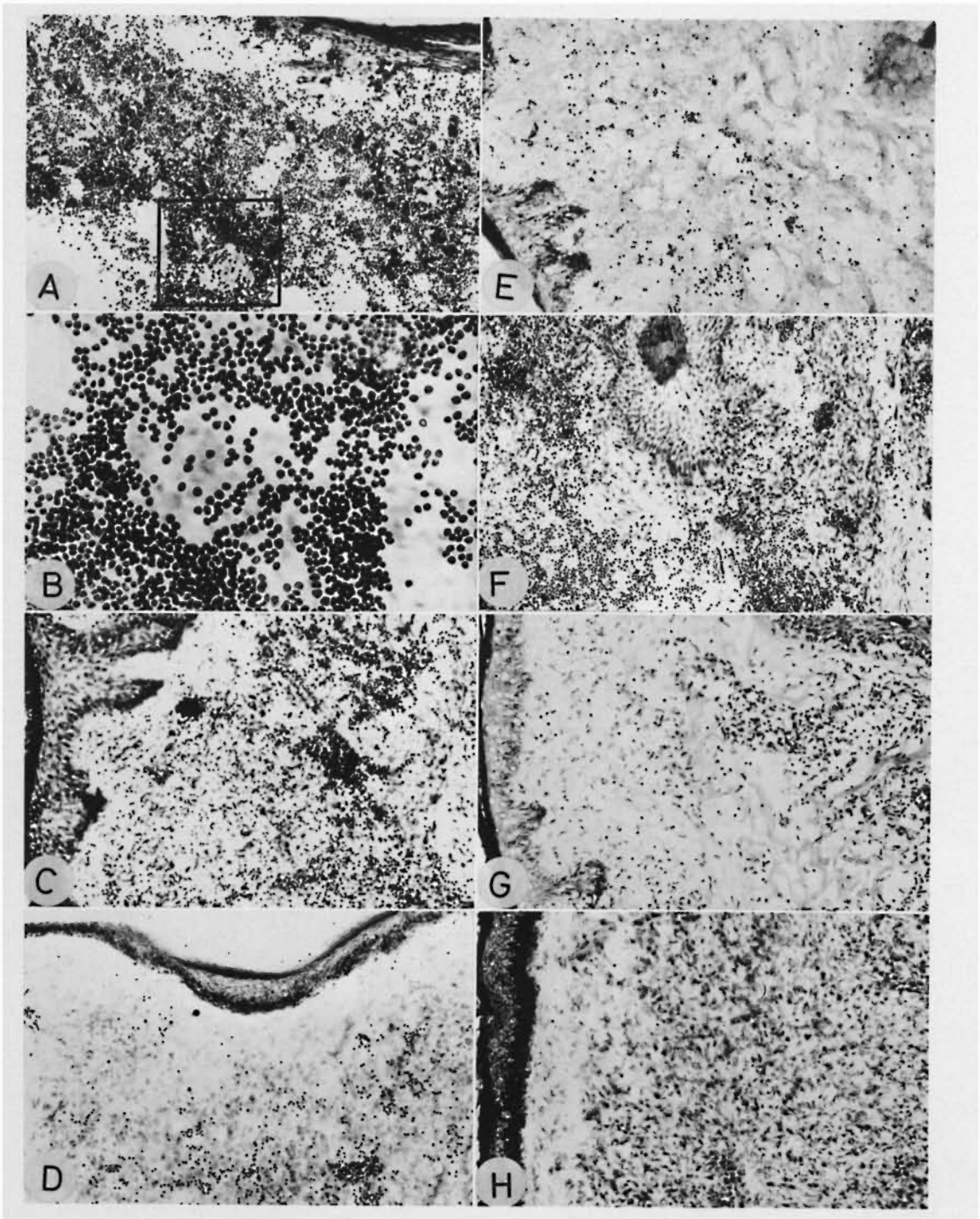

FIG. 1. Adherence of sheep erythrocytes (SRBC) to lymphocytic infiltrates of dermal lesions across the leprosy spectrum. Cryostat sections of skin biopsies were layered with AET-coated erythrocytes at 4°C, and subsequently stained with benzidine, followed by 1% toluidine blue. The quantity of T cell infiltration in the dermal lesions is visualized by erythrocyte adherence under low power magnification.

A. Polar tuberculoid leprosy (TT) showing maximal adherence of SRBC (6+) to lymphocytes around epithelioid cell granulomas (×120).

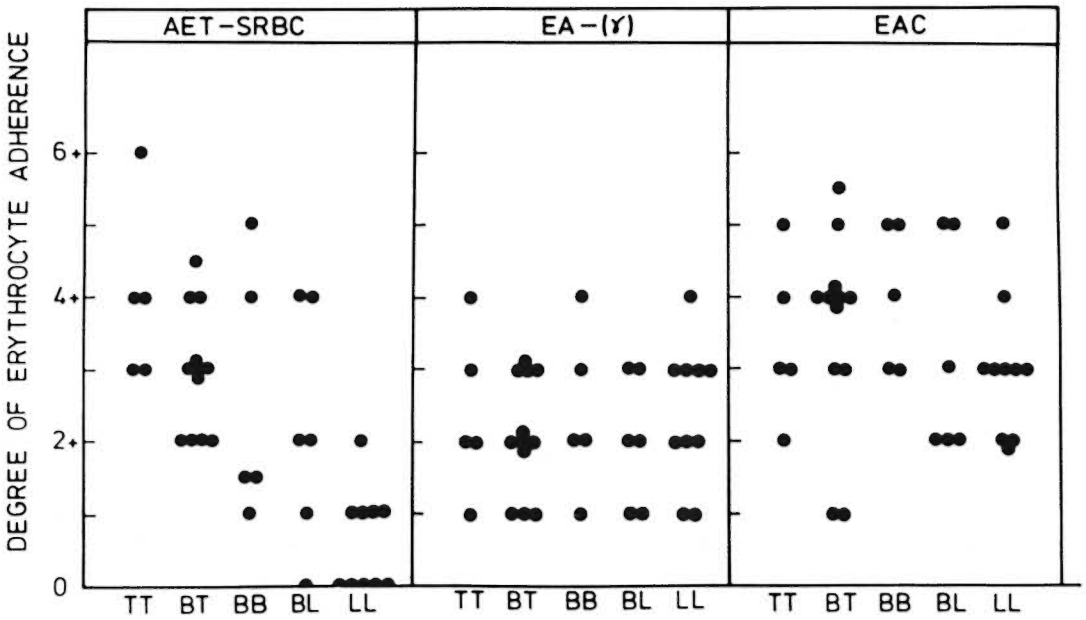

FIG. 2. Extent of infiltration by T cells, cells bearing receptors for the Fc portion of IgG, and C3 in the dermal lesions of 38 untreated leprosy patients as assessed by the degree of adherence of erythrocytes respectively treated with AET (AET-SRBC), IgG antibody (EA-γ), and hemolysin and complement (EAC).

TT = Polar tuberculoid leprosy, 5 patients studied;
BT = Borderline tuberculoid leprosy, 12 patients studied;
BB = Borderline leprosy, 5 patients studied;
BL = Borderline lepromatous leprosy, 6 patients studied;
LL = Polar lepromatous leprosy, 10 patients studied.

formly observed to the same degree in all types of leprosy. Thus, both the epithelioid cell granulomas of tuberculoid leprosy and the foamy cell macrophages containing acid-fast bacilli showed adherence to EA and EAC.

The presence of nonspecific esterase was noted to the same degree in all types of leprosy. However, the pattern of staining was heterogenous, and varied from intense diffuse staining in some cells to pale/negatively stained cells. In the larger granulomas, the central cells stained more strongly than the peripheral cells. In other granulomas, esterase negative cells closely interspersed with the positive cells were seen in the same areas. Some of the smaller granulomas, which had epithelioid cells as judged by the

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B. Higher magnification of area defined in A, showing central collections of pale epithelioid cells and peripheral localization of SRBC over the lymphocytic infiltrates (×300).

C, D. Borderline tuberculoid leprosy (BT) biopsies from two patients with varying degrees of dermal lymphocytic infiltration are depicted. C shows moderate adherence of SRBC (4+) to lymphocytes in a predominately epithelioid cell granulomatous lesion (×120). D depicts scattered collections of SRBC (2+) over the small numbers of lymphocytes in the lesion (×120).

E. Borderline leprosy (BB) showing focal collections of SRBC (1+) over the small numbers of lymphocytes in the dermal granulomas (×120).

F, G. Borderline lepromatous leprosy (BL). Dermal lesions from two patients with varying degrees of lymphocytic infiltration are depicted. F shows moderate numbers of SRBC (4+) localized to the lymphocytes in the granuloma (×120). G shows scattered SRBC (1+) adherent to the few lymphocytes in the lesion (×120).

H. Polar lepromatous leprosy (LL) showing occasional SRBC (0) over a predominantly foam cell macrophage granuloma devoid of lymphocytes (×120).

hematoxylin eosin stained serial sections, failed to stain for esterase. This was particularly observed in the smaller lesions at the tuberculoid end of the spectrum.

In some sections, esterase staining was done after processing for adherence by EA and EAC. In individual sections, no correlation was observed between esterase staining of cells and the degree of EA and EAC adherence to the cells.

DISCUSSION

The present investigation draws attention to the utility of using suitably modified erythrocytes for the identification of mononuclear cells in granulomas in general, and in the dermal lesions of leprosy in particular. In earlier years, the identification of T cells in tissues by means of overlaying SRBC met with mixed success ([7, 20, 21, 22]). Treatment of SRBC with AET increases the binding of sheep erythrocytes to T cells in blood ([10]) and in our hands gave uniformly successful results in the dermal lesions of leprosy. Reproducible localization of T cells by adherence of AET-SRBC was observed in serial sections of the same biopsy. The improvement obtained in tissues by AET and neuraminidase-treated sheep erythrocytes has also been reported by other workers ([2, 3]).

In general, it was observed that AET-SRBC adherence in the skin lesions correlated with the presence of lymphocytes in the granulomas. The highest degree of adherence, indicating the presence of T cells in the highest proportion, was uniformly observed in polar tuberculoid leprosy. Minimal or no adherence was seen in polar lepromatous leprosy. There was a graded reduction in the adherence of AET-SRBC across the leprosy spectrum, and variability in the density of the indicator cells was seen in individual patients in borderline leprosy. Thus, it would appear that the predominant lymphocyte in the leprosy lesion is a T cell. These T cells are mostly associated with granulomas of the epithelioid cell variety as in tuberculoid, borderline tuberculoid, and in some patients with borderline leprosy. A paucity of T cells was observed in the foamy macrophage granulomas of lepromatous leprosy. These results are in conformity with immunological studies of cellular immunity in leprosy ([8, 15, 25]). However, two patients with borderline leprosy, which is known to have poor T cell functions, showed significant focal areas of adherence of AET-SRBC indicating thereby the presence of T cells in the skin. Whether these focal collections of T lymphocytes are due to infiltration by a subset of T cells not involved in the expression of cell-mediated immunity or due to suppressor mechanisms operative in this disease ([16, 17]) cannot be answered by the present study.

The cells of the mononuclear phagocyte series (MPS) in the granulomas across the leprosy spectrum appear to bind uniformly to EAC and EA, thus indicating the presence of C3 and Fc receptors, respectively. Individual variability in the degree of adherence was observed in general over the whole spectrum. Ridley, *et al.* ([20]) have reported an absence of Fc receptors on the epithelioid cells of tuberculoid leprosy. This is in contrast to our present studies in which Fc receptors were uniformly found in tuberculoid and lepromatous lesions. These workers appeared to have used unpurified hemolysin to coat the indicator cells and this may not adequately demonstrate the Fc receptors on the cells of the mononuclear phagocytes series. Using the purified IgG fraction of anti-ox erythrocyte antibody to demonstrate the Fc receptors, we did not observe any significant differences in Fc receptor density on macrophages across the leprosy spectrum. It is interesting to note that both paucibacillary epithelioid cells and bacilli-loaded foamy macrophages had C3 and Fc receptors. Moreover, in large granulomas of both types, cells bearing a higher density of these receptors were more peripherally located. This was not due to technical variability since the red cells were uniformly distributed over the tissue section during the incubation period. It may be possible that the receptor density is related to the maturity of the cells of the mononuclear phagocytic series in the granulomas. Experimental studies over the years have indicated that young monocytes from the peripheral blood reach the tissues and mature into macrophages in an inflammatory focus ([5, 24]). Thus, the peripheral localization of cells with a high density of re-

ceptors may be related to the localization of younger macrophages in the peripheral areas of the granulomas.

Further confirmation of the heterogeneity of the cells of mononuclear phagocyte series which form the granulomas came from the studies of nonspecific esterase activity. Similar to the above results, the presence of nonspecific esterase was uniformly observed in the lesions across the leprosy spectrum. This is in conformity with earlier studies on lysosomal enzyme activity in leprosy lesions ([9]). However, the granulomas *per se* consisted of a variety of cells, from intensely staining to negatively staining epithelioid/foamy macrophages. The smaller granulomas contained fewer positive cells, and the larger granulomas had more positive cells in the center of the lesion. *In vitro* studies on cultures of human blood derived macrophages from our laboratory had shown increasing levels of activity of nonspecific esterase over a three-week period ([18]). Thus, these differences in enzyme activity and receptor density may indicate that the cells of the mononuclear phagocyte series in the different regions of the leprosy granulomas are in varying stages of maturity. These features do not appear to be related to the presence of T cells or to the presence of intracellular bacilli, since they were uniformly observed in all types of granulomas. Recent reports on experimental BCG and *M. leprae*-induced granulomas in guinea pigs ([13]) and tubercular granulomas in rabbits ([6]) have also shown varying degrees of enzyme activity in the different regions of the granulomas.

In conclusion, it would appear that the quantity of T cells in the dermal lesions of leprosy constitutes the single major factor for the diversity in the granulomas across the leprosy spectrum. Moreover, the presence of large numbers of T cells was observed to be associated with phagocytic cells capable of eliminating *M. leprae* (*i.e.*, epithelioid cells). Their absence was uniformly observed in the granulomas of lepromatous leprosy consisting of bacilli-laden foamy macrophages. The C3 and Fc receptor densities and enzyme content of the mononuclear phagocytes showed a heterogeneity among the individual cells of the granulomas but there were no significant differences related to the leprosy spectrum. Thus, the functional differences between epithelioid cells and foamy macrophages may be related to T cell influences which are too subtle to be distinguished by the presently available methodologies.

SUMMARY

The *in situ* nature of mononuclear cell infiltrates in the dermal lesions of 38 untreated leprosy patients was studied by means of conventional surface marker techniques using erythrocytes coated with AET, anti-erythrocyte IgG antibody, hemolysin and complement for the identification of T cells, and cells bearing Fc and C3 receptors, respectively. In general, T cells were the predominant lymphocytes in the leprosy lesions. They were mostly seen to be associated with epithelioid cell granulomas and showed maximal density in tuberculoid leprosy. A graded reduction of T cells was observed in borderline leprosy with a severe reduction/absence in polar lepromatous leprosy. The cells of the mononuclear phagocyte series in the various granulomas of the leprosy spectrum showed the presence of Fc and C3 receptors. Cells bearing a higher density of these receptors had a peripheral localization; whereas cells showing diffuse staining for nonspecific esterase were located more in the central regions of the granulomas. The differences in the individual cells of the phagocytic series appeared to be related to cell maturity; whereas the quantity of T cell infiltration in the lesions showed a correlation with the leprosy spectrum.

RESUMEN

Se estudió la naturaleza de los infiltrados de células mononucleares en las lesiones dérmicas de 38 pacientes con lepra sin tratamiento. Se utilizaron eritrocitos AET, eritrocitos sensibilizados con hemolisina IgG, y eritrocitos sensibilizados con hemolisina y con complemento, para identificar a los linfocitos T, a las células con receptores para Fc y aquellas con receptores para Fc y C3, respectivamente. En general, las células T fueron los linfocitos predominantes en las lesiones leprosas. Estas células se vieron asociadas con los granulomas de células epitelioides y mostraron su máxima densidad en la lepra tuberculoide. En la lepra intermedia (BB) se observó una reducción gradual en el número de células T mientras que en la lepra lepromatosa polar éstas estuvieron severamente disminuí-

das o ausentes. Las células de la serie fagocítica mononuclear en los diversos granulomas del espectro mostraron la presencia de receptores para Fc y C3. Las células con una alta densidad de estos receptores tuvieron una localización periférica mientras que las células con tinción difusa de esterasa se localizaron en las regiones más centrales de los granulomas. Las diferencias en las células individuales de la series fagocítica parecieron estar relacionadas con la maduración celular mientras que la cantidad de células T infiltrantes en las lesiones mostraron una correlación con el espectro de la lepra.

RÉSUMÉ

En vue d'étudier *in situ* la nature des infiltrats à cellules mononucléaires dans les lésions du derme, chez 38 malades atteints de lèpre non traitée, on a eu recours à des techniques faisant appel à un marqueur conventionnel de surface. Ce marqueur était basé sur l'utilisation, d'une part, d'érythrocytes recouverts d'AET, d'anticorps IgG anti-erythrocytes, d'hémolysine, et du complément pour l'identification des cellules T, et d'autre part, des cellules portant respectivement les récepteurs Fc et C3. En général, les cellules T représentaient le type de lymphocytes prédominant dans les lésions de lèpre. L'observation a montré qu'ils étaient principalement associés aux granulomes à cellules épithélioides, et qu'ils présentaient une densité maximale dans la lèpre tuberculoide. Une réduction progressive des cellules T a été observée dans la lèpre dimorphe, alors que dans la lèpre lépromateuse polaire on notait leur diminution très marquée ou même leur absence. Les cellules de la série phagocytaire mononucléaire présentaient dans les divers granulomes du spectre clinique de la lèpre, des récepteurs Fc et C3. Les cellules qui témoignaient de la densité la plus élevée de ces récepteurs étaient localisées périphériquement, alors que les cellules montrant une coloration diffuse pour l'estérase non spécifique étaient situées davantage dans les régions centrales des granulomes. Les différences notées dans les cellules individuelles de la série phagocytaire semblaient être en relation avec la maturité des cellules, alors que la quantité de l'infiltration à cellules T dans les lésions présentait de son côté une corrélation avec le sprectre de la lèpre.

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