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Defective Monocyte Chemotaxis in Active Lepromatous Leprosy[1]

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Lepromatous leprosy (LL) is characterized by a large number of tissue macrophages laden with *Mycobacterium leprae* as well as several immunologic abnormalities. These include deficiencies of macrophage-activating cytokines as well as interleukin-2 (IL-2) and gamma interferon (γ-interferon) ([6, 7, 14]), a deficiency of OKT4/Leu3 positive helper T cells in lepromatous lesions ([19]), and cell migration abnormalities ([1, 3, 11, 20, 21]).

Previous studies have reported a deficiency in neutrophil chemotaxis secondary to a serum chemotaxis inhibitor in multibacillary patients with active disease ([20, 21]). Masuda and Scheinberg ([11]) have reported diminished monocyte chemotaxis to a lymphocyte-derived chemotactic factor in both lepromatous and tuberculoid patients compared to normal controls. In addition, Azulay ([1]) reported a decrease in monocyte chemotaxis to casein in four LL patients compared to normal controls.

This study was designed to observe the relationships between chemotaxis, bacterial index (BI), therapy, and serum antibodies to the *M. leprae*-specific phenolic glycolipid-I (PGL-I) antigen in a large, well-defined population of leprosy patients. A newly designed 48-well chemotaxis chamber combined with the use of image analysis makes a suitable system for clinical research on chemotaxis.

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MATERIALS AND METHODS

Patient description and disease classification. All patients were enrolled in the New York Regional Hansen's Disease Program. Over half the patients were of Hispanic origin from the Caribbean; approximately one third were Asian ([10]). All patients were treated with multidrug regimens ([8]). Leprosy patients were clinically and histologically classified according to the Ridley-Jopling scale ([16]). The BI was measured on a semiquantitative scale (0–6+) approximating that of Ridley ([15]). Histology (including BI) was reported from the GWL Hansen's Disease Center, Carville, Louisiana, U.S.A., by two pathologists (C. K. Job, J. A. Freeman) whose agreement on BI was good ($r = 0.94$). Patients with a BI of 1+ or greater are referred to as bacillary active.

Monocyte/macrophage cell isolation. Blood was drawn from healthy donors and leprosy patients on the same day in heparinized tubes. Isolation of mononuclear cells was by the method of Boyum ([2]). Blood was layered over Ficoll-Paque (Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey, U.S.A.) and centrifuged. The interphase cell layer was removed and washed three times with RPMI 1640 media with glutamine (GIBCO, Grand Island, New York, U.S.A.), penicillin (100 IU/ml), streptomycin (100 μg/ml), and gentamicin sulfate (0.5 ml per 100 ml media). Viability was better than 95% as measured by trypan blue dye exclusion. Total leukocyte recovery was 1×10^6 to 3×10^6 cells per ml of whole blood. Differential counts were made after staining the cell suspension with acridine orange (Fisher Scientific Co., Fair Lawn, New Jersey, U.S.A.). Fifty μl of the cell suspension with a monocyte concentration of approximately 2×10^6 ml was mixed with 10 μl of acridine orange (0.3 mg/ml) in phosphate-buffered saline (PBS), and was allowed to incubate at room temperature for

about 5 min. Ten μl was then placed on a microscope slide, a coverslip was added, and the cells were observed under a fluorescence microscope. Monocytes were distinguishable by their bright red cytoplasmic fluorescence (300 cells counted), and the numbers thus determined were used to standardize the monocyte number added per well. The results of this method correlated closely with the counting of monocytes by other criteria: a) Differential counts determined on whole blood smears stained with Wright stain (Harleco, Philadelphia, Pennsylvania, U.S.A.), and b) comprehensive blood stain CBC using a Coulter Counter model S plus IV and V (Coulter Electronics, Inc., Hialeah, Florida, U.S.A.). The cell suspensions usually contained 15% to 35% monocytes, 65% to 85% lymphocytes, and less than 1% granulocytes.

Chemotaxis assay. The chemotaxis assay was done in a multiwell chamber as described by Falk, *et al.* ([5]). The bottom wells were filled with 25–30 μl of the attractant solution diluted in Gey's balanced salt solution (GBSS). A nucleopore filter sheet (polyvinyl pyrrolidone coated, 10 μm thick, with a 5 μm pore size; Neuroprobe, Inc., Bethesda, Maryland, U.S.A.) was placed over the wells. A gasket was placed over the filter followed by the top plate. Fifty μl of the cell suspension adjusted to contain 100,000 monocytes was added to each top well. The chamber was then incubated for 90 min in moist air containing 5% CO_2. After the incubation period, the filter was removed and the residual cells remaining on the top side of the filter were wiped off. The filter was fixed in methanol and stained in Diff Quick (American Scientific Products, McGaw Park, Illinois, U.S.A.). The migrated cells on the filter sheets were evaluated by means of an Optomax image analyzer (Optomax, Inc., Hollis, New Hampshire, U.S.A.). The number of migrated cells was measured in five fields per triplicate using a 40× objective, and the mean number of cells per mm^2 of the filter was calculated. The coefficient of variation within the five fields was in the range of 0.10–0.24. The coefficient of variation among the triplicate wells was always less than 0.25. In order to compare the chemotactic response of patients with healthy controls, chemotaxis is expressed as:

$$\frac{\text{mean number of patient monocytes/mm}^2\text{ of filter}}{\text{mean number of control monocytes/mm}^2\text{ of filter}} \times 100$$

The data are also expressed as mean number of cells per mm^2 of the filter (Fig. 3).

Chemotactic factor. N-Formylmethionyl-leucyl-phenylalanine (FMLP), a synthetic peptide, was purchased from Miles-Yeda Ltd., Miles Laboratories, Elkhart, Indiana, U.S.A.). A stock solution (10^{-4} M) was prepared and stored at −20°C. The dilution which gave optimal chemotaxis is 10^{-8} M (data not shown).

Effect of serum on chemotaxis. Serum, undiluted or diluted to 20% in GBSS, from borderline tuberculoid (BT) and LL patients with and without erythema nodosum leprosum (ENL) and healthy controls was preincubated with cells for 2 hr at either room temperature or 37°C. Total viable cell counts, using a hemocytometer and trypan blue, as well as monocyte counts by acridine orange fluorescence were done before and after the 2-hr incubation. The monocyte count was adjusted to 2×10^6 monocytes/ml prior to assay.

Assay for IgM antibodies to PGL-I. IgM antibodies to PGL-I were assayed as previously described ([9]). PGL-I (armadillo-derived PGL-I was kindly provided by P. J. Brennan), sphingomyelin, cholesterol, and dicetyl phosphate (0.1:2.0:1.5:0.2, molar ratio) were combined, and the lipid mixture was sonicated in Tris-buffered saline. Control liposomes without PGL-I were prepared as above. Flat-bottomed polystyrene microtiter plates (Dynatech, Springfield, Virginia, U.S.A.) were coated by incubation with 100 μl of the PGL-I-liposome suspension (diluted to 2.5 μg PGL-I/ml in Tris-buffered saline) or with the control-liposome suspension for 18 hr at 37°C. After washing three times with PBS, the plates were incubated (for 1.5 hr at 37°C) with 200 μl of 3% (w/v) bovine serum albumin (BSA; Sigma Chemical Company, St. Louis, Missouri, U.S.A.) in PBS (BSA-PBS). BSA-PBS was replaced by 100 μl of test serum (in duplicate) diluted 1:20 in BSA-PBS. A positive lepromatous serum pool was included as reference on each plate. After incubation with test serum for 1.5 hr at 37°C, the plates were again washed three times with PBS,

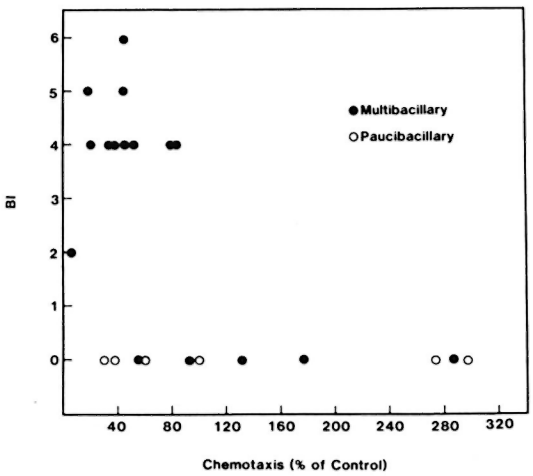

FIG. 1. Chemotaxis, expressed as percent of healthy controls, versus bacterial index; ○ = paucibacillary, ● = multibacillary ($p < 0.05$). For this study, multibacillary are BL and LL patients as defined by the Ridley-Jopling classification ([16]) and paucibacillary are BT and BB patients without regard to bacterial index.

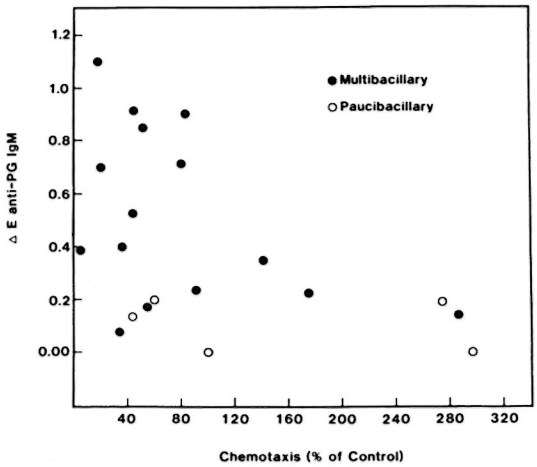

FIG. 2. Chemotaxis, expressed as percent of healthy controls, versus serum antibody levels of anti-PGL-I IgM; ○ = paucibacillary, ● = multibacillary ($p < 0.05$).

and incubated (for 1.5 hr at 37°C) with 100 μl of peroxidase-conjugated goat anti-human IgM (Cappel Laboratories, Downington, Pennsylvania, U.S.A.) diluted 1:1000 in BSA-PBS. After washing three times with PBS, 100 μl of substrate solution containing 1.8 mM 2, 2′-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Boehringer Mannheim Corp., New York, New York, U.S.A.) and 0.1 mM H_2O_2 in 0.1 M phosphate buffer, pH 7.0, was added for 1 hr at room temperature. The reaction was stopped with 100 μl of 0.32% (w/v) NaF, and the extinction (E) was read at 405 nm using a Titertek microtiter plate reader. The results were expressed as E = E(PGL-I-liposome coat) − E(control-liposome coat).

RESULTS

Patient monocyte chemotaxis. There was a significant inverse correlation ($p < 0.05$) between chemotaxis, as measured by percent of control, and the BI (N = 22) (Fig. 1). Our initial data showed that the higher the BI, the greater the reduction in the ability of the patient's monocytes to respond to the chemotactic factor FMLP. In addition, there was also a significant inverse correlation ($p < 0.05$) between chemotaxis and serum levels of anti-PGL-I IgM antibodies (N = 20) (Fig. 2).

Subsequent to our initial findings, we increased the number of patients studied including patients receiving thalidomide. Our data show a significant ($p < 0.001$) increase in monocyte chemotaxis in bacteriologically active patients (BI = 1–5) receiving thalidomide compared to the non-thalido-

THE TABLE. *Monocyte chemotaxis and anti-PGL-I IgM in thalidomide-treated and untreated patients (mean ± S.D.).*

Treatment	BI = 0		BI = 1–5	
	CTX[a]	PGM[b]	CTX	PGM
Thalidomide (N = 13)	137.5 ± 81.9	0.40 ± 0.24[c]	200.8 ± 113.6[d]	0.21 ± 0.6[e]
No thalidomide (N = 20)	139.5 ± 102	0.17 ± 0.1	42.0 ± 23.8	0.6 ± 0.3

[a] CTX = monocyte chemotaxis.
[b] PGM = serum levels of anti-PGL-I IgM.
[c] $p < 0.05$ (anti-PGL-I IgM; BI = 0) thalidomide vs non-thalidomide treated.
[d] $p < 0.001$ (CTX; BI = 1–5) thalidomide vs non-thalidomide treated.
[e] $p < 0.01$ (anti-PGL-I IgM; BI = 1–5) thalidomide vs non-thalidomide treated.

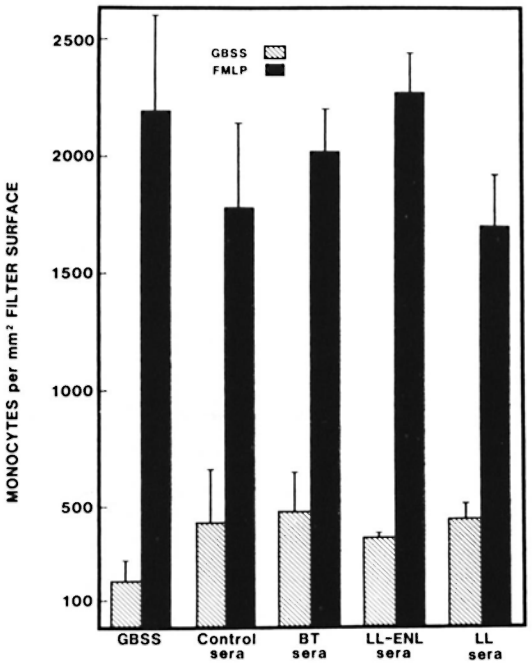

FIG. 3. Normal monocytes were incubated in either GBSS or undiluted sera from healthy controls or patients (three pooled sera per group) at room temperature for 2 hr prior to the assay. GBSS and FMLP are the chemotactic factors; bars represent the mean ± S.D. of triplicate wells of individual sera. (This figure is representative of the 18 sera tested.)

mide-treated group (The Table). There was no difference in the chemotaxis between the thalidomide- and non-thalidomide-treated group when the BI = 0. In addition, serum levels of anti-PGL-I IgM in the thalidomide-treated group were significantly ($p < 0.05$) elevated compared to the thalidomide-treated group when the BI = 0. However, when the BI = 1–5, the serum levels of anti-PGL-I IgM were significantly ($p < 0.01$) decreased in the thalidomide-treated group compared to the non-thalidomide-treated group.

Effect of serum on monocyte chemotaxis. We did not find a decrease in the chemotaxis of monocytes from healthy controls when preincubated with serum from leprosy patients. Of 18 sera tested, including LL with ENL, LL with high serum anti-PGL-I IgM, BT, and controls, there was no significant decrease in monocyte migration to FMLP (chemotaxis) or GBSS (random movement) (Fig. 3). Neither the serum dilution (undiluted or diluted 20% in GBSS) nor the temperature for preincubation (room temperature or 37°C) altered monocyte migration. The chemotactic response of the monocytes of BT serum donors was not significantly different from the chemotactic response of healthy controls. In contrast, the chemotactic response of the monocytes of the LL-ENL and LL with high serum anti-PGL-I IgM serum donors was significantly reduced ($p < 0.05$) compared to the control chemotactic response (data not shown).

Incubation of normal cells in certain sera at either room temperature or 37°C decreased the total cell yield and selectively decreased the percentage of monocytes post-incubation. Monocyte yields were 50% lower after incubation with either sera from LL-ENL patients or from patients with high anti-PGL-I IgM when compared to monocyte yields after incubation in GBSS, control, or BT serum. Thus, all incubated cells were adjusted to the same monocyte concentration for the chemotaxis assay.

DISCUSSION

Our study shows a significant decrease in the ability of peripheral blood monocytes from bacteriologically active LL patients not treated with thalidomide to migrate *in vitro* when compared to normal healthy controls and patients with a BI of 0. The serum level of anti-PGL-I IgM was significantly decreased in patients taking thalidomide (BI = 1–5) compared to the non-thalidomide group. This is probably due to ENL and/or thalidomide treatment. Patients (BI = 1–5) taking thalidomide demonstrated significantly higher chemotaxis activity than patients with the same BI not taking thalidomide. In keeping with these findings, recent work by Nielsen and Bennike ([13]) showed that monocyte chemotaxis was enhanced after 1 week of thalidomide treatment. These findings may also relate to the ability of thalidomide to augment macrophage function as shown by the thalidomide-induced enhancement of IgM antibody synthesis in mice to DNP-Ficoll ([17]).

In contrast to the work of Campbell, *et al.* ([4]), we did not find a decrease in the chemotaxis of monocytes from healthy controls after preincubation with serum from

18 patients. The reasons for the differences in observations on the effect of sera are not known. Patient variation is possible, as are differences in the assay procedure. Since cell number is important in the assessment of chemotaxis, a difference in the final absolute number of monocytes is an additional possible difference between the studies. Our findings are similar to those of Smith, *et al.* ([18]) who found defective monocyte chemotaxis in seven patients with acquired immune deficiency syndrome (AIDS). Five of these 7 patients had Kaposi's sarcoma, and 6 of the 7 had opportunistic infections, including 3 with *M. avium-intracellulare* infections. Sera from these AIDS patients did not contain a factor that inhibited monocyte function.

A variety of lymphokine deficiencies have been found in lepromatous leprosy, including a decrease in IL-2 and γ-interferon ([7]) and a decrease in monocyte-activating lymphokines in concanavalin A (ConA) stimulated supernatants ([14]). However, circulating monocytes of leprosy patients have been found to be intrinsically intact for their ability to diminish the growth of intracellular legionella when activated by leukocyte culture supernatants containing monocyte-activating lymphokines ([8]). More recently, Nathan, *et al.* ([12]) have found an intrinsically low level of H_2O_2 production in the circulating monocytes of lepromatous leprosy patients prior to a clinical trial with low doses of recombinant γ-interferon. The H_2O_2 production of the circulating monocytes normalized during the administration of as little as 1 μg to 10 μg of recombinant γ-interferon. Defective chemotaxis in active lepromatous patients may represent an additional intrinsic abnormality of circulating monocytes in lepromatous leprosy. Alternatively, one could argue that neither diminished H_2O_2 production nor diminished chemotaxis is an intrinsic monocyte defect since both are known to be lymphokine dependent (γ-interferon and lymphocyte-derived chemotactic factor, respectively) and that the circulating monocytes are lethargic due to *in vivo* lymphokine depletion. Regardless of the mechanism, diminished chemotaxis represents a functional deficiency of circulating monocytes in active lepromatous leprosy and could play a significant role in the pathogenesis of leprosy infections.

SUMMARY

This study of monocyte chemotaxis in leprosy patients showed a significant inverse correlation ($p < 0.05$) of chemotaxis and the bacterial index (BI) ($N = 22$). In addition, there was a significant inverse correlation ($p < 0.05$) between chemotaxis and the serum levels of anti-phenolic glycolipid-I IgM antibodies ($N = 20$). Patients taking thalidomide who had a BI ≥ 1 had a significantly greater ($p < 0.001$) chemotaxis response than that of patients with the same BI who were not taking thalidomide. No significant decrease in chemotaxis of monocytes from healthy donors was observed when the cells were pre-incubated with serum from 18 leprosy patients. We conclude that monocytes from patients with active lepromatous leprosy not receiving thalidomide have an intrinsic abnormality when assessed by chemotaxis.

RESUMEN

Este estudio sobre la quimiotáxis de monocitos en pacientes con lepra mostró una significante correlación inversa ($p < 0.05$) entre quimiotáxis e índice bacteriano (IB) ($N = 22$). Además, también hubo una significante correlación inversa entre la quimiotáxis y los niveles séricos de anticuerpos IgM contra el glicolípido fenólico-I ($N = 20$). Los pacientes bajo tratamiento con talidomida que tenían un IB ≥ 1 tuvieron una respuesta quimiotáctica significativamente mayor ($p < 0.001$) que los pacientes con el mismo IB que no estaban tratados con talidomida. No se observó una disminución significativa en la quimiotáxis de monocitos de donadores sanos cuando las células se preincubaron con sueros de 18 pacientes con lepra. Se concluye que los monocitos de pacientes con lepra lepromatosa activa que no estan bajo tratamiento con talidomida tienen una anormalidad intrínseca en su actividad quimiotáctica.

RÉSUMÉ

Cette étude de la chimiotaxie des monocytes chez des malades de la lèpre a mis en évidence une corrélation négative significative ($p < 0{,}05$) de la chimiotaxie et de l'index bactériologique (BI) dans un échantillon de 22 malades. De plus, on a observé une corrélation négative significative ($p < 0{,}05$) entre la chimiotaxie et les taux sériques des anticorps IgM contre l'antigène glycolipide phénolique-I, dans un échantillon de 20 malades. Les malades qui étaient traités par

la thalidomide, et qui avaient un BI ≥ 1, présentaient une réponse chimiotactique significativement plus élevée ($p < 0{,}001$) que ceux ayant le même Index Bactériologique, mais qui ne prenaient pas de thalidomide. Aucune diminution significative n'a été observée dans chimiotaxie des monocytes provenant de donneurs sains, lorsque les cellules étaient soumises à une incubation préalable dans du sérum obtenu chez 18 malades de la lèpre. On peut en conclure que les monocytes des malades souffrant de lèpre lépromateuse active et qui ne reçoivent pas de thalidomide, présentent des anomalies intrinsèques lorsqu'on étudie leur chimiotaxie.

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