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Polymorphonuclear Cell Function in the Various Polar Types of Leprosy and Erythema Nodosum Leprosum

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Polymorphonuclear leukocyte motility, both in vivo and in vitro, and reduction of Nitro Blue Tetrazolium was studied in tuberculoid and lepromatous leprosy patients and a group of lepromatous patients with erythema nodosum leprosum (ENL). A profound defect in random migration, chemotaxis, and chemokinesis was found in lepromatous patients with and without complicating ENL, and marked depletion of skin window migration confirmed these in vitro findings. Tuberculoid patients exhibited a mild defect in polymorphonuclear leukocyte motility. Serum inhibitors of normal polymorphonuclear leukocyte chemotaxis were found in all types of leprosy, but sera from lepromatous and ENL patients were most inhibitory. Resting levels of Nitro Blue Tetrazolium reduction were normal in all three groups. Reconstitution of polymorphonuclear leukocyte cells from normal and ENL patients with ENL serum, however, showed increased Nitro Blue Tetrazolium reduction well above the normal range, whereas reconstitution with normal, lepromatous, and tuberculoid sera failed to increase Nitro Blue Tetrazolium reduction above the normal values.

Impaired cell-mediated immune responses both in vivo and in vitro are well documented in lepromatous leprosy (3, 7, 9, 17, 21). Defective macrophage phagocytosis in lepromatous leprosy has been reported previously (2), but these findings have been disputed by Drutz et al. (5), who reported normal phagocytic and antimicrobial activities in the monocytes, macrophages, and polymorphonuclear leukocytes (PMN) in patients with tuberculoid and lepromatous leprosy.

Defective neutrophil motility in Boyden chambers and markedly decreased accumulation of PMN in Rebuck skin windows have been described in certain infections associated with an absence of delayed hypersensitivity responses (1, 20). Bullock et al. (4) have reported depressed mobilization of leukocytes into an area of skin abraded to induce a nonspecific inflammatory response in patients with leprosy. Furthermore, Ward et al. (22) have described the presence of an inhibitor which inactivates various leukoattractants in the serum of patients with lepromatous leprosy. Random migration and chemotactic responses of neutrophils of patients with the various types of leprosy and erythema nodosum leprosum (ENL) have been incompletely described. This paper examines in detail random motility, stimulated random motility (chemokinesis), and true directional motility (chemotaxis) of neutrophils from patients with tuberculoid and leptromatous leprosy and complicating ENL. Nonactivated sera from patients were also tested for spontaneous chemotactic activity against autologous neutrophils. The effect of sera from each patient group on the locomotor responses of normal PMN cells was also evaluated.

Analysis of these effects has been made possible by the advent of the technique of Zigmond and Hirsch (24) which employs varying concentrations of chemotactic factor above and below the filter, enabling dissociation of random from truly directional motility. The semiquantitative reduction of Nitro Blue Tetrazolium (NBT) was also evaluated.

MATERIALS AND METHODS

Patients and controls. Patients were South African Negroes hospitalized at Westfort leprosarium, which is situated 40 miles from the laboratory of investigation. All investigations began within 90 min postvenesection.

Patients were classified both clinically and histologically, by the Ridley and Jopling (14) classification, into five groups: true tuberculoid, borderline tuberculoid, borderline, borderline lepromatous, and true lepromatous. For this investigation patients were divided into two main groups. Patients designated as tuberculoid were in reality borderline tuberculoid cases because of the rarity of true tuberculoid patients, and those designated as lepromatous were both borderline

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and true lepromatous patients. All of the lepromatous patients had negative Mitsuda reactions and other in vitro manifestations of defective cell-mediated immunity. Lepromatous leprosy patients manifesting ENL, diagnosed clinically and by biopsy of skin lesions, were investigated as a separate group. These patients were not receiving steroids or other anti-inflammatory drugs during the period of investigation or at any time before the onset of the ENL. Tuberculoid and lepromatous patients were investigated on admission to Westfort before antileprosy treatment was commenced. Healthy male and female laboratory workers served as normal controls.

Neutrophils. Neutrophils were obtained from preservative-free heparinized whole blood (5 U of heparin per ml) donated by patients and normal controls. After sedimentation at 37°C, the buffy layer was centrifuged at 250 × *g* for 10 min. The resultant cell pellet was washed three times with medium 199 (Grand Island Biological Co., Grand Island, N.Y.), pH 7.2, and suspended to the appropriate concentration according to the cell requirements of the various neutrophil function tests.

Cell motility studies. The motility studies were of two types, chemotaxis and random migration. These were initially assessed by the modified method of Boyden (23), whereby PMN which accomplish complete trans-filter passage are counted microscopically. Where defective neutrophil movement was evident, the leading-front method of Zigmond and Hirsch (24) was employed to ascertain the exact type of cell motility affected.

Leukoattractants. Two chemoattractants were used: (i) fresh human serum (patient and control), activated by 500 μg of bacterial lipopolisaccharide (EAS; *Escherichia coli* O127:B8 [Difco Laboratories, Detroit, Mich.]) per ml. The mixture was incubated for 30 min at 37°C, followed by a fourfold dilution with medium 199; and (ii) denatured casein at a concentration of 5 mg/ml, prepared by alkali hydrolysis with subsequent readjustment to pH 7.2. This preparation was stored in aliquots at −20°C, and the same batch was used for the entire investigation.

Chemotaxis assay. PMN chemotaxis was measured with a modified Boyden chamber (23), which utilizes 0.2 ml of patient or control cell suspensions (3×10^6 cells per ml) in the upper chamber separated from 1 ml of leukoattractant solution by a 5-μm pore size membrane filter (Millipore Corp., Bedford, Mass.). Chambers were incubated at 37°C for 3 h, after which filters were removed, fixed with methanol, stained with hematoxylin, dehydrated, and cleared with xylene. The average number of neutrophils per high-power field (HPF) reaching the lower surface of the filter was determined and expressed as an average per triplicate filters.

Random migration was assessed by measuring the extent of cell migration across the 5-μm pore size filter in the absence of a leukotactic gradient. Cells were suspended to a concentration of 3×10^6 per ml in tissue culture medium 199 containing 5% heat-inactivated fetal calf serum (Wellcome Research Laboratories). The same medium was placed in the bottom compartment, and chambers were incubated and filters were processed as for chemotaxis studies.

When these techniques were used, the normal ranges for chemotaxis using EAS and casein as chemoattractants were 198 to 256 and 120 to 162 cells per HPF, respectively; in random migration systems the range was 15 to 30 cells per HPF.

Simultaneous assessment of neutrophil random and truly directional motility. Patient and control PMN random migration, chemokinesis (stimulated random migration), and true chemotaxis were assessed when varying concentrations of EAS (1.5, 3.8, 6, and 12%) derived from normal serum were placed above and below the filter in all possible combinations by the method of Zigmond and Hirsch (24). Furthermore, to assess the effects of the sera of patients on specific types of neutrophil motility, similar systems were constructed, using normal PMN incubated with varying concentrations of EAS derived from the sera of patients. Chambers were incubated for 40 min at 37°C. Filters were processed as above and mounted with the top side uppermost. Neutrophil migration was expressed as the distance travelled (in micrometers) as measured with an optical micrometer on the fine focus of a microscope. Results are expressed in tabular form (checkerboard).

Chemotaxis inhibition studies. Patient and control sera were heat inactivated at 56°C for 30 min and added to normal neutrophils to give a final serum concentration of 20%. Endotoxin-activated normal serum was used as the chemoattractant in a 3-h incubation system at 37°C.

Semiquantitative NBT test. The semiquantitative NBT test was performed by the method of Sher et al. (16). Heparinized blood was allowed to sediment spontaneously at 37°C. A 0.1-ml quantity of the leukocyte-rich supernatant was incubated with an equal volume of NBT solution for 15 min at 37°C, followed by a further incubation at room temperature for 15 min. Smears of the mixtures were made on glass slides, fixed in methanol, and stained for 5 min in dilute hematoxylin. Slides were evaluated microscopically, and results were expressed as the percentage of NBT (reduced)-positive PMN.

Reconstituted semiquantitative NBT test. PMN cells were obtained as above, then gently spun, washed six times with Hanks tris(hydroxymethyl)aminomethane buffer, and made up to a concentration of 10^7 cells per ml. A 0.1-ml quantity of serum from controls and from the various patient groups was added to 0.1 ml of cell suspension and incubated for 10 min at 37°C. A 0.2-ml quantity of NBT solution was then added to the mixture. This was incubated for 15 min at 37°C, followed by a further 15 min at room temperature. Staining and counting of slides were carried out as above.

Skin window migration. Skin window migration was performed on 10 patients from each group (tuberculoid, lepromatous, and ENL) by the method of Rebuck and Crowley (13). A drop of sterile egg white, covered by a sterile microscope slide, was placed in a small abraded area of the skin of the volar forearm. Slides were removed at 1.5, 3, and 5 h, and the total number of neutrophils was counted in areas of greatest cellular concentration per HPF. The slides were read blindly by two observers, and the mean of the two readings was recorded.

RESULTS

NBT test. Resting NBT tests in all patients were within normal limits (normal range, 0 to 10%). Results of reconstituting various PMN cells with normal serum and serum from the different groups of patients are shown in Table 1. Although tuberculoid and lepromatous sera produced a statistically significant increase in the number of NBT-positive neutrophils ($P < 0.05$, and < 0.001, respectively), these increased levels were either within or just above the normal range. ENL sera, however, had a marked stimulatory effect on both normal and ENL PMN cells ($P < 0.001$), well above the normal values.

In vitro cell motility studies. Neutrophils from all three groups of patients manifested depressed chemotaxis to endotoxin-activated normal and autologous sera and hydrolyzed casein in the classical Boyden assay system (Table 2).

The severity of the defect correlated with the type of leprosy. The inhibition in ascending order of severity was tuberculoid, lepromatous, and ENL. Likewise, normal neutrophils showed depressed motility when endotoxin-activated sera of patients were used as leukoattractants. Nonactivated sera of patients manifested spontaneous chemotactic ability which was much less than the corresponding spontaneous activity of normal nonactivated serum. This could be due to the presence of inhibitors of cell motility in the serum. The degree of severity was again related to the patient group from which the serum was obtained. Random migration was similarly depressed in all patient groups (Table 2).

The results of the experiments utilizing different concentrations of control and patient EAS above and below the filter with matched neutrophils are shown in Fig. 1. It is evident that inhibition of PMN motility of all patient groups occurred in the absence of a gradient (chemokinesis, within the diagonal). Percent inhibition of chemokinesis (by comparison with control values) was 0, 13, 25, and 20% for ENL; 31, 20, 55, and 51% for lepromatous; and 17, 20, 30, and 25% for tuberculoid for ascending equal concentrations of EAS on either side of the filter.

To assess the extent of directional motility, the true increments in chemotaxis between control and patient cells were compared. The true chemotactic increment is calculated by substracting the expected value for migration in positive gradients from the observed experimental value. Results of these calculations from the data shown in Fig. 1 are summarized in Table 3. By comparison with the control, the ENL and lepromatous groups manifested no directional response, whereas the tuberculoid group showed about a 50% reduction compared with control

TABLE 2. *Chemotactic responsiveness of neutrophils of patients to various chemotactic stimuli*

Chemotaxis system[a]	No. of neutrophils per HPF in the following patient groups[b]:		
	Tuberculoid	Lepromatous	ENL
PC/PS	119 ± 7.8[c]	43.4 ± 8.1	18.8 ± 6.9
PC/NS	151 ± 9.0	61.6 ± 8.4	34.3 ± 13.6
NC/PS	150.7 ± 12.3	100.2 ± 11.6	51.7 ± 9.0
NC/PS (nonactivated)	70.7 ± 24.1	44.0 ± 0.59	38.3 ± 6.9
PC/casein	139 ± 21.8	107.7 ± 10.8	61.2 ± 4.3
Random migration	17 ± 4.3	11.0 ± 1.7	13.9 ± 2.4

[a] PC and NC, Cells of patients and normal cells, respectively, present in the upper chamber. PS and NS, Endotoxin-activated patients and normal serum, respectively, present in the lower chamber. The mean value ± standard error of the mean for NC/NS was 215.6 ± 16.0; normal random migration, 15 to 30 cells per HPF (mean, 25); normal casein migration 120 to 165 cells per HPF (mean, 145).

[b] Number of cases tested in each group was: tuberculoid, 12; lepromatous, 11; and ENL, 12.

[c] Mean ± standard error of the mean.

TABLE 1. *NBT results after reconstitution with various sera*

PMN cells[a]	Resting NBT tests levels	No. of NBT-positive neutrophils after reconstitution with:			
		Normal serum	Tuberculoid serum	Lepromatous serum	ENL serum
Normal	4.1 ± 2.0[b]	5.2 ± 3.1[b]	8.8 ± 3.5 ($P < 0.05$)[c]	12.1 ± 2.7 ($P < 0.001$)	28.6 ± 5.9 ($P < 0.001$)
Tuberculoid	1.7 ± 1.7	7.8 ± 4.3	6.2 ± 4.1		
Lepromatous	3.4 ± 3.3	5.0 ± 3.2		8.5 ± 4.4	
ENL	3.5 ± 2.7	8.4 ± 3.2			32.1 ± 10.7 ($P < 0.001$)

[a] The number of cases tested in the various groups were: normal, 17; tuberculoid, 11; lepromatous, 10; and ENL, 8.

[b] These figures represent the mean ± standard deviation of NBT-positive neutrophils.

[c] The stimulatory effect of the various sera was compared with the effect of normal serum by the Student *t* test.

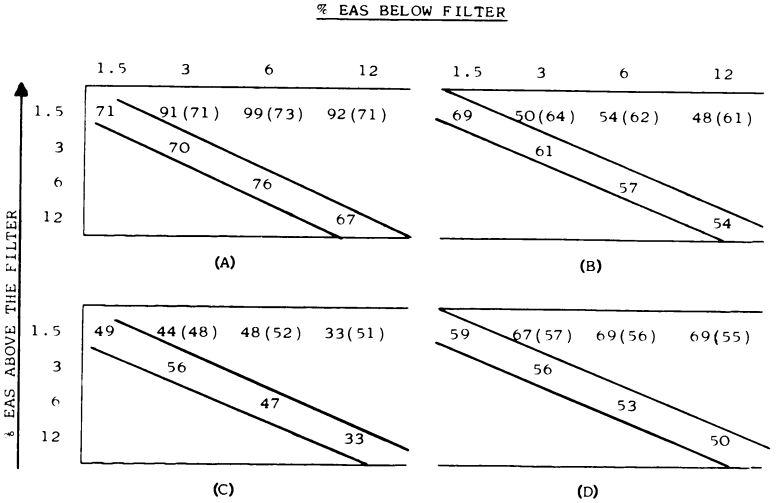

FIG. 1. *Effect of control and patient neutrophil motility of varying concentration gradients and absolute concentration of EAS derived from autologous serum. Figures along the diagonal from upper left to lower right show the distance migrated (in micrometers) in increasing concentrations of the chemoattractant in the absence of a concentration gradient. Above the diagonal cells are moving in a positive gradient. The figures in parentheses are estimates of the expected migratory values in positive gradients, assuming that the cells detected only the absolute concentration of the chemoattractant (i.e., random migration) but not the gradient. (A) Control, (B) ENL, (C) lepromatous, and (D) tuberculoid.*

TABLE 3. *Analysis of the chemotactic responsiveness of control and patient neutrophils by comparison of true chemotactic increments from results shown in Table 3*

Positive gradients		True chemotactic increments (μm) in:			
% of EAS above the filter	% of EAS below the filter	Control group	Tuberculoid group	Lepromatous group	ENL group
1.5	3	20	10 (50)[a]	0	0
1.5	6	26	13 (50)	0	0
1.5	12	21	14 (66)	0	0

[a] Figures in parentheses are percentages of control chemotaxis for the corresponding positive gradient.

values. Impaired chemokinesis and true chemotaxis were evident in all three groups. These results indicate that the defect in neutrophil locomotion was a combined defect of both random and directed motility, which was most pronounced in the lepromatous group.

Serum inhibitors. Normal PMN cells incubated with heat-inactivated sera from each group of patients showed impaired chemotactic responsiveness to endotoxin-activated autologous serum (Table 4). The inhibition profile was once more related to the type of leprosy, being most severe in the lepromatous group.

Skin window migration. Rebuck skin windows indicated a marked decrease in neutrophil accumulation in the 3- and 5-h slides in patients with lepromatous leprosy and in those with

TABLE 4. *Serum inhibition of normal PMN chemotaxis*

No. of PMN cells per HPF with the following types of serum in the upper chamber[a]:			
Autologous normal	Tuberculoid	Lepromatous	ENL
243 ± 40[b]	142 ± 47	59 ± 23	54 ± 22

[a] The number of PMN cells per HPF with normal chemotaxis was 233 ± 30 (mean ± standard deviation). In each instance, six cases were tested.
[b] Mean ± standard deviation.

TABLE 5. *Neutrophil accumulation in skin windows*

Patient group	No. of patients	No. of PMN cells per HPF in areas of maximum cell conc at:		
		1.5 h	3 h	5 h
Tuberculoid	10	0	50.8 ± 9.5[a]	160 ± 20.73
Lepromatous	10	0	5 ± 2.5	16.4 ± 4.18
ENL	10	0	8.75 ± 3.14	39.75 ± 15.76
Normal	10	0	57.2 ± 8.7	162.6 ± 20.49

[a] Mean ± standard error of the mean.

ENL. Tuberculoid patients, however, were within the normal range (Table 5). A variation in the number of cells accumulating in ENL cases was observed in the 5-h period. This may be related to the degree of ENL.

Figures 2 and 3 illustrate areas of maximal cellular concentrations in the skin windows of a lepromatous and a tuberculoid patient, respectively.

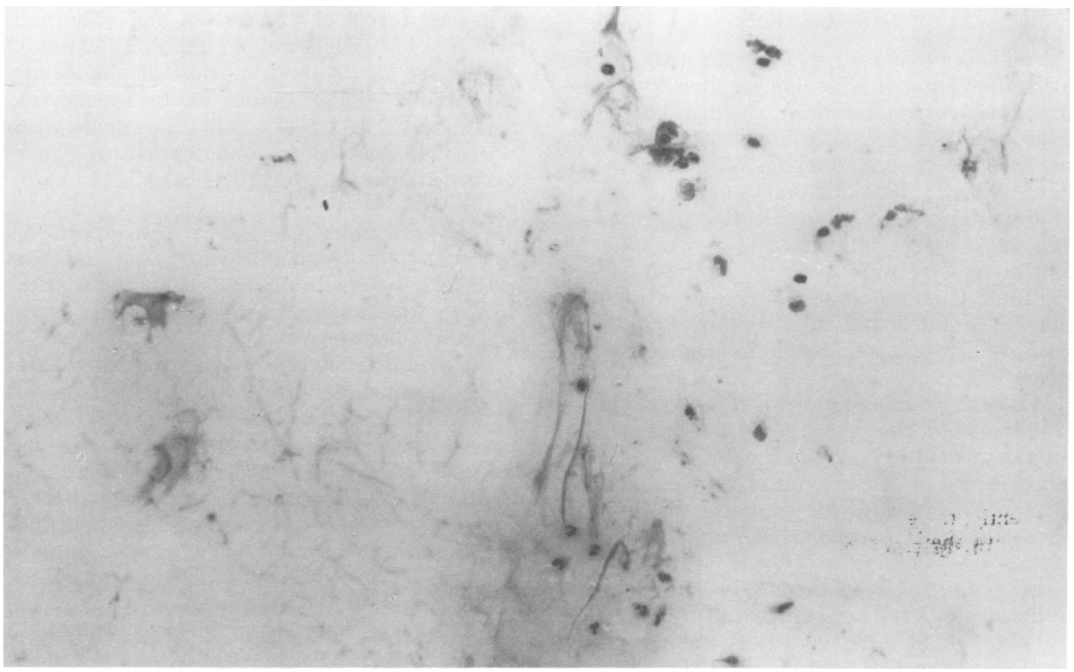

FIG. 2. *Skin window migration of a patient with lepromatous leprosy from an area of maximum cellular concentration. (×100).*

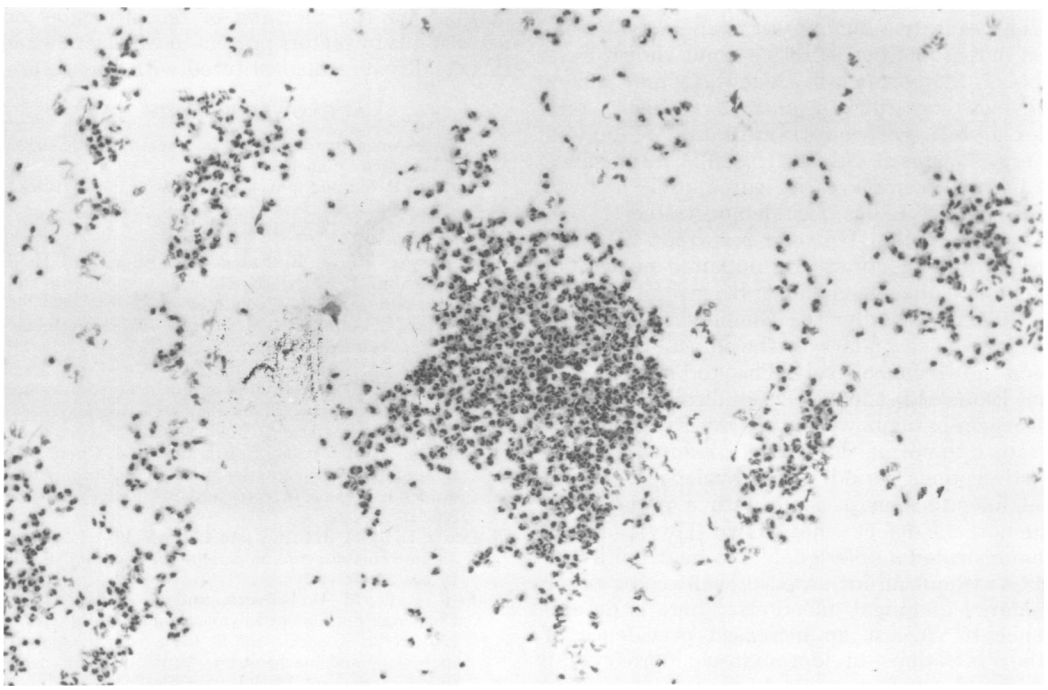

FIG. 3. *Skin window migration from a patient with tuberculoid leprosy from an area of maximum cellular concentration. (×100).*

DISCUSSION

Previous in vivo (4) and in vitro (22) studies have shown impaired leukocyte motility in lepromatous leprosy. However, the in vitro studies have been incomplete in analyzing the precise defects and the relationship of the degree of motility to the type of leprosy.

The present study has indicated the existence of a severe neutrophil-intrinsic chemotactic defect associated with a serum inhibitor of cell motility. This impairment was most evident in the lepromatous patients, in those exhibiting ENL, and to a lesser degree in tuberculoid patients.

When the technique of Zigmond and Hirsch (24) was used, the defect could be attributed to an impairment of both the random and directed components of the locomotor response. In the lepromatous group the impairment of true chemotaxis was particularly severe. The nature of the serum inhibitor of cell motility has not been characterized in this study. However, the inhibitory profile is dissimilar to that described by Ward et al. (22), who documented a high level of serum leukoattractant inhibitor in patients with lepromatous leprosy. The present study indicates the existence of a cell-directed inhibitor of leukocyte motility.

The causative factors in the chemotactic defect in lepromatous leprosy are not known. Possibly, an impairment in chemotaxis may reflect previous ingestion of antibody-antigen complexes, with consequent utilization of limited energy sources of the cells (15, 19). The presence of immune complexes in lepromatous leprosy, especially ENL, has been demonstrated (11).

An association between recurrent bacterial and fungal infections and impaired neutrophil chemotaxis has been shown by several workers (6, 10, 12). Whether the chemotactic defect is primary or secondary to the infection has not been shown conclusively. The studies of Mowat and Baum (12) and similar studies in this laboratory on patients with acute infections showed a return to normal chemotactic responses in the postinfectious period. The prevalence of bacterial infections in patients with a preinfection chemotactic defect is not known. This study has demonstrated a severe defect in neutrophil motility without an increased prevalence of either bacterial or fungal infections. There is no evidence to suggest an increased prevalence of other infections in lepromatous leprosy (18). Several host factors in lepromatous leprosy could be playing a role in the prophylaxis against bacterial infections. Such factors include high levels of lysozyme, low serum zinc and iron, decreased levels of vitamin A, and abnormal fatty acid levels (Sher et al., unpublished data).

Drutz et al. (5) reported normal phagocytic and antimicrobial activities in the monocytes, macrophages, and PMN cells in patients with tuberculoid and lepromatous leprosy. We have confirmed these findings (Sher et al., unpublished data).

We were unable to confirm the reports of Goihman-Yahr et al. (8), who found elevated NBT levels (unstimulated) in patients with ENL. In this study resting NBT levels were normal in all groups.

The addition of ENL serum to repeatedly washed normal and ENL neutrophils induced marked NBT stimulation in these cells. These findings suggest the existence of factors in the serum of ENL patients capable of stimulating neutrophil NBT reduction. Immune complexes, elevated lysozyme levels, and antigens released into the serum may be such factors. Normal levels of NBT reduction in ENL neutrophils could be explained by the presence of blocking factors such as immune complexes, antibodies, or a high antigen load. Washing of PMN may remove these blocking factors, allowing the ENL serum to cause increased NBT reduction. It is possible that our in vitro conditions were not optimal for the blocking of receptor sites on neutrophils by factors present in ENL sera when these cells were reconstituted with this serum.

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