

Lesional modulation of peripheral monocyte leucotactic responsiveness in leprosy

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SUMMARY

Because the accumulation and activation of mononuclear phagocytes are critical to the host response to intracellular microbial pathogens, we evaluated mechanisms of peripheral monocyte leucotactic regulation in leprosy. Plasma from 53 of 67 patients was found to inhibit the locomotion of normal human monocytes. Neither the prevalence nor the magnitude of plasma leucotactic inhibitory activity correlated with disease histology or duration, type or duration of chemotherapy, or history of erythema nodosum leprosum. Plasma leucotactic inhibitory activity resided principally in a non-immunoglobulin, cell-directed inhibitor of 230,000 daltons molecular weight. Fractionation of plasma from patients with lepromatous leprosy revealed an additional, immunoglobulin-containing inhibitor of approximately 400,000 daltons weight, possibly an IgG-IgA immune complex. Production of leucotactic inhibitors by unstimulated and concanavalin A-stimulated peripheral mononuclear cells was normal; however, cutaneous explants from these patients spontaneously produced the 230,000 dalton leucotactic inhibitor *in vitro*. The ability of the lesions of leprosy to impede monocyte traffic may be an important pathogenetic mechanism.

Keywords leprosy monocyte leucotaxis inhibitor

INTRODUCTION

Active infection with *Mycobacterium leprae* is characterized by a broad spectrum of host responses, marked by great variability in histopathology, cellular immune reactivity, and clinical course of infection. The pathophysiological basis for this variability is not yet clear though genetic influences seem likely. Nevertheless, as *M. leprae* resides primarily in mononuclear phagocytes, the ability of these cells to cope with the leprosy bacillus is an issue central to the understanding of host resistance to infection.

Many leprosy patients manifest defects of phagocyte locomotion, due at least in part to leucotactic inhibitors in plasma and serum (Masuda & Scheinberg, 1980; Azulay, 1982; Ward, Goralnik & Bullock, 1976). We have found that peripheral monocytes from patients infected with *Mycobacterium tuberculosis* also display defective leucotactic responsiveness, due to the increased activity in plasma of a specific, cell-directed inhibitor of monocyte leucotaxis (Campbell, 1979). In our subsequent studies, we have found that such inhibitory activity reflects the pathological

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activation by mycobacterial products of a subpopulation of peripheral blood mononuclear cells with natural killer-like characteristics. Since a microbial mechanism for subverting the mobilization and activation of mononuclear phagocytes could be a powerful determinant of the outcome of host-parasite interactions in other mycobacterioses, we have initiated studies of the regulation of monocyte and macrophage traffic in leprosy patients.

MATERIALS AND METHODS

Subjects. The study subjects were drawn from the inpatient and outpatient populations of the Gillis Long Hansen's Disease Center and participated after granting signed, informed consent. Disease staging was based upon clinical criteria as well as classification of skin biopsies according to the scheme of Ridley & Jopling (1966). The study population included patients in the tuberculoid (TT) and polar lepromatous (LL) stages of disease as well as patients with borderline tuberculoid (BT) and borderline lepromatous (BL) leprosy. No patient had manifestations of erythema nodosum leprosum or a reversal reaction at the time of study. The normal control subjects were employees of the Hansen's Disease Center and the East Carolina University School of Medicine who were free of known disease and who were taking no medication.

Generation of leucotactic inhibitors. Heparinized peripheral venous blood was separated on Ficoll-Hypaque gradients (Böyum, 1968). After two washings, the mononuclear cells were suspended in RPMI 1640 (Grand Island Biological Co., Grand Island, NY) containing 10% heat-inactivated newborn calf serum, 50 U of penicillin and 50 µg of streptomycin per ml, and 2 mM L-glutamine with or without 100 µg/ml of concanavalin A (Pharmacia Fine Chemicals, Piscataway, NJ). After 5 h, the cells were centrifuged and resuspended in stimulant-free medium to complete a 72 h incubation at 37°C in humidified 5% CO₂ before harvest and freezing of the supernatant.

Punch biopsies (2 mm) of clinically apparent cutaneous lesions were taken and cultured in 3 ml volumes of medium. The entire supernatant was harvested daily and the culture volume restored with fresh medium. Whenever possible, biopsies for culture were taken from sites adjacent to those used to stage the disease.

Assay of leucotactic inhibitory activity. Samples of normal human mononuclear cells were incubated for 30 min in either TC Medium 199 containing 2% bovine serum albumin (BSA, United States Biochemical Corp., Cleveland, OH) or in undiluted culture supernatant or heparinized (10 U per ml of blood) plasma (Campbell, Thomas & Tolson, 1982). Each sample was then resuspended at 1×10^6 monocytes per ml of TC 199/2% BSA and leucotactic responsiveness to 10% zymosan-activated normal human serum determined in blind-well leucotaxis chambers divided by a sandwich of a 5 µm pore size polycarbonate (Nucleopore, A.H. Thomas Co., Philadelphia, PA) and a 0.45 µm pore size cellulose nitrate (Sartorius Membranfilter, Science Essentials Co., Anaheim, CA). The responses of the supernatant (or plasma)-preincubated (ML_{XSPN}) and BSA-preincubated (ML_{XBSA}) cells were compared and inhibitory activity calculated by:

$$\% \text{ inhibition} = (1 - \text{ML}_{\text{XSPN}}/\text{ML}_{\text{XBSA}}) \times 100.$$

In some experiments, leucotactic responsiveness was also assessed to 10^{-7} M formyl-methionylleucylphenylalanine (Sigma) and to the 72 h supernatants of normal peripheral blood lymphocytes (1×10^6 /ml of RPMI 1640 with supplements) stimulated with 10 µg/ml of concanavalin A.

Chromatographic techniques. Samples of plasma and culture supernatants were applied to 0.9 × 15 cm columns of a goat anti-total human immunoglobulins preparation (G, A, and M, Cappel Laboratories, Cochranville, PA) coupled to Sepharose 4B (Pharmacia) at 5 mg of antibody per ml of gel. These columns had a specific capacity of approximately 20 mg of IgG. The columns were washed with 0.1 M NaHCO₃/0.5 M NaCl (excluded fraction) and the retained material eluted with 0.1 M glycine hydrochloride/0.5 M NaCl, pH 2.5 and the eluate pH adjusted to 7.0 with 1 M NaOH (eluted fractions). The fractions were dialysed against 0.01 M phosphate-buffered saline (PBS), pH 7.0, before further use.

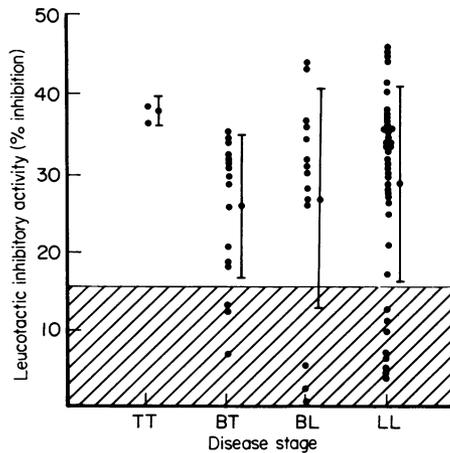


Fig. 1. Plasma leucotactic inhibitory activity in patients with polar tuberculoid (TT), borderline tuberculoid (BT), borderline lepromatous (BL), and polar lepromatous (LL) leprosy. The vertical bars represent the group means ± 1 s.d. and the shaded area the normal mean ± 2 s.d.

The immunoaffinity chromatography fractions were concentrated 10-fold by pressure ultrafiltration in Amicon chambers fitted with YM10 Diaflo membranes (Amicon Corp., Lexington, MA) before application to 1.5×80 cm columns of Bio-Gel A-1.5 m (Bio-Rad Laboratories, Richmond, CA) calibrated with urease (Miles Laboratories, Elkhart, IN), catalase (Pharmacia), immunoglobulin G (Sigma Chemical Corp., St Louis, MO), and bovine serum albumin (Sigma). The columns were eluted with PBS.

Statistics. The results were evaluated by Chi square analysis and by rank sum (Mann-Whitney) and rank order testing (Colton, 1974).

RESULTS

Plasma leucotactic inhibitory activity. Plasma from 79% of the 67 patients contained supernormal leucotactic inhibitory activity. While preincubation of normal monocytes in 15

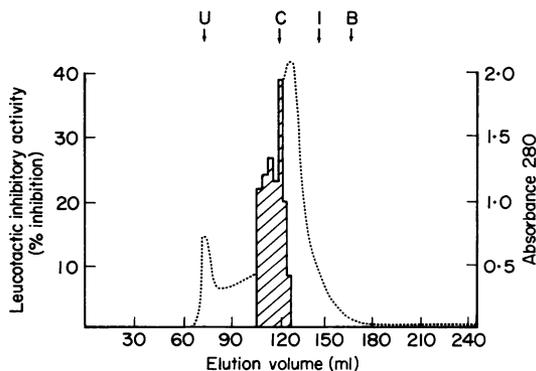


Fig. 2. A representative chromatogram of plasma from a patient with tuberculoid leprosy. The plasma was first depleted of immunoglobulin by immunoaffinity chromatography and then applied to a column of Bio-Gel A-1.5m calibrated with urease (U, 483,000 daltons), catalase (C, 235,000 daltons), IgG (I, 150,000 daltons), and bovine serum albumin (B, 67,000 daltons). Inhibitory activity is indicated by the shaded area and eluate absorbance at 280 nm by the dotted line. Inhibitory activity in the unfractionated plasma was 37.0%.

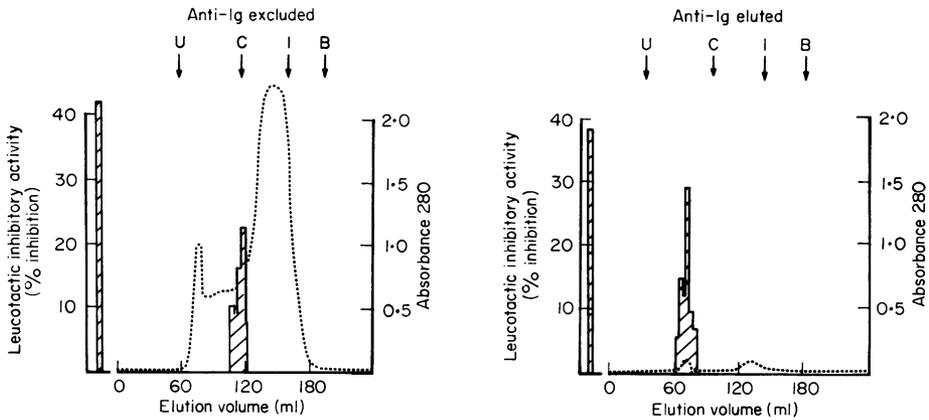


Fig. 3. A representative fractionation of plasma from a patient with lepromatous leprosy. Inhibitory activity in the unfractionated plasma was 33.9%. (Left) Data from the non-immunoglobulin fraction; (right) the immunoglobulin fraction initially retained by the anti-immunoglobulin column. Inhibitory activity is indicated by the shaded areas and eluate absorbance at 280 nm by the broken lines. The shaded bar at the left of each panel represents the activity in that fraction before application to the Bio-Gel column.

samples of normal plasma inhibited leucotactic responsiveness to zymosan-activated normal human serum by $5.6 \pm 4.5\%$ (mean \pm 1 s.d.), significant inhibitory activity ($> 15\%$), ranging from 17.0 to 45.5%, was detected in the plasma of 53 patients.

The prevalence and magnitude of plasma leucotactic inhibitory activity were unrelated to the position of the patient in the leprosy spectrum. As shown in Fig. 1, there were no significant differences among the two TT, 15 BT, 13 BL, and 37 LL patients. Similarly, neither the prevalence nor magnitude of inhibitory activity was related to disease duration, treatment status, inclusion of prednisone in disease therapy, or, in the lepromatous patients, a history of antecedent erythema nodosum leprosum.

Characterization of the plasma leucotactic inhibitors. Samples of plasma from three (two for polar tuberculoid) patients representative of each histological classification were sequentially

Table 1. Monocyte leucotactic inhibitory activity in the 72 h culture supernatants of peripheral blood mononuclear cells

Donors (N)	Supernatant activity*	
	Unstimulated	Concanavalin A (100 μ g/ml)
Normal (15)	5.4 ± 7.6	38.6 ± 10.2
Leprosy (27)	2.7 ± 6.1 †	34.2 ± 8.1 †
TT (1)	0.0	30.5
BT (6)	0.0 ± 0.0 †	30.3 ± 8.4 †
BL (4)	3.5 ± 7.0 †	36.6 ± 9.2 †
LL (16)	2.1 ± 4.3 †	35.3 ± 8.0 †

* Expressed as the percentage inhibition of leucotactic responsiveness of normal monocytes preincubated in the indicated supernatant compared to the responsiveness of monocytes preincubated in 2% bovine serum albumin.

† $P > 0.05$ compared to normal values.

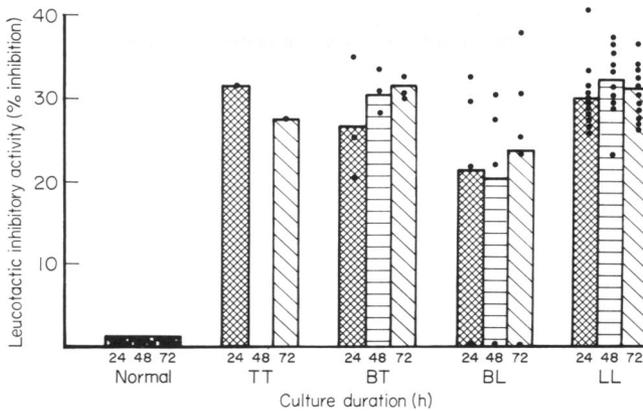


Fig. 4. Explant culture supernatant leucotactic inhibitory activity in normal controls (NL) and in patients with leprosy. The vertical bars represent the group means. The explants were cultured in 3 ml volumes of tissue culture medium and the supernatants harvested daily.

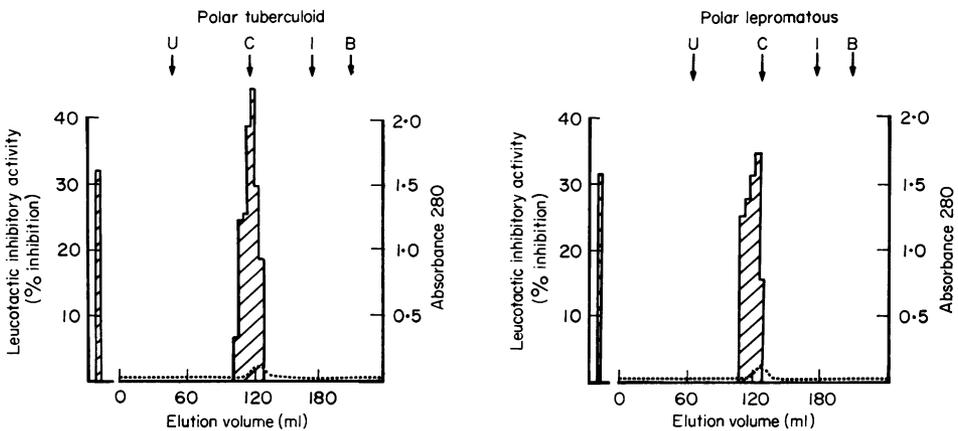


Fig. 5. Representative Bio-Gel A-1.5m fractionations of culture supernatants of cutaneous biopsies from one patient each with (left) polar tuberculoid disease, and (right) polar lepromatous disease. Inhibitory activity is indicated by the shaded areas and eluate absorbance at 280 nm by the broken lines. The shaded bar at the left of each panel represents the activity in that fraction before application to the Bio-Gel column.

fractionated by anti-immunoglobulin immunoaffinity and gel chromatography. Plasma from the polar and borderline tuberculoid and the borderline lepromatous patients yielded a single, non-immunoglobulin species of inhibitor which eluted from the Bio-Gel A-1.5m columns with an approximate molecular weight of 230,000 daltons. This inhibitor is similar to the cell-directed inhibitor of monocyte leucotaxis, CDI-Mlx, which is devoid of intrinsic leucotactic activity and which acts to inhibit locomotor responses to a variety of soluble mediators, including C5a, leucotactic lymphokines, and formyl-methionylleucylphenylalanine (FMLP, Campbell & Tolson, 1986). To address this relationship more directly, pools of the gel chromatography fractions containing this inhibitor were also tested for their leucotactic activity. In three experiments, no significant monocyte leucotactic activity was detected (2.5 ± 0.7 monocytes per field *vs* 2.3 ± 0.3 with TC 199 *vs* 21.5 ± 1.7 with zymosan-activated serum). No inhibitory activity was detectable in the fractions eluted from the immunoaffinity columns with these samples.

Plasma from lepromatous leprosy patients contained the non-immunoglobulin leucotactic inhibitor as well as a second inhibitor, which was retained by the anti-immunoglobulin columns and had an approximate molecular weight of 400,000 daltons. This immunoglobulin inhibitor was present both in lepromatous patients with and without a history of antecedent erythema nodosum leprosum. On double immunodiffusion, this 400,000 dalton fraction gave a line of partial identity with IgA and IgG with an IgG spur. No IgM or C5 was detected in that fraction. Representative chromatographs are presented in Figs 2 and 3. In contrast with the non-immunoglobulin inhibitor, this 400,000 dalton inhibitor was intrinsically leucotactic, displaying activity equivalent to that of zymosan-activated normal human serum. Preincubation of normal monocytes in the 400,000 dalton fraction deactivated the cells for migration toward zymosan-activated serum but did not impair responses to leucotactic lymphokines present in the supernatants of concanavalin A-stimulated normal lymphocytes or FMLP.

Generation of leucotactic inhibitors by peripheral blood mononuclear cells. We next sought to ascertain whether the increased plasma leucotactic inhibitory activity detected in these patients reflected spontaneous production by peripheral mononuclear cells, as is the case in patients infected with *Mycobacterium tuberculosis*. The 72 h supernatants of unstimulated and concanavalin A-stimulated peripheral mononuclear cells from 27 patients were assayed for inhibitory activity. As shown in Table 1, there was no evidence for spontaneous production of leucotactic inhibitory activity by peripheral blood mononuclear cells of leprosy patients. Moreover, the inhibitory activity in mitogen-stimulated cultures from these patients did not differ from that in cultures from 15 normal subjects. No significant differences emerged when these data were analysed by disease classification.

Spontaneous inhibitor production by explanted cutaneous lesions. Since a peripheral origin for the increased plasma leucotactic inhibitory could not be identified, we next evaluated whether such activity might reflect inhibitor production within the foci of infection. Punch biopsies of cutaneous lesions from 19 patients were taken and culture supernatants harvested daily for three days. As shown in Fig. 4, the culture supernatants of cutaneous explants from six normal controls contained no inhibitory activity. By contrast, the 24 h supernatants from 18 of the patient biopsies inhibited the leucotactic responsiveness of normal monocytes by a mean of $29.9 \pm 4.8\%$. Leucotactic inhibitory activity in the supernatants obtained on the second and third days of culture were similar (30.9 ± 4.3 and $30.9 \pm 3.4\%$ inhibition, respectively). The mean leucotactic inhibitory culture in plasma from these 18 patients was $32.8 \pm 2.8\%$. No significant differences were detected when the inhibitory activities in the supernatants from the first, second, or third days of culture were compared among the one TT, three BT, three BL, and eleven LL biopsies. No inhibitory activity was detected in any supernatant from the nineteenth patient, a 75-year-old male with borderline lepromatous disease of 10 years' duration whose plasma inhibited normal monocyte leucotaxis by 36.1%.

Characterization of the explant supernatant leucotactic inhibitors. Pools were made of the culture supernatants of explanted cutaneous lesions from patients representative of each histological disease type of leprosy and then fractionated by immunoaffinity and gel chromatography as before. In contrast with the plasma fractionations, which disclosed either one or, in polar lepromatous disease, two leucotactic inhibitors, each of the culture supernatants contained only a single species of inhibitor. As shown in the typical fractionations presented in Fig. 5, a non-immunoglobulin inhibitor with a molecular weight of 230,000 daltons accounted for all of the activities in supernatants from polar tuberculoid and polar lepromatous biopsies. Supernatants from borderline patients yielded similar patterns.

DISCUSSION

The data presented here detail an abnormality of inflammatory regulation in patients infected with *Mycobacterium leprae*. Plasma from the majority of such patients was found to contain pathological concentrations of a non-immunoglobulin leucotactic inhibitor of 230,000 daltons molecular weight. This leucotactic regulator is probably identical to CDI-MLx, the monocyte-specific, cell-directed inhibitor we have detected in plasma from patients with active tuberculosis (Campbell,

1979). Although the frequency and magnitude of plasma inhibitory activity did not correlate with disease staging, fractionation of plasma from patients with polar lepromatous disease revealed the presence of an additional, immunoglobulin-containing inhibitor of approximately 400,000 daltons molecular weight. Peripheral mononuclear cell production of leucotactic regulators was normal; however, cutaneous explants spontaneously elaborated the 230,000 dalton inhibitor during culture *in vitro*, suggesting that the increased plasma CDI-MLx activity reflects production of this substance within foci of disease.

These data complement previous observations of defective phagocyte locomotion in leprosy patients. Ward and co-workers detected abnormalities of leucotactic factor inactivators in patients with lepromatous disease (Ward *et al.*, 1976). Subsequently, Masuda & Scheinberg (1980) reported impaired monocyte leucotactic responses to lymphokines in both polar tuberculoid and lepromatous patients. Cell-directed leucotactic inhibitory activity was detected in plasma from those patients, but was not characterized further. Defects of neutrophil locomotion, particularly marked in patients with lepromatous disease and in those manifesting erythema nodosum leprosum, have also been recognized and appear also to be related to circulating cell-directed inhibitors (Wahba, Cohen & Sheskin, 1980). However, it is possible that these neutrophil defects may be an epiphenomenon of hyperimmunoglobulinaemia in these patients since immunoglobulin G has been shown to inhibit neutrophil locomotion *in vitro* (Woronick, Malnick & Maderazo, 1981).

Our previous studies have indicated that the production of CDI-MLx by peripheral blood mononuclear cells is an attribute exclusive to a non-cytotoxic subpopulation of natural killer-like cells bearing the OKM1 and Leu 7 but not the Leu 11 membrane antigens (Campbell & Tolson, 1986). Indeed, such cells appear capable of secreting a variety of immunoregulatory cytokines, including interleukins 1 and 2, α - and γ -interferons, and B cell growth factor (Scala *et al.*, 1984; Kasahara *et al.*, 1983; Djeu *et al.*, 1982; Pistoia *et al.*, 1985). They may present antigen to T cells and can act to suppress T and B cell functions *in vitro* (Scala *et al.*, 1985; Tilden, Abo & Balch, 1983; Arai *et al.*, 1983). Thus, dysfunction of these immunoregulatory cells could have great relevance to the pathogenesis of leprosy. Little is known, however, of natural killer cell numbers and function in leprosy although Humphres, Gelber & Kraherbuhl (1982) noted abnormally low cytolytic activity of these cells in peripheral blood from patients with concurrent erythema nodosum leprosum.

The leucotactic inhibitor detected in these patients probably has immunoregulatory actions beyond the modulation of monocyte locomotion. We have recently found that CDI-MLx induces monocyte prostanoid production *in vitro* and that this effect, as well as its effect on leucotaxis, can be blocked with inhibitors of cyclooxygenase and thromboxane synthetase. Further, preincubation in these agents *in vitro* or in the thromboxane A₂ receptor antagonist, SQ 29,548, restores the leucotactic responsiveness of tuberculous monocytes. Prostaglandin E₂ and thromboxane A₂ have been shown to modulate the phagocytic and secretory function of mononuclear phagocytes as well as Ia antigen display, actions which might critically influence the outcome of host-mycobacterial interactions *in vivo* (Razin, Bauminger & Globerson, 1978; Cahill & Hopper, 1982; Snyder, Beller & Unanue, 1982).

The nature and origins of the immunoglobulin-containing inhibitor detected in lepromatous plasma are not yet clear. While the concentration of circulating immune complexes is increased in many patients with leprosy, the composition of the complexes varies with the disease classification (Ramanatha *et al.*, 1984). It may be relevant to the observations detailed here that complexes from lepromatous patients are particularly likely to contain C-reactive protein, an acute-phase reactant which exerts diverse effects on immune functions, including those of natural killer cells and monocytes (Baum *et al.*, 1983; Mortensen & Duszkievicz, 1977).

The pathophysiological significance of the observations detailed here is uncertain. Our studies have shown that CDI-MLx is one element in an immunoregulatory loop which includes natural killer-like cells and monocytes and which causes the activation of monocyte arachidonic acid metabolism. This loop might be supposed to act physiologically to focus mononuclear phagocytes within inflammatory foci and to modulate the function of these immobilized cells. Our studies in progress further suggest that the detection of increased CDI-MLx activity in plasma is a pathological event. On the one hand, peripheral monocyte leucotactic responsiveness and plasma CDI-MLx activity are normal in patients with deep mycoses due to *Histoplasma capsulatum* and

Blastomyces dermatitidis. In tuberculosis, however, defective locomotor responsiveness and increased inhibitor activity reflect the activation of this cellular cascade by circulating mycobacterial products. The data presented here imply that increased plasma CDI-MLx in leprosy patients reflects spillage from cutaneous foci. Whether such spillage impedes monocyte immigration into inflammatory foci is speculative. If, however, CDI-MLx modulates monocyte maturation, these observations may have great relevance to the ability of mononuclear phagocytes to deal with these intracellular pathogens.

Inasmuch as the prevalence and magnitude of plasma inhibitory activity did not correlate with histopathology, these data do not yet allow the conclusion that the detection of increased CDI-MLx in leprosy has the same connotation as in other mycobacterial infections. These data do, however, provide some preliminary insight into the potential of the lesions of leprosy to modulate systemic immune responses. Immunoglobulin production, including the production of specific anti-mycobacterial antibodies, by explanted lesions has previously been demonstrated (Lai A Fat *et al.*, 1979, 1980). The present study extends that perspective to include the elaboration of inflammatory regulators by these lesions and suggests the possibility of a similar origin for soluble inhibitors of other immune responses in these patients (Nelson *et al.*, 1975).

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