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Presence of soluble, *Mycobacterium leprae*-derived antigen in the inflammatory exudate of reactional lepromatous leprosy

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Summary By immunofluorescence techniques, immunocomplexes deposition in the wall and periphery of dermal blood vessels have been demonstrated in 8 leprosy-reaction lesions (4 ENL, 4 Lucio's phenomena). Two additional ENL lesions were negative for the presence of immunocomplexes with anti-IgM, IgG, IgA, C3 and C1q antisera. The 10 leprosy reaction lesions, however, were positive for the presence of *Mycobacterium leprae*-derived soluble antigen. This antigen, visualized with a potent human anti-*M. leprae* antiserum, was often found in and around the dermal blood vessels showing vasculitis and always in the macrophages (Virchow's cells) present in the leprous granulomas. This finding was independent of the presence of intact or fragmented *M. leprae* in those locations.

The role of mycobacteria derived material in the genesis of type-2 leprosy reactions is discussed.

Introduction

From previous work in our Department on immunological abnormalities in patients with reactional lepromatous leprosy, a considerable incidence of circulating immune complexes (CIC) has been found to be associated with the presence of erythema nodosum leprosum (ENL) and Lucio's phenomenon.[1–3] In one study, a group of patients suffering from diffuse lepromatous leprosy complicated by

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Lucio's phenomenon showed severe impairment in several of their immunological parameters. [2-3] Their levels of total serum proteins were elevated mainly due to an increase in the alpha-2 and gammaglobulin fractions. All of the immunoglobulin classes were elevated but the IgG and IgM classes were the most altered. The complement components C3 and C4 were normal and the 50% haemolytic complement activity was only slightly elevated. The C-reactive protein and rheumatoid factors tests were positive in around 50% of the cases. Thirty-three percent of the patients had circulating immune complexes and all of them had circulating antimycobacterial antibodies.

In general, the patients with reactional lepromatous leprosy showed low numbers of T-lymphocytes (E-rosettes) and, in some cases, B-lymphocytes (EAC-rosettes) were increased. A great majority of patients gave negative leucocyte inhibition factor (LIF) tests with lepromin as the antigen. The LIF tests with PPD and the response to the intradermal injection of PPD and other antigens were comparable both in lepromatous and normal groups.

We have extended our studies on reactional lepromatous leprosy and in this paper we present our results on the presence of soluble immune complexes and antigen at the lesion sites and suggest a role for these materials in the genesis of reactional type-2 leprosy lesions.

Materials and methods

PATIENTS

Six lepromatous patients with ENL and 4 patients with Lucio's phenomenon were studied. They were patients attending regularly at the Centro Dermatológico Pascua (Mexico City) for medical care, or residents of the leprosarium 'Hospital Dr P López' (Zoquiapan, México). All the patients were adult males or females having an old leprosy infection, and they were under conventional anti-leprosy (but not anti-reactional) treatment (DDS) at the time of the study. Five other, age-matched, non-reactional lepromatous patients were also included in the study as a control group. Within this group, 3 patients were under DDS-treatment for over 3 years, one had just begun treatment and the last one was an untreated case.

SKIN BIOPSIES

They were taken with a 5 mm-wide circular punch in a manner deep enough to include all of the dermis and part of the subjacent fatty tissue. Each biopsy was divided into two halves. One half was formalin-fixed and processed for routine histology, and the other was included in 'tissue-teck', frozen on dry ice, and sectioned thereafter as described below.

STAINING OF TISSUE SECTIONS

Five microns-thick tissue sections were prepared in a cryostat (Tissue Teck II, Miles), fixed for 10 min in cold acetone, washed 3 × with phosphate-buffered saline (PBS) and stained with fluoresceinated anti-human IgA, IgM, IgG, C3, C1q, or anti-*Mycobacterium leprae* gamma globulin (see below). Except for the fluoresceinated anti-C1q (which was previously prepared in goat in our laboratory) and anti-*M. leprae* (to be described here) antibodies, all of the other immunoreagents were purchased from Hyland and Ortho (Behring). Staining of tissue sections pretreated for 60 min with an enzyme mixture containing 8 mg egg lysozyme (Sigma L6876) and 8 mg wheatgerm lipase (Sigma L3001) per 40 ml of 0·06 M phosphate buffer, pH 6·8, was performed according to conventional procedures.

FLUORESCEINATED HUMAN ANTI-*M. LEPRAE* ANTIBODY

Gammaglobulins (HGG) were precipitated from a pool of sera from lepromatous patients that were strongly positive in the counterimmunoelectrophoresis (CIE) test[4] with a *M. lepraemurium* extract as the antigen. HGG were precipitated with ammonium sulphate at 1/3 of saturation, exhaustively dialysed against saline borate solution, pH 8·6 (H_3BO_3 0·309 g; $Na_2BO_4O_7 \cdot 10\ H_2O$ 0·477 g; NaCl 8·3 g, and water to 1000 ml), and labelled with fluorescein isothiosyanate (FITC) according to Nairn.[5] Briefly, to each 4 ml of a 2% protein solution, 1·0 ml of 0·2 M Na_2HPO_4, pH 9·0, were added. The pH in the mixture was adjusted to 9·5 with 0·1 M $Na_3\ PO_4$ and the volume brought to 8·0 ml with 0·145 M NaCl. After incubation for 60 min at room temperature in a dark place, the reaction was stopped by cooling the mixture in an ice-bath for a few minutes. Free fluorescein was separated by chromatography through a G-25 Sephadex column (2 × 25 cm), eluting with 0·01 M phosphate buffer, pH 7·2. Labelled protein was collected, dialysed against the above buffer and applied to a DEAE- cellulose column. DEAE chromatography was performed according to Wood[6] using a 2·6 × 13 cm column per each 200 mg of protein. To separate fractions optimally labelled from those under- or over-labelled, elution with 0·01 M, 0·03 M and 0·05 M phosphate buffer, pH 7·2, was performed. Most of our FITC-labelled protein (495/280 ratio = 0·72) was eluted with 0·01 M phosphate buffer, although a considerable amount of conjugate (495/280 ratio = 0·83) was eluted with 0·3 M phosphate buffer. Both fractions, however, showed absence of non-specific staining ability and were highly brilliant under ultraviolet light. The FITC-labelled fraction used in the present experiments was that eluted with 0·01 M phosphate buffer and had a protein concentration of 13 mg per ml. To use, it was diluted 1:5 with saline phosphate buffer, pH 7·2 (NaCl, 0·85 g; Na_2HPO_4, 1·07 g; $NaH_2PO_4 \cdot 1H_2O$, 0·35 g, water to 1000 ml). The antibody specificity of this FITC-labelled fraction was demonstrated by treating mouse liver, spleen, kidney, heart, brain, striated muscle and skin tissue sections, 5 microns-thick, with the antibody preparation for 60 min.

Results

SPECIFICITY OF ANTI-*M. LEPRAE* ANTIBODY

When treatment of normal mouse liver, spleen, heart, brain, striated muscle and skin tissue sections with our anti-*M. leprae* preparation was performed, no staining was observed except for the weak, natural self fluorescence of collagen. On the contrary, a very bright specific fluorescent staining was observed associated to the granulomatous collections in the livers and spleens of mice bearing a 3 month infection with *M. lepraemurium* (Figure 1). The same antibody preparation did stain enzyme-treated smears of purified *M. lepraemurium* suspensions.

IMMUNOHISTOLOGIC STUDY OF BIOPSIES

Immunofluorescence studies in 8 leprosy reaction biopsies (4 ENL, 4 Lucio's phenomena) indicated the presence of immunocomplexes in the walls of the dermal blood vessels or in their periphery (Figure 2). In two cases of ENL we were unable to demonstrate deposition of immunocomplexes in or around the dermal vessels which, however, presented vasculitis of variable intensity and other inflammatory changes including oedema and infiltration of PMN leucocytes. In these two cases the results were negative for the presence of IgM, IgG, IgA, C3 and C1q, despite that fluorescent antisera from different sources were used. These negative results in regard to the demonstration of immunocomplexes in the lesions could possibly be explained assuming that the biopsies were taken from old or healing lesions rather than from early ones. At this time, immunocomplexes could well be phagocytosed and degraded. Alternative explanations are possible as we will underline below. Others[7] have also been unable to demonstrate immunocomplexes in the vessel walls or in the granulomatous infiltrates of 19 biopsies of skin lesions of patients with borderline lepromatous (10) or lepromatous (9) leprosy showing a recent reaction.

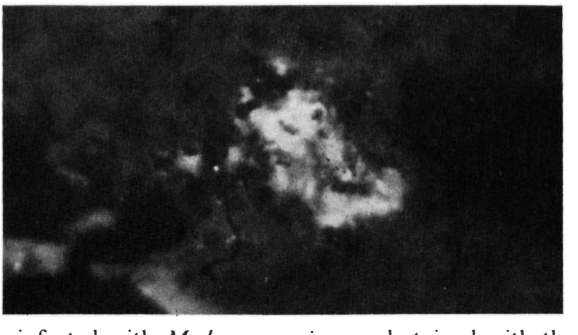

Figure 1. Mouse liver infected with *M. lepraemurium* and stained with the fluorescein-labelled human anti-*M. leprae* immunoglobulin. The specific staining of hepatic macrophages found in a small leprous granuloma is shown (ca. ×600).

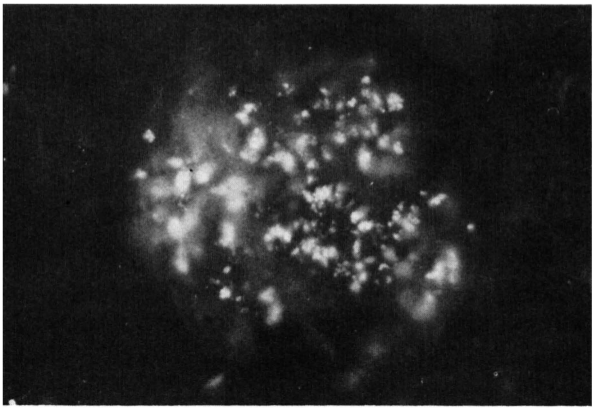

Figure 2. Deposition of immune complexes in a dermal blood vessel of a Lucio's phenomenon as visualized through their reaction with a fluoresceinated rabbit anti-human IgG antiserum. Notice the fluorescent material filling and obstructing the light of the small vessel (× 480).

An interesting observation was the absence of demonstrable *M. leprae* within the inflammatory cells in some cases showing severe vascular lesions. On the other hand, we have observed in all of the biopsies taken from both ENL (6) and Lucio's phenomenon (4), the presence of intracellular soluble mycobacterial antigen.

A granular or homogeneous pattern of fluorescent reaction was consistently observed in the cytoplasm of macrophages and PMN leucocytes around the affected blood vessels and within macrophages and Virchow's cells of the granulomas, this indicating the presence of *M. leprae* soluble antigen (Figures 3–5). This finding was independent of the identification of acid-fast bacilli by the Fite–Faraco staining method. In several cases, the fluorescent tissue was composed of PMN and macrophages full of bacillary fragments. Many cells in the infiltrate showed morphological evidences of degeneration.

Within the control group, a close correlation between the presence of acid fast bacilli and a positive fluorescent anti-mycobacterial test was observed. Two cases with abundant *M. leprae*-laden Virchow's cells gave a positive fluorescent anti-mycobacterial test. The remaining 3 cases contained lesser amounts of Virchow's cells, most of them free of bacilli, and they were negative for the presence of soluble mycobacterial material. In the absence of leprosy reaction, control cases were negative in the anti-C1q fluorescent test.

Discussion

Erythema nodosum leprosum (ENL) and erythema necrotisans or Lucio's phenomenon, are manifestations of the type 2 leprosy reactions.[8]

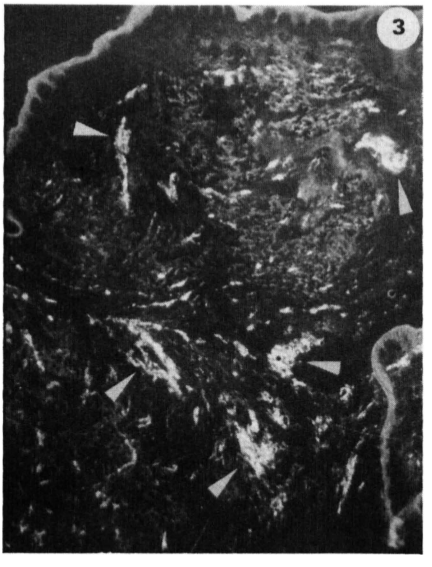

Figure 3. A section of a leprosy reaction type ENL stained with the fluoresceinated human anti-*M. leprae* antibody. A great number of macrophages containing intact or fragmented *M. leprae* or *M. leprae*-derived antigens is seen throughout the dermal lesion both affecting blood vessels and in the leprous granulomas (Virchow's cells) (arrowheads) (× 125).

ENL appears in nearly 50% of lepromatous patients under treatment although it appears also in patients without it. It appears less frequently in subpolar or borderline lepromatous leprosy but it does not appear in the other types of leprosy.

From the clinical point of view, ENL typically presents as collections in the 'normal' skin of small nodules, slightly raised, tender, erythematous, warm and recurrent, that blanch under finger pressure. These nodules last for some days (usually 3 to 7), then vanish leaving no trace unless they reappear on or adjacent to a previous lesion. In this case, they disappear leaving a bluish and fibrotic scar of the thickened skin. Because of their dermal and hypodermal localization, the nodules cannot be rolled under the skin. Occasionally, several isolated lesions fuse with each other and even more seldom they become necrotic and suppurative. This happens especially on the extensor surfaces giving origin to a form of necrotising erythema nodosum.

The Lucio's phenomenon or erythema necrotisans is another form of type 2 leprosy reaction. This is a complication of the diffuse lepromatous leprosy of Lucio and Latapi but it can appear in some cases of advanced nodular lepromatous leprosy, especially when such cases are under irregular treatment or without it. Although this type of leprosy reaction is frequent in patients from Mexico and Central American countries, it is not limited to them and isolated cases of Lucio's phenomenon have been reported in other parts of the world. The lesions appear in the skin as erythematous spots, tender or slightly indurated,

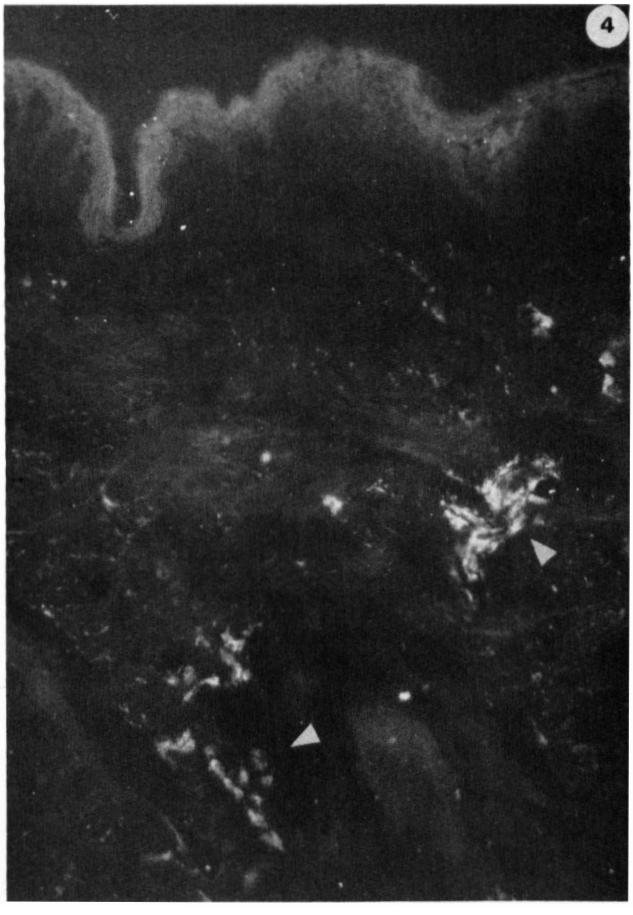

Figure 4. A tissue section from a Lucio's phenomenon stained with a fluoresceinated anti-*M. leprae* antibody, showing the presence of soluble *M. leprae*-derived antigen in the papillar and reticular dermis. Notice the fluorescent material in and around dermal blood vessels and in the Virchow's cells of the lepromatous granuloma (arrowheads) (× 120).

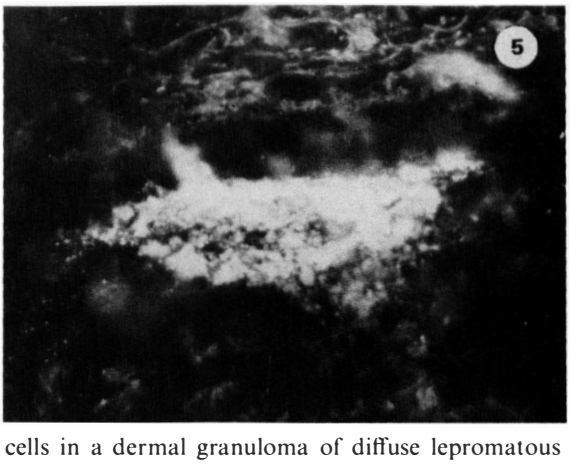

Figure 5. Virchow's cells in a dermal granuloma of diffuse lepromatous leprosy with Lucio's phenomenon stained with a fluoresceinated human anti-*M. leprae* antibody (× 480).

painful, that soon become purpuric and finally necrotic (some of them become ulcerated) leaving an irregular or stellate crust of dark brown colour which eventually comes off leaving an hypo-or hyperpigmented atrophic scar. Although the lesions appear fundamentally on the limbs (and those of the legs are the most severe and long lasting), they may appear on other parts of the body.

The factors responsible for these type 2-leprosy reactions are not known for sure, and because of this, multiple etiologies have been considered. Although there is no definite proof of it, it is most probable that leprosy reactions are a manifestation of the host immune reactivity. It is also possible that massive destruction of bacteria due to the host defensive mechanisms, to the effect of antileprosy drugs, or to other causes, can also constitute a highly toxigenic and therefore injuring mechanism. The 'toxic etiology' of the leprosy reactions can accompany their 'immunological etiology'. This type of leprosy reaction is triggered by very diverse factors: pregnancy, successful vaccination, abrupt climatological changes (higher incidence during the cold or rainy months of the year), intercurrent infections, physical or mental stress, anaemia, diverse parasitosis, etc.[9]

The histologic study of leprosy reactions type 2 in both ENL and Lucio's phenomenon, has revealed the existence of an inflammatory process that complicates the lepromatous skin histology. The inflammatory exudate, essentially made up of PMN leucocytes and variable numbers of lymphocytes and plasma cells, extends through the whole dermis and accumulates around the blood vessels and in the vicinity of the foamy histiocytes of the granulomatous tissue. In general, polymorphs are free of intact bacilli but they may contain fragmented bacillary material. There is an intense vasculitis, sometimes with fibrinoid necrosis and endothelial thickening of the deep vessels, as well as capillary necrosis in the more superficial lesions. Some oedema may be observed in the tissue adjacent to the injured vessels. In ENL, the inflammatory changes (vasculitis and cellular infiltrate) affect, principally, the deep dermis and hypodermis. The affected vessels are those of medium calibre and the changes are, mainly endothelial thickening. In Lucio's phenomenon, the inflammatory changes affect the whole dermis. The epidermal lesions are secondary to the lesions that affect the dermal and hypodermal blood vessels. In this case, small and medium calibre vessels are affected. They often present thickening, obstruction and necrosis.[10]

Such a type of inflammatory infiltrate and vasculitis resembles the histology of the experimental Arthus reaction. In the Arthus' reaction, the observed changes have been explained on the basis of a type III hypersensitivity reaction[11] which is triggered by the presence of immunocomplexes after their deposition on the vascular endothelia. Complement and PMN also participate. The above-mentioned resemblance stimulated several researchers to look for elements of the immune response in the lesions that characterize this type of leprosy reactions. With the aid of immunofluorescent techniques, granular deposits of IgG and IgM

immunoglobulins and C3 have been found in 50% to 60% of the studied ENL biopsies.[11, 12] The same biopsies that were positive for immunoglobulins were so for C3 complement, and in 35% of the biopsies, mycobacterial antigen was detected by its reaction with an undiluted goat antiserum to *Mycobacterium tuberculosis*.[13] In a more recent study, Ridley & Ridley,[14] using an anti-BCG antibody, have also shown the presence of mycobacterial antigen (in the form of immunocomplexes) both extracellular and within neutrophils and macrophages in skin biopsies of patients with ENL.

Granular deposition of IgG and IgM immunoglobulins and complement (C3 and C1q) have been detected on the walls of the dermal blood vessels and in perivascular areas in the biopsies from 2 patients with Lucio's phenomenon.[15]

Apart from these findings, the immunological etiology of the leprosy reaction type 2, finds some support in the demonstration of a higher incidence of circulating immunocomplexes in lepromatous patients undergoing an ENL or Lucio's reaction.[1, 16] Very often, a leprosy reaction of ENL or Lucio's phenomenon type does not appear alone but associated with some of the following systemic manifestations: fever, adenopathy, arthralgias, neuritis, asthenia, iridocyclitis, epididymo-orchitis and some others. These systemic manifestations are analogous to those found in serum sickness which is known to be associated to the presence of circulating immunocomplexes. Other relevant associations with type 2 leprosy reaction are the high incidence of mixed cryoglobulinemia,[14] alterations in the complement levels, often with elevation of C2[17] and C3,[11] the elevated concentrations of IgG euglobulin,[18] and glomerulonephritis.[19, 20]

Based on our observations (see Results), we suggest that *apart* from the immunocomplexes deposition, other factors could participate in the triggering of the injuring type-2 leprosy reactions. One of those factors could be the massive bacterial load (intact or degraded bacilli) inside the tissue PMN and macrophages that leads to the disruption of these cells releasing chemotactic, hydrolytic and other inflammatory factors that eventually cause vasculitis. The role of macrophage secretory products in chronic inflammatory processes has already been clearly established[21] and there is also the possibility that glycolipid and peptidoglycan components of mycobacterial walls also trigger release of mediators, without causing cell death. Additionally, immunocomplexes-mediated tissue damage would also account for the vasculitis that characterizes these type-2 leprosy reactions.

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