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An Immunoperoxidase Study of Immunological Factors in Skin Lesions Across the Spectrum of Leprosy[1]

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Although immunity in leprosy is known to be primarily cell-mediated, humoral factors have been postulated to play a significant role in the modulation or depression of the immune response, especially in lepromatous disease ([2, 18]) and in the induction of reactions ([2]). Interest in this aspect of immunology has been reflected in recent work. All reports agree that there is a progressive increase, from BT to LL, in the incidence of serum antibodies which react with mycobacterial antigens ([11,15,24]). Similarly, serum levels of immunoglobulins are raised in leprosy by comparison with healthy controls, though more so in the lepromatous than the tuberculoid form, and least in borderline ([12, 20, 21]). Other authors have confirmed the rise in the lepromatous form, or failed to detect it in tuberculoid patients ([4]). Levels of circulating complement (C3) vary widely, but the means are lower in lepromatous leprosy than in control sera ([14,21]). C3d is not significantly increased in lepromatous leprosy except during episodes of erythema nodosum leprosum (ENL), and never in the tuberculoid form; but Clq binding activity was increased in all groups ([1]). Alpha-1-antitrypsin was found to be elevated in lepromatous sera, and the levels did not correlate with those of immunoglobulin ([6]).

Immunological factors present in lesions are at least as relevant as those in serum, probably more so. Faber and Leiker ([9]) attempted to assay the immunoglobulin and complement in skin lesions by immunofluorescence, but were able to demonstrate few or no specifically staining cells in the lesion, though there were some deposits in the skin structures. The immunoperoxidase technique affords a more sensitive means of investigating the immunological factors in the tissue lesions. It has not so far been exploited, and we have found no reference to its application in leprosy apart from the study of lysozyme by Rea and Taylor ([16]). In the present study we employ this technique for an evaluation of immunoglobulins, complement components, and other immunological agents across the leprosy spectrum. A comparison between the results in lepromatous leprosy and those in ENL will be the subject of a separate communication.

MATERIALS AND METHODS

Patients. Twenty-eight patients throughout the leprosy spectrum (24 untreated, 4 treated) were included in the study. Skin biopsies were received from the Medical Research Council Units at Sungei Buloh, Malaysia, and Addis Ababa, Ethiopia. They comprised material from men and women of different racial origin and age. Patients were classified according to the method described by Ridley ([17]). There were 4 TT patients, 4 BT, 5 BB, 5 BL, 6 LL active, and 4 LL regressing. Patients in reaction were excluded from the study.

Method. Biopsies were fixed in FMA ([17]). The mercuric chloride and acetic acid present in this fixative are ideal for immunoperoxidase ([8]). Specimens were processed routinely for hematoxylin eosin and a modified Fite-Faraco stain for acid-fast bacilli ([13]), which was superimposed on positive immunoperoxidase stained sections.

Immunoperoxidase. Sections were cut at 4 μm and air dried. Antisera used were directed against IgG, IgM, C3, Clq, C3d, plasminogen, muramidase (lysozyme), C-reactive protein, and α-1-antitrypsin (Mercia).

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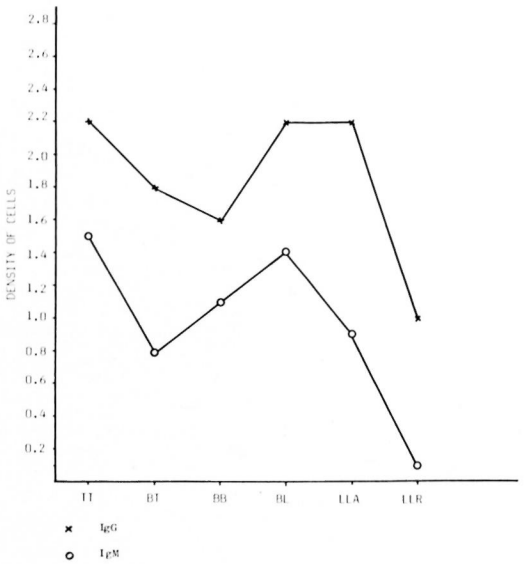

FIG. 1. The immunoperoxidase index (density of positive-staining cells on a 0 to +++ scale) for immunoglobulins in the granuloma across the leprosy spectrum. LLA = active LL. LLR = regressing LL.

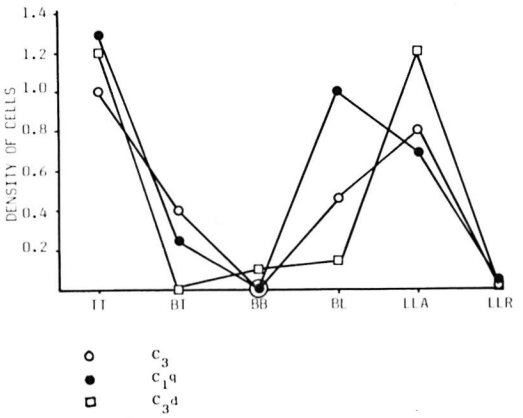

FIG. 2. The immunoperoxidase index (density of positive-staining cells on a 0–+++ scale) for complement components in the granuloma across the leprosy spectrum. LLA = active LL. LLR = regressing LL.

The peroxidase-anti-peroxidase technique of Burns (5) was followed with minor modifications. Trypsination was not found to be of value and was not included in the method finally adopted. The sections had to be flat and thoroughly dried at 37°C. Optimum dilution of antisera was determined by using skin sections fixed in FMA. Control sections included normal rabbit serum in place of antisera. Sections for Ia antigen were treated differently from the others by the two stage indirect method, the reason being that the antibody was prepared in mice instead of rabbits. Positive results were analyzed and recorded on an antibody scale of 0 to +++ and the means of each group plotted on a graph. Particular attention was given to cells of the granuloma, lymphocytes, mononuclear phagocytes, and fibroblasts.

RESULTS

The immunoperoxidase staining was found to be reproducible from one section to another, and the assessment of the results was also consistent from one observer to another. There was a slight difficulty in comparing one part of the spectrum with another insofar as some of the immunological factors were distributed differently in the lesion, but any errors on this score must be small. Plasma cells which contained large amounts of immunoglobulin reacted also for other factors, in particular C3, Clq, plasminogen, and α-1-antitrypsin. At least in part this staining of plasma cells was thought to be non-specific and it was discounted. However, it was not possible to be certain of the identity of every cell, and we report the main cell types. The results were fairly consistent between the different patients in each of the five groups of the spectrum and in LL in regression.

The overall scores for each of the factors tested throughout the spectrum are shown graphically in Figures 1, 2, and 3. The scores refer to staining in or close to the granuloma. The results for the connective tissue and epidermis are dealt with separately.

Immunoglobulins. There were two significant peaks of comparable intensity for both IgG and IgM, one at TT, the other at BL or LL. The IgG level was consistently higher than IgM throughout. In TT, BT, and BB, IgG and IgM were found mainly in the cytoplasm of certain lymphocytes (Figs. 4 and 5), although in the case of the smaller lymphocytes the immunoglobulins appeared to be on the cell surface. The higher score in TT than BT correlated with the larger total number of lymphocytes in the former. Plasma cells were present in

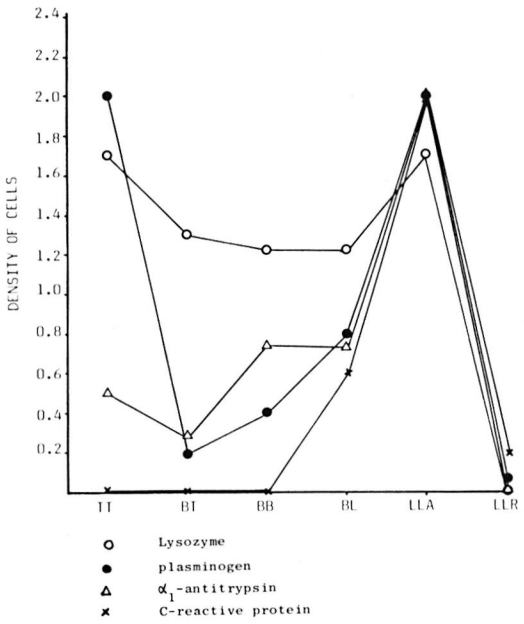

FIG. 3. The immunoperoxidase index (density of positive-staining cells on a 0 to +++ scale) for immunological mediators across the leprosy spectrum. LLA = active LL. LLR = regressing LL.

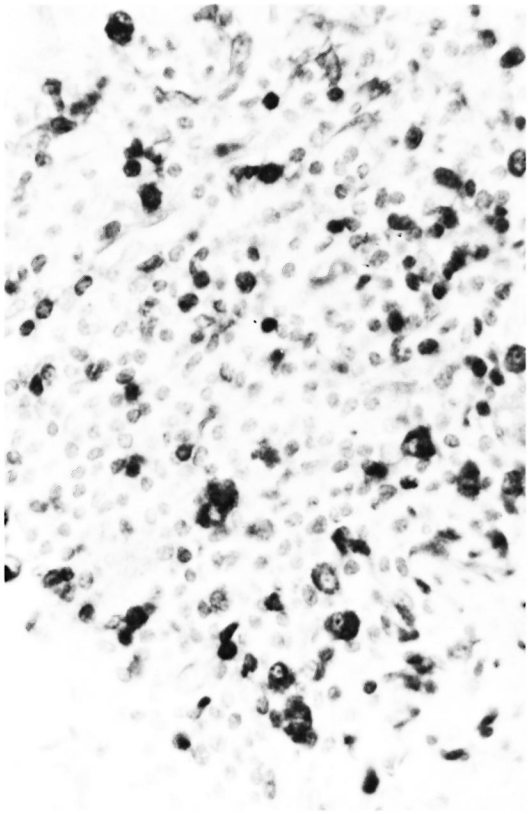

FIG. 4. IgG producing cells present in considerable numbers among the lymphocytes on the periphery of a TT granuloma (H & E superimposed on anti-IgG immunoperoxidase ×300).

smaller numbers, but all contained immunoglobulins. The immunoglobulin containing cells were situated on the periphery of granulomas, especially in the sub-epidermal zone and around nerves.

In BL and LL, IgG and IgM were found mainly in plasma cells but also in some lymphocytes and in certain macrophages with small numbers of ingested bacilli. The IgG containing cells were present within the granuloma. In active LL, there was also some deposition of immunoglobulin intercellularly or around blood vessels. Kappa and lamba chains were demonstrated in about equal numbers of cells.

Complement components. All three components of complement (C3, C3d and Clq) showed peaks at TT and at BL or LL, the highest being in C3 at LL (Fig. 6). C3 was present mainly in macrophages, to a lesser extent in some giant cells, but not in epithelioid cells. In TT it was found in mononuclear cells situated towards the periphery of each epithelioid cell granuloma mass. In BL and LL the complement-containing macrophages were distinct cells, devoid of acid-fast bacilli (AFB) which were present in moderate numbers throughout the granuloma. C3d (Fig. 7) was present in smaller amounts in cells similar to those with C3. Clq was present in the same cells as C3, and also in epithelioid cells in TT in addition to the intercellular spaces of the granuloma (Fig. 8).

Other factors. Plasminogen was present to a small extent in epithelioid cells with more in giant cells, and in the macrophages of lepromas, especially those with globi. The greatest peak was at TT, where it was also found extracellularly and on the surface of some lymphocytes. Alpha-1-antitrypsin was present mainly in active LL lesions, in macrophages with AFB present, more at the periphery than the center. Lysozyme was found mainly in TT in the form of granules in epithelioid cells (Fig. 9) and in LL in the macrophages without AFB. C-reactive protein, found in macrophages

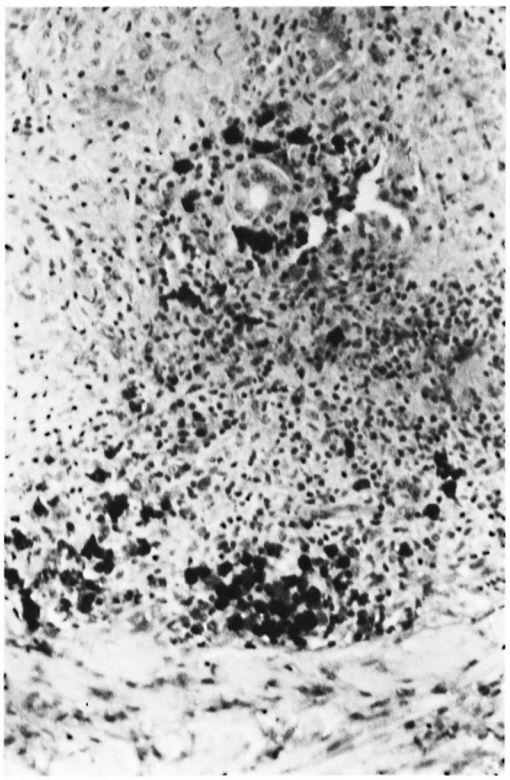

FIG. 5. Clusters of IgG producing cells in a BT granuloma (H & E superimposed on anti-C3 immunoperoxidase ×100).

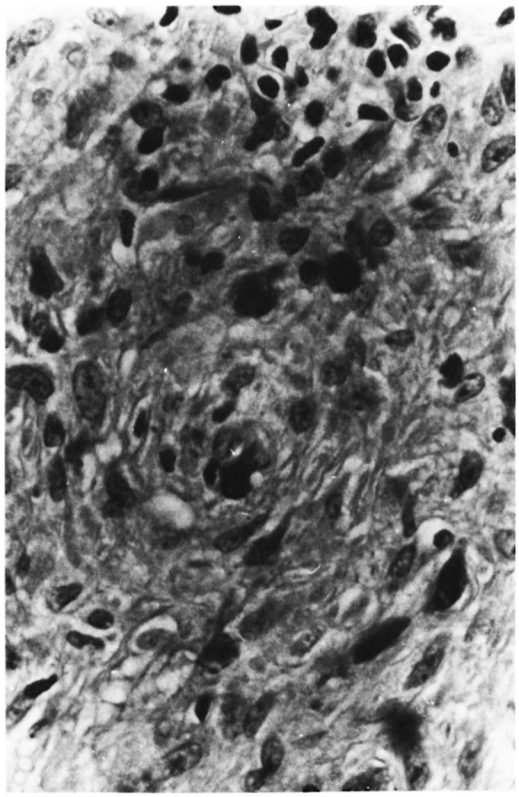

FIG. 6. C3 producing macrophages near a capillary in an LL granuloma (H & E superimposed on anti-C_3 immunoperoxidase ×600).

or other host cells, was related directly to the presence of bacilli. Ia antigen was found in 4 out of 4 TT lesions, and not in any other cases throughout the spectrum. It was present mainly in large pale cells with dendritic processes among the lymphocytic exudate below the epidermis, also in giant cells and a few epithelioid cells.

Regressing lesions. The foregoing results apply to active lesions. The only lesions examined in regression were those of LL patients. These showed a conspicuous drop in the level of all immunological factors by comparison with active lesions of the same group.

Epidermis and connective tissue. IgG was seen as an intercellular deposit along the basement membrane of the epidermis whenever it was present in large amounts (TT and LL). IgG, and to a lesser extent IgM, was present also in the active fibroblasts (not fibrocytes) in the dermis in all groups, especially around the periphery of an active granuloma. Complement (C3 and Clq) was observed in active fibroblasts, mainly in BB; and Clq was seen in cells of the epidermis. All these factors were absent from the fibroblasts in fibrotic, non-infective lesions used as controls.

DISCUSSION

The immunoperoxidase technique is a valuable tool for the investigation of a variety of immunological factors in cells or intercellular spaces of a granulomatous lesion, but it cannot be used uncritically. As Heyderman ([10]) points out, with presently available commercial antisera the label on the vial may be taken to indicate that the designated antibody is present, but not that other antibodies are necessarily absent. In our own work it seemed clear that a number of the antisera employed, especially those for complement, reacted with high levels of immunoglobulins in plasma cells, though these cells are known not to be major pro-

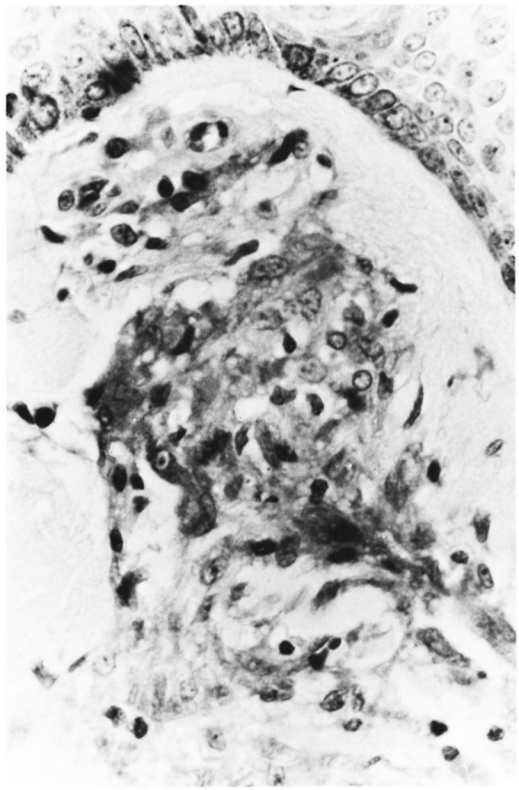

FIG. 7. C3d present as a faint deposit in macrophages around a papillary vessel in LL (H & E superimposed on anti-C3d immunoperoxidase ×500).

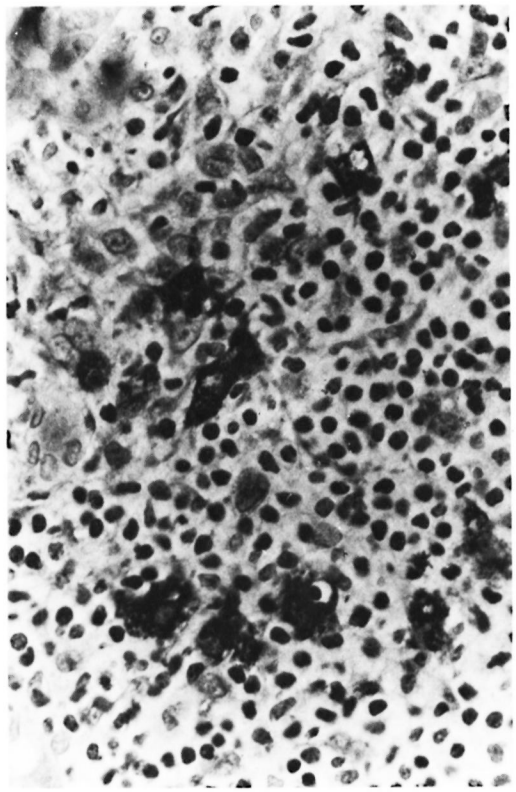

FIG. 8. Clq in epithelioid cells (or cells of mononuclear origin) in a TT granuloma. Note the diffuse outlines due to export into the extracellular spaces (H & E superimposed on anti-Clq immunoperoxidase ×500).

ducers of complement ([22]). We decided to discount these results.

The present results suggest that the levels of immunoglobulins at the site of skin lesions in leprosy parallels fairly closely the circulating levels, though the two have not been compared in individual patients. Thus our findings in lesions are in close agreement with those of several serum studies ([12, 20, 21]), from which it appears that IgG, and perhaps IgM, is elevated in TT leprosy almost to the level of LL, while there is an appreciable dip in the BT-BB region. We have not tested for specific mycobacterial antibodies in lesions, but it is interesting that two groups of workers ([11, 15]) have found a dip in serum antibody levels in BT or even in the TT-BT position, which has received little comment. The gradient from BT to LL is a straight increase. Thus it would appear that there is an elevation of antibodies in polar tuberculoid leprosy (TT) which is paradoxical to the antigen gradient. On our results it appears to be associated with the large number of lymphocytes in the lesions of the TT group, many of which are immunoglobulin producing, and therefore presumably B cells. On these results it would appear that in TT lesions there is a relative antibody excess by comparison with LL in which there is a relative antigen excess. In the BT-BB region the levels of antigen and antibody are moderate or low and the ratio between the two is equivocal.

Complement (C3) in our skin lesions produced peaks in TT and LL similar to those of the immunoglobulins, but it does not seem to correlate with the reported levels of circulating C3. There is more in the lesions. Thus, circulating C3 was found to be decreased in leprosy by comparison with controls ([21]), while serum C3d was seldom increased in lepromatous leprosy (except

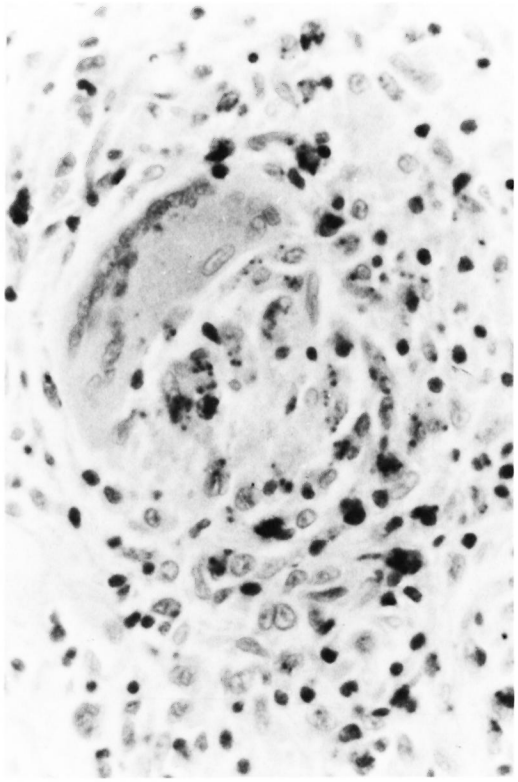

FIG. 9. Muramidase (lysozyme) present as granules in mononuclears, epithelioid and giant cells in TT (H & E superimposed on anti-muramidase immunoperoxidase ×300).

during reactions) and never in tuberculoid disease ([1]). The explanation, no doubt, is that circulating C3 is synthesized primarily in the liver ([7]), whereas in tissue it can be synthesized by macrophages ([3, 12, 22, 23]), which is what we observed. Since we also found C3d in similar areas of the same lesions, it can be concluded that C3 is synthesized, fragmented, and utilized in leprosy lesions, especially TT and LL. The cells involved appeared to be mononuclear cells or young macrophages which had not yet ingested bacilli (synthesizing macrophages). Clq was found in the same cell types and also in epithelioid cells.

The fact that complement and bacilli were usually found in different sorts of macrophages, and that immunoglobulin was mainly in plasma cells or lymphocytes, indicates that the substances we have been staining with immunoperoxidase are not for the most part immune complexes. Nevertheless, some macrophages with a few bacilli appeared to have taken in immunoglobulin. It would be surprising if complexes did not form in the intercellular spaces of the granuloma, where there was usually some diffuse staining due to exocytosis.

The other factors which we investigated were lysozyme, plasminogen, C-reactive protein, and α-1-antitrypsin. All four produced a peak in LL, but the last two did not peak in TT. Thus the curves for lysozyme and plasminogen were parallel to those of immunoglobulins and complement and are in agreement with the results of Rea and Taylor ([16]) for lysozyme. These substances were present mainly in cells of the mononuclear phagocyte series. As far as we know, plasminogen, as opposed to plasminogen activator, has not previously been demonstrated in macrophages.

There was a noticeable tendency for all these factors, including immunoglobulins and complement, to be distributed in cells mainly near the periphery of the granuloma in tuberculoid lesions and in cells around blood vessels near the center of a granuloma in lepromatous lesions. There was an impression that in lepromas the influx of cells built up around small blood vessels, while in tuberculoid lesions the central vessels tended to be blocked by endothelial swelling or they were obliterated, and the influx came from the periphery. This aspect is to be explored in a separate report.

Every one of the immunological factors for which we tested was found to decline during regression of lepromatous infections. But as regards active infections there was in every instance an increase across the spectrum from BT to LL, which was broadly parallel to the increase in the bacterial load. As regards immunoglobulins, it is reasonable to suppose that the increase was a secondary response to the antigenic load. The other factors, including complement, all appear to be synthesized by the cells of the mononuclear phagocyte series within the lesion. Some of the increase here could also perhaps be a secondary response, arising from the nature of macrophages in chronic inflammation to set up a self-activating and self-perpetuating system ([18,19]). The present results are consistent with this hypothesis, though they do little to prove it. What these results emphasize

is that whereas the spectrum from BT to LL illustrates a single mode of response whose strength is determined by the level of the antigenic load, the response in TT lesions is of a different order and independent of the antigenic load, which is minimal. This suggests that although there are important differences between BT and LL arising from the differences in the antigenic load, BT (the common form of tuberculoid leprosy) shares with LL the defect which leads to immunological failure in susceptible people. TT by contrast (a rare group) alone appears to exemplify the sort of response which might perhaps be regarded as adequate and normal for the majority of the population. If this is true it would imply that for some purposes the distinction between lepromatous and "tuberculoid" (usually BT) is not the one that matters.

SUMMARY

The immunoperoxidase technique was used to assess the quantity and situation of various immunological factors in 24 skin biopsies which represented the leprosy spectrum from TT to LL. The factors were immunoglobulins (IgG and IgM), complement components (C3, C3d and Clq), plasminogen, muramidase (lysozyme), C-reactive protein and α-1-antitrypsin. The results were compared with previous reports on the assessment of these factors in serum.

The quantities of these factors in the lesions produced peaks at TT and LL, with a dip in the BT-BB region (C-reactive protein and α-1-antitrypsin excepted). The immunoglobulins, present mainly in plasma cells and lymphocytes, correlated in general with reports of serum levels. The complement components were present in appreciable amounts, though the serum levels are depressed; they were seen in young mononuclear cells with a low bacterial load. All factors produced an ascending gradient in active lesions from BT to BL or LL, which correlated with the bacterial load and its viability. In regression (studied only in LL) there was a decrease in all factors. In TT there was an increase in most factors which did not correlate with the antigen load, and which probably resulted in an excess of antibody over antigen. In active LL there is probably an antigen excess.

The results suggest the possibility that there is a common defect from BT to LL, in which the generation of immunological factors within the lesion is a secondary response to the antigenic load. In TT alone (a rare group) is there an enhanced immunological response unrelated to the antigen load. In support of this was the finding of Ia antigen only in TT lesions.

RESUMEN

Se usó la técnica de la inmunoperoxidasa para buscar la cantidad y situación de varios factores inmunológicos en 24 biopsias de piel que comprendían a todo el espectro de la lepra desde el extremo TT hasta el extremo LL. Los factores fueron inmunoglobulinas (IgG e IgM), componentes del complemento (C3, C3d y Clq), plasminógeno, muramidasa (lisozima), proteína C-reactiva y α-1-antitripsina. Los resultados se compararon con reportes previos sobre la determinación de estos factores en suero.

Las concentraciones de estos factores en las lesiones mostraron picos en las regiones TT y LL, con un valle en la región BT-BB (excepto para proteína C-reactiva y α-1-antitripsina) del espectro. Las inmunoglobulinas, presentes principalmente en células plasmáticas y en linfocitos, correlacionaron en general con los reportes sobre sus niveles séricos. Los componentes del complemento se encontraron en cantidades apreciables, aunque los niveles séricos estuvieron deprimidos; estos componentes se observaron en las células mononucleares jóvenes con baja carga bacteriana. Todos los factores conformaron un gradiente ascendente en las lesiones activas de la porción BT a la BL o LL del espectro, que correlacionó con la carga bacteriana y con su viabilidad. En regresión (estudiada sólo en LL) hubo una disminución de todos los factores. En TT hubo un incremento en la mayoría de los factores que no correlacionó con la carga antigénica. Esto, probablemente, dió como resultado un exceso de anticuerpo sobre la cantidad de antígeno presente. En LL activa hay probablemente un exceso de antígeno.

Los resultados sugieren la posibilidad de que haya un defecto comun de BT a LL, en el cual la generación de factores inmunológicos dentro de la lesión es una respuesta secundaria a la carga antigénica. En TT (un grupo raro) hay una respuesta inmunológica incrementada sin relación con la carga antigénica. En apoyo de esto estuvo el hallazgo de antígenos Ia sólo en las lesiones TT.

RÉSUMÉ

On a eu recours à une technique par immunoperoxydase pour évaluer la quantité et la localisation de divers facteurs immunologiques dans 24 biopsies cutanées, provenant de malades représentant tous le spectre de la lèpre, depuis la forme TT jusqu'à LL. Les facteurs recherchés étaient les suivants: les immunoglobulines (IgG et IgM), les constituants du com-

plément (C3, C3d et Clq), le plasminogène, la muramidase (lysosyme), la protéine C-réactionnelle, et l'antiytripsine α-1. Les résultats ont été comparés avec des études antérieures portant sur l'évaluation de ces facteurs dans le sérum.

Les concentrations de ces facteurs dans les lésions ont révélées des valeurs maximales pour les malades TT et LL, avec des fréquences abaissées dans la région BT-BB, à l'exception toutefois de la protéine C-réactionnelle et de l'antitrypsine α-1. Les immunoglobulines, trouvées principalement dans les plasmocytes et dans les lymphocytes, présentaient en général une corrélation avec les taux sériques. Les constituants du complément étaient présents, en quantité notable, malgré que les taux de sérum étaient abaissés; on les a observés dans les cellules mononucléaires jeunes avec une charge bactérienne faible. Tous ces facteurs montraient une valeur ascendante dans les lésions actives, de BT jusqu'à BL ou LL; ces valeurs étaient en relation avec la charge bactérienne et avec la viabilité des bacilles. Lorsque l'on a procédé à une étude de régression, qui n'a par ailleurs été pratiquée que pour les échantillons provenant de malades LL, on a observé une diminution pour tous ces facteurs. Pour les échantillons TT, on a noté une augmentation de la plupart des facteurs, sans corrélation avec la charge antigénique, et qui était probablement la conséquence d'un excès d'anticorps par rapport aux antigènes. Dans les échantillons LL actifs, il y avait probablement un excès d'antigènes.

Ces résultats suggèrent qu'il pourrait exister un défaut commun, qui touche les malades allant de la forme BT à la forme LL, et qui consisterait en la production de facteurs immunologiques à l'intérieur de la lésion comme réponse secondaire à la charge antigénique. C'est seulement dans les échantillons TT (qui constituent d'ailleurs un groupe rare), qu'il existe une stimulation de la réponse immunologique sans relation avec la charge antigénique. L'observation de l'antigène Ia renforce cette hypothèse seulement dans les lésions TT.

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