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Muramidase (Lysozyme) Findings in Sural and Radial Nerve Biopsies in Leprosy Patients After Varying Periods of Treatment[1]

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In tissues and secretions, Sir Alexander Flemming (6) was the first to show that lysozyme was the bacteriolytic element. Since the enzyme acts on muramic acid linkages in bacterial cell walls, the term muramidase has also been used as a synonym of the enzyme. Interest in this enzyme has remained high since the enzyme has been found to be raised in several diseases, indicating that it might be of diagnostic and/or prognostic value.

Recently, immunohistological techniques have been used to study the distribution of muramidase in tissues. Mason and Taylor (12) have thus shown that the enzyme could be found in serous salivary acinar cells, lactating mammary tissue, Paneth's cells, renal tubular cells, myeloid cells (including eosinophils), and histiocytic cells. In pathological tissues, they found marked positivity in reactive histiocytic cells such as found in granulomatous conditions like tuberculosis and Crohn's disease. The distribution pattern of the enzyme intracellularly was not described.

Other studies have demonstrated elevated serum muramidase levels in tuberculosis (9, 11, 17), sarcoidosis (16), inflammatory bowel diseases (3, 4), and myeloproliferative diseases (20, 25). Blennerhassett and Papadimitriou (1) studied the enzyme in several granulomatous reactions, and Rea and Taylor (18) studied its levels in serum and its distribution in tissues across the leprosy spectrum. In untreated leprosy patients, serum muramidase levels were elevated and particularly high levels were found in patients with severe reversal reaction or Lucio's phenomenon. These levels tended to decline with prolonged sulfone therapy. In tissues, two distinct staining patterns for intracellular muramidase were described. A granular pattern was seen in epithelioid and giant cells; whereas a saccular pattern was found in the lepromatous histocytes. These findings were later confirmed by Cologlu (2). In both studies, peripheral nerves were not included. Our study was therefore designed to study muramidase distribution in nerves and to compare the findings with those described in the skin of leprosy patients. We also stained several of the biopsies with Congo red to detect the presence of amyloid deposits in these nerves.

MATERIALS AND METHODS

Patients were randomly selected from among those admitted to the All Africa Leprosy and Rehabilitation Training Center (ALERT), Addis Ababa, Ethiopia. All of the patients had had oral sulfone (dapsone, DDS) 100 mg daily for varying periods of time, ranging from one month to 20 years, and others had received rifampin in addition to DDS (Tables 1 and 2). Patients with reversal reaction were given prednisolone. Compliance to drug treatment was difficult to assess, but a review of the patients' medical records indicates that many of them had almost certainly been irregular in their drug intake. We studied 34 Ethiopian patients, 24 males and 10 females, ranging in age from 15–60 years.

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TABLE 1. *Seventeen patients with lepromatous (LL) and borderline lepromatous (BL) leprosy.*

Biopsy no.	Sex	Age	Therapy		Bacilli		Histological diagnosis		Muramidase[b]	
			Drug(s)[a]	Duration (yr)	Skin	Nerve	Skin	Nerve	Skin	Nerve
1	F	46	DDS	1	NA[c]	0	NA	LL	NA	Granular +
2	M	31	DDS	10	Few	0	LL	LL	Saccular +	Saccular +
3	M	57	DDS	7	Few	0	LL		0	Doubtful
4	M	63	DDS	12	Many	Many	LL	LL	Saccular ++	Doubtful
5	M	38	DDS/RMP	1	Many	Many	LL	LL	0	Saccular +++
6	M	23	DDS/RMP	1	Many	Many	BL	LL[d]	Saccular ++	Saccular +++
7	M	38	DDS	15	Few	Few	LL	LL	0	Saccular +
8	M	23	DDS	10	Few	Many	LL	LL	0	Saccular ++
9	F	10	DDS/RMP	12	Many	Many	LL active	LL active	Saccular	Saccular +++
10	M	60	DDS	6	Few	Many	NA	LL	0	Doubtful
11	M	16	DDS	1	Many	Many	LL	LL	Saccular ++	Saccular ++
12	M	12	DDS/RMP	2	0	0	LL	LL	Saccular +	Saccular ++
13	M	31	DDS	1	Many	Many	LL relapse	LL relapse	Saccular ++	Saccular +++
14	M	42	DDS/RMP	16	Many	Many	LL relapse	LL relapse	Saccular ++	Saccular ++
15	F	26	DDS	17	NA	Many	NA	LL	NA	Saccular ++
16	M	51	DDS	10	NA	Many	NA	LL	NA	Saccular ++
17	F	60	DDS	20	NA	0	NA	LL	NA	0

[a] DDS = dapsone, RMP = rifampin.
[b] 0 = negative, + = faintly positive, ++ = positive, +++ = intensely positive.
[c] NA = not available.
[d] LLs = subpolar lepromatous.

TABLE 2. *Twelve patients with borderline tuberculoid (BT) and tuberculoid (TT) leprosy.*

Biopsy no.	Sex	Age	Therapy		Bacilli		Histology		Muramidase[b]	
			Drug(s)[a]	Duration	Skin	Nerve	Skin	Nerve	Skin	Nerve
18	F	25	DDS	3 yr	0	0	BT	BT	Granular +	Granular ++
19	M	16	DDS	2 yr	0	0	BT/TT	Normal	Granular ++	Negative
20	F	23	DDS/Pred.	2 yr	0	0	Not diagnostic	BT	Negative	Negative
21	F	40	DDS/Pred.	3 mo	0	0	Inadequate material	BT	Negative	Granular ++
22	M	28	DDS/Pred.	5 yr	0	0	Not diagnostic	BT	Negative	Granular +
23	M	37	DDS	2 yr	0	0	BT	TT	Granular +	Granular ++
24	M	24	DDS	3 yr	0	0	BT	TT	Granular +	Granular ++
25	M	60	DDS	15 yr	0	0	Normal	TT	Negative	Granular +
26	M	33	DDS	10 yr	0	0	NA[c]	BT	NA	Granular ++
27	M	20	DDS	2 mo	0	Few	BB[d]	BB	Granular/ Saccular	Granular/ Saccular
28	M	22	DDS/RMP	2 yr	0	Many	Not diagnostic	BB/BT	Negative	Vacuolated +++
29	F	23	DDS/Pred.	4 mo	0	0	NA	BT	NA	Granular ++

[a] DDS = dapsone, RMP = rifampin, Pred. = prednisolone.
[b] 0 = negative, + = faintly positive, ++ = positive, +++ = intensely positive.
[c] NA = not available.
[d] BB = mid-borderline.

TABLE 3. *Five patients with reversal (delayed hypersensitivity) reactions.*

Biopsy no.	Sex	Age	Therapy		Bacilli in nerve	Histology[b]		Muramidase[c]	
			Drug(s)[a]	Duration (mo)		Skin	Nerve	Skin	Nerve
30	F	33	DDS	1	Few	BB-BT	BB-BT	Granular ++	Granular ++
31	F	27	DDS/Pred.	2	Few	BB-BT	BB-BT	Granular +++	Granular +++
32	M	16	DDS/Pred.	9	0	BB-BT	BB-BT	Granular +++	Granular +++
33	M	17	DDS/Pred.	4	0	BB-BT	BB-BT	Granular +++	Granular +++
34	M	46	DDS/Pred.	1	0	BB-BT	BB-BT	Granular +	Granular +

[a] DDS = dapsone, Pred. = prednisolone.
[b] BB = mid-borderline, BT = borderline tuberculoid.
[c] 0 = negative, + = faintly positive, ++ = positive, +++ = intensely positive.

Skin biopsies were taken from active lesions; whereas the sural nerve biopsies were taken from either of the two legs, irrespective of the presence or absence of a cutaneous lesion over the course of the nerve. The technique of nerve biopsy, fixation, processing, and staining with hematoxylin and eosin, as well as with a Fite-Faraco modification of Ziehl-Neelsen staining, is described elsewhere ([8]).

The peroxidase-antiperoxidase (PAP) immunohistological staining technique ([22]) was used to demonstrate muramidase enzyme in tissues. Briefly, this included deparaffinization of sections and taking to water, blocking endogenous peroxidase with absolute methanol containing 0.3% hydrogen peroxidase, application of 1:5 diluted normal swine serum, followed by incubation with rabbit anti-human muramidase antibody. The antibody was used at a 1:1000 dilution. Swine anti-rabbit serum immunoglobulin at a 1:20 dilution was then applied before incubation with horseradish peroxidase–rabbit antiperoxidase (PAP) complex. The PAP complex was used as a 1:100 dilution. Localization of the enzyme activity was revealed by a reaction with diaminobenzidine-tetrahydrochloride (DAB) containing 0.01% hydrogen peroxide. This reaction gives a brown product at the site of the antigen-antibody reaction. Sections were counterstained with hematoxylin, dehydrated, cleared, and mounted in DPX. The substitution of normal serum for the specific anti-muramidase antibodies provided control sections. A few blocks of histologically normal nerves were retrieved from the histology archives and processed in a similar way as those from leprosy patients.

All dilutions were done in Tris buffer (pH 7.6), while washes were done in Tris-saline buffer (a 1:10 dilution of Tris buffer in normal saline). Antibodies were purchased from Dakopatts Immunoglobulin A/S, Copenhagen, Denmark; DAB was from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

RESULTS

Using the Ridley-Jopling system ([19]), there were 16 lepromatous (LL), 1 borderline lepromatous (BL), 4 mid-borderline (BB), and 13 borderline tuberculoid (BT) or tuberculoid (TT) patients. Tables 1, 2, and 3 summarize our findings on the examination of nerve biopsies from these patients for muramidase. The criteria for describing the pattern as either saccular or granular were as close as possible to those described by Rea and Taylor ([18]) and by Cologlu ([2]) and as illustrated in their publications.

Two distinct patterns of muramidase staining were observed. In the saccular pattern, positive histiocytes and globi demonstrated a homogenous stain within vesicles or their lumen (Figs. 1 and 2). This form was characteristically seen in globi. On the other hand, the granular pattern consisted of uniformly distributed, intracytoplasmic, fine brown granules (Figs. 3 and 4), most intensely found in epithelioid cells and in giant cells when present.

In seven instances, skin material was not available for examination, and in a further three we found it impossible to use the terms

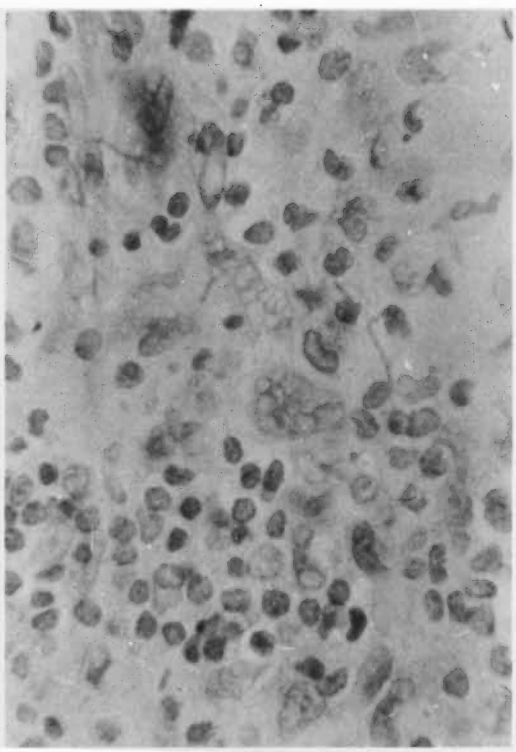

FIG. 1. Saccular pattern of muramidase staining in a number of globi (nerve biopsy from a lepromatous leprosy patient).

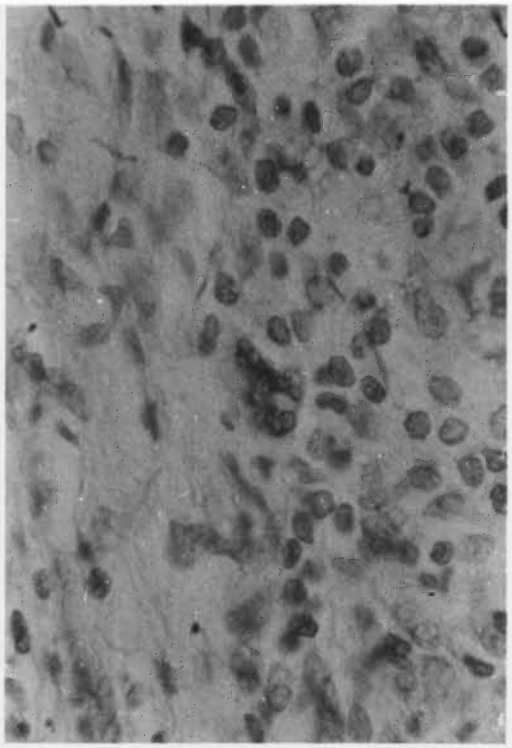

FIG. 2. Saccular pattern of muramidase staining in lepromatous leprosy nerve biopsy with emphasis on a globus.

saccular or granular with confidence and have recorded the results as "doubtful." A high overall agreement was obtained with previous publications in that a saccular pattern was found in all but one of the lepromatous cases (which anomalously had a granular pattern), and a granular pattern was found in 13 out of 16 (81%) of the borderline and tuberculoid cases. The most intense patterns of granular staining were found in the nerves of patients who had reversal reactions at the time of biopsy, as noted by Rea and Taylor in the skin. Schwann cells did not stain for muramidase. No muramidase-positive cells were found in the normal nerves.

Staining for amyloid by Congo red in 26 biopsies from the series of 34 (76.5%) was negative in all cases.

DISCUSSION

We set out to study the muramidase activity in nerves of leprosy patients. In the process, we found a surprisingly high percentage of gross abnormalities in the sural nerve biopsies, even in the absence of lesions involving the overlying skin. These findings have been described in detail in another publication ([8]).

The pattern of muramidase reaction in the nerves was found to parallel the reaction in the skin. In the nerves, however, the muramidase staining was more intense than that found in the skin of the same patient. Furthermore, in some patients the skin was negative for muramidase staining while the nerves showed a positive reaction, indicating that the enzyme tended to persist longer in the nerves than in the skin. While in some patients there was a correlation between the duration of treatment and the intensity of muramidase staining, a close scrutiny revealed an association between the number of acid-fast organisms and the muramidase staining. This could be due to the fact that most of our patients were irregular in their

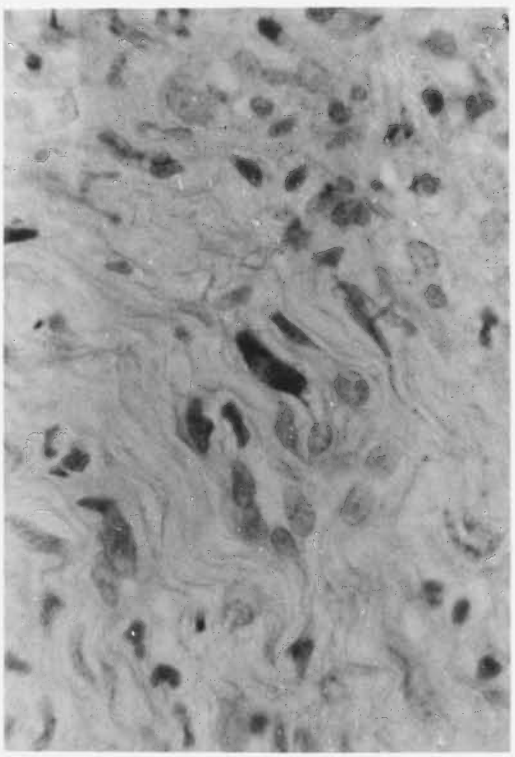

FIG. 3. Granular pattern of muramidase staining in a histiocyte (nerve biopsy from a tuberculoid leprosy patient).

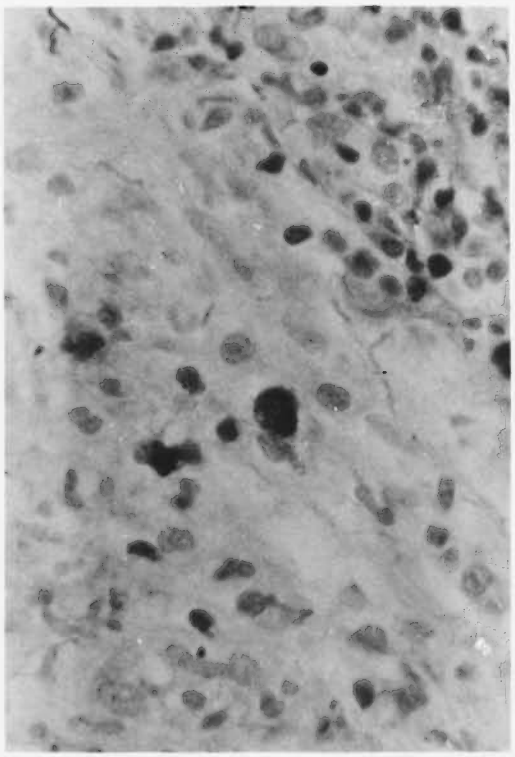

FIG. 4. Granular pattern of muramidase staining in a histiocyte (nerve biopsy from a tuberculoid leprosy patient).

drug intake and, thus, even a long period of treatment is not equivalent to a reduction in bacillary load. In general, patients with high bacillary loads had a more intense muramidase staining. This finding is interesting in the light of our knowledge that even when bacilli are not detectable in the skin, solid-staining, presumably viable, bacilli can be demonstrated in the Schwann cells of peripheral nerves ([21]). Since we were not able to demonstrate muramidase activity in Schwann cells, it is tempting to suggest that a deficiency of muramidase enzyme in these cells could confer a survival advantage to *Mycobacterium leprae* in these cells. On the other hand, it has been shown that *M. leprae* antigens persist for long periods in peripheral nerves ([14]) and, therefore, it is possible that these antigens could induce muramidase activity in phagocytic cells found in the nerves. This could perhaps explain the persistence of muramidase-positive cells in the nerves from borderline patients, even though no bacilli were seen in a majority of those patients.

The irregular distribution of muramidase among the cells of the granuloma examined may reflect the observation of Mason and Taylor ([12]) and Gordon, *et al.* ([7]) that muramidase is synthesized predominantly in "reactive" histiocytes rather than in the "resting" histiocytes. The strong intensity of muramidase staining during reversal reaction would tend to support this concept. During these reactions there is an increase in lymphocytes transformable by *M. leprae,* indicating that the reaction is a delayed-type hypersensitivity to *M. leprae.* These sensitized lymphocytes could, through mediators, be activating resident cells like epithelioid cells. After such an activation, these cells could then become strongly positive for muramidase. This suggestion was first put forward by Rea and Taylor ([18]).

Our inability to demonstrate muramidase activity in cells of neuronal origin suggests

that the cells infiltrating the peripheral nerves in leprosy are derived from circulating blood cells. This would, in turn, tend to support the suggestion that peripheral nerve involvement in leprosy is secondary to hematogenous spread ([5]).

As reported previously ([2, 18]), we saw two different patterns of muramidase staining. A saccular pattern was seen in the lepromatous end of the spectrum. The enzyme was visualized as a brown homogeneous material within the walls of small vesicles or saccules. A granular pattern, on the other hand, was found in the tuberculoid end of the spectrum and was particularly obvious during reversal reactions. Our findings are thus in accord with previous publications ([2, 18]) and do not support the findings of Yamashita, *et al.* ([24]), who were unable to demonstrate muramidase activity in tuberculoid patients.

Naik, *et al.* ([15]) have shown that serum lysozyme was reduced in patients treated with dapsone as compared with untreated patients, and this correlated well with the improvement in their clinical and bacteriological status. We also saw a reduced tissue lysozyme reaction in those patients who were successfully treated, as evidenced by a good histological response with absent, or very few, solid-staining viable bacilli.

The function of muramidase is not clearly understood. It is generally accepted to be bacteriolytic, but as Yamashita, *et al.* ([24]) have suggested, the presence of considerable lysozymal activity in macrophages filled with leprosy bacilli in patients with progressive lepromatous leprosy challenges the active role of lysozymal enzyme in effectively combating this particular infection. Kanetsuna ([10]), in his *in vitro* study on the effect of lysozyme on the growth of several strains of mycobacteria, has shown that lysozyme acts only on cell wall mucopeptide. Although hydrolysis of the cell wall did not result in the immediate death of mycobacteria, he believes that the hydrolysis by lysozyme may provide a first step in the complicated destruction of the bacilli.

The significance of the two patterns of muramidase activity in leprosy patients remains unknown. However, just as the peculiar distribution of T lymphocyte subsets in the various forms of leprosy may reflect microanatomical regulation of the immune response ([13]), it is possible that the different muramidase patterns reflect various levels of activation in resident cells of a leprosy granuloma. It might also be a manifestation of the kind of activation signals these cells are receiving. However, all of these points remain purely speculative, and further studies are required to understand the importance of these specific muramidase patterns.

In our study, which consisted of patients with long-standing leprosy, a thorough search for amyloid deposition in the skin and nerve tissues by the Congo red staining technique did not reveal any positivity. Williams and Cathcart ([23]), in their study of secondary amyloidosis among leprosy patients at Carville, U.S.A., and Guadalajara, Mexico, were impressed by the significant difference in the incidence of secondary amyloidosis in the two groups. Using similar investigatory methodology and patient selection, a high positivity of 31% in the American patients was discovered compared to only 6% in the Mexican group. Our findings, although involving only a small number of patients, may suggest a rather low incidence of secondary amyloidosis among our patients, at least in so far as nerve and skin tissues are concerned. This compares well with the Mexican population. Could this therefore imply a genetic susceptibility to amyloid deposition?

SUMMARY

Using the immunoperoxidase staining method, tissue muramidase (lysozyme) activity was studied in 34 nerve biopsies from leprosy patients and compared to findings in the skin. In a majority of lepromatous and borderline-lepromatous leprosy patients, the enzyme was seen to form a saccular pattern within the cells; whereas a granular pattern was found at the tuberculoid end of the leprosy spectrum, as well as during reversal reactions. Indeed, the most intense enzymatic activity was found in four patients with reversal reactions. Compared to the skin, muramidase activity was found to be more intense and persisted longer in the nerves. Successful antileprosy treatment reduced the enzymatic activity in both the nerves and the skin, but more so in the skin. Schwann cells and axons did not show muramidase activity, indicating that the muramidase-positive cells are not of neuronal

origin. Our results suggest that a high percentage of mononuclear cells infiltrating the peripheral nerves in leprosy are derived from blood monocytes.

The function of tissue muramidase in leprosy is not yet clear. Its peculiar intracellular distribution pattern in the different forms of leprosy, however, warrants further study to elucidate its role in the pathogenesis of the disease.

RESUMEN

Usando el método de la inmunoperoxidasa, se estudió la actividad de muramidasa (lisozima) tisular en 34 biopsias de nervios de pacientes con lepra y se comparó con la actividad encontrada en la piel. En la mayoría de los pacientes lepromatosos y lepromatosos-intermedios, la enzima se encontró con un patrón sacular dentro de las células, a diferencia del patrón granular encontrado en el extremo tuberculoide del espectro y durante las "reacciones reversas". De hecho, la más intensa actividad enzimática se encontró en 4 pacientes con reacciones reversas. Comparando con la piel, la actividad de muramidasa fue más intensa y persistió más tiempo en los nervios. El tratamiento antileproso exitoso redujo la actividad enzimática tanto en los nervios como en la piel pero ésto fue más marcado en la piel. Las células de Schwann y los axones no mostraron actividad de muramidasa, indicando que las células muramidasa positivas no son de orígen neuronal. Nuestros resultados sugieren que una alta proporción de las células mononucleares infiltrantes derivan de los monocitos de la sangre.

La función de la muramidasa tisular en lepra aún no está clara. Su patrón peculiar de distribución intracelular en las diferentes formas de lepra, justifican su estudio posterior para dilucidar su papel en la patogénesis de la enfermedad.

RÉSUMÉ

On a eu recours à la méthode de coloration par l'immunoperoxydase, pour étudier l'activité de la muramidase (lysosyme) des tissus dans 34 biopsies nerveuses prélevées chez des malades de la lèpre. On a comparé ces observations à celles faites au niveau de la peau. Chez la majorité des patients atteints de lèpre lépromateuse ou dimorphe-lépromateuse, on a constaté que l'enzyme présentait un profil en sac à l'intérieur des cellules. Par contre, un profil granulaire a été observé chez les malades atteints de lèpre tuberculoïde, qui se situe à l'autre extrémité du spectre clinique de la lèpre, de même qu'au cours des réactions de réversion inverse (reversal reactions). En fait, l'activité enzymatique la plus prononcée a été relevée chez 4 malades présentant de ces réactions inverses. Par comparaison aux observations faites au niveau de la peau, l'activité en muramidase était plus intense au niveau des nerfs; elle persistait également plus longtemps. Un traitement efficace contre la lèpre a permis de réduire l'activité enzymatique à la fois dans les nerfs et dans la peau. Cet effet était cependant plus prononcé dans la peau. Les cellules de Schwann, de même que les axones n'ont pas présenté d'activité typique de la muramidase, ce qui indique que les positives pour la muramidase ne sont pas d'origine nerveuse. Ces résultats suggèrent qu'une forte proportion des cellules mononucléaires qui infiltrent les nerfs périphériques au cours de la lèpre, sont dérivées des cellules monocytes du sang. La fonction de la muramidase tissulaire dans la lèpre n'est pas encore éclaircie. Les profils particuliers de la distribution intra-cellulaire de cette enzyme dans les différentes formes de lèpre soulignent cependant la nécessité de procéder à des études complémentaires, afin d'élucider son rôle dans la pathogénèse de la maladie.

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