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Acid Mucopolysaccharide Metabolism in Leprosy 2. Subcellular Localization of Hyaluronic Acid and ß-Glucuronidase in Leprous Infiltrates Suggestive of a Host-*Mycobacterium leprae* Metabolic Relationship[1, 2]

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In a preceding study ([22]), we extended the study of the distribution of hyaluronidase-labile and nonsulfated acid mucopolysaccharide (AMPS), previously reported by Hollander and Sommers ([15]) to be present in lepromas, to other types of leprosy. The most significant AMPS deposition consonant with hyaluronic acid (HA), as noted by Mabalay *et al* ([20]) and by us ([22]), was observed in the lepromas of active lepromatous leprosy and was present to a lesser extent only in the central portions of early granulomas of tuberculoid leprosy. There were intermediate degrees of distribution of AMPS between the poles corresponding to the case distribution within the immunopathologic spectrum. This distribution of AMPS seems to be in inverse proportion to the degree of cell-mediated immunity (CMI) in leprosy, just as lipid storage in lepromatous leprosy has been repeatedly reported by many investigators to present a similar inverse ratio ([9, 11, 12, 33, 34, 36]). As the deposited lipid in leprosy has been regarded as derived from *Mycobacterium leprae* ([11, 12, 33, 34, 36]), so the origin of HA should be determined in order to pursue understanding of its biological activity. If HA is derived from the host and *M. leprae* tend to grow in locales where HA is abundant, then HA might be regarded as a possible important nutrient for *M. leprae*. If, on the other hand, *M. leprae* produces HA as do type A streptococcus ([6, 17, 18, 19, 47]) and *Treponema pallidum* ([42]), then it might be regarded as a substance interfering with the host's defense mechanisms against leprosy. These two possibilities were raised in a previous study ([22]). Accordingly, biopsy specimens of leprosy skin were studied to determine the subcellular localization of AMPS and β-glucuronidase. Beta-glucuronidase as well as N-acetyl-β-glucosaminidase activity in leprosy lesions were also examined histochemically to differentiate the activities of the two enzymes since the latter may also play some part in the degradation of hyaluronic acid.

MATERIALS AND METHODS

Leprosy skin biopsies were processed for the observation of AMPS and β-glucuronidase by electron microscopy as well as for morphologic evaluation by light microscopy of the distribution of β-glucuronidase, N-acetyl-β-glucosaminidase and for evaluation by acid-fast and hematoxylin-eosin stains.

Sources of material. Wedge biopsies from active lepromatous (7 cases), borderline (3 cases) and tuberculoid (3 cases) leprosy were obtained from outpatients in Hong Kong, Okinawa and Saigon. The cases were classified according to the criteria of Ridley and Jopling ([29, 30]). All patients had well developed lesions and were untreated within the last five years or not treated at all. The biopsies were usually cut into two pieces of almost equal size. One piece was fixed in formol chloral hydrate ([39]) and the other in glutaraldehyde ([32]), after cutting into 1-2 mm bits. Glutaraldehyde fixed tissue was then washed with and preserved in cacodylate buffer containing 7% sucrose ([39]) and the tissues trans-

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ported on wet ice.

Electron microscopic histochemistry. Tissues were minced on a Sorvall TC-2 tissue sectioner by the method of Smith and Farquhar [38] at 20 and 40 microns respectively following either formol chloral hydrate or glutaraldehyde fixation. The sectioned tissues were then stained overnight with either Rinehart and Abul-Haj's modification of Hale's iron solution [31, 40] for glutaraldehyde fixed tissue to detect AMPS, or for one hour with 0.08 mM naphthol-AS-BI-β-D-glucuronide solution according to the method of Smith and Fishman [39] for detecting β-glucuronidase. The processed sections were post-fixed with phosphate buffered and plain osmic acid [39] respectively and embedded in epoxy resin after alcohol dehydration [3]. Foci of cellular infiltration were chosen by light microscopic observation of Paragon [41] stained sections. After sectioning on a Porter Bloom MT2-B ultramicrotome followed by uranyl acetate [44] and lead citrate [28], the tissues were examined with a Zeiss EM9 S-2 electron microscope having a fixed acceleration voltage of 60 kilovolt.

Light microscopic histochemistry. Portions of skin biopsies preserved in cacodylate buffer after fixation with formol chloral hydrate were sectioned at about six microns on a cryostat at −20°C and stained with the method for β-glucuronidase [39] followed by counterstaining with methyl green according to the method of Hayashi *et al* [14]. In selected cases the sections were also stained for N-acetyl-glucosaminidase according to the method of Hayashi [13].

Histopathology. Portions of formol chloral hydrate fixed tissue were embedded in paraffin, cut and stained with hematoxylin and eosin [25], Triff [46] and modified Ziehl-Neelsen [5] stains. These served to confirm the

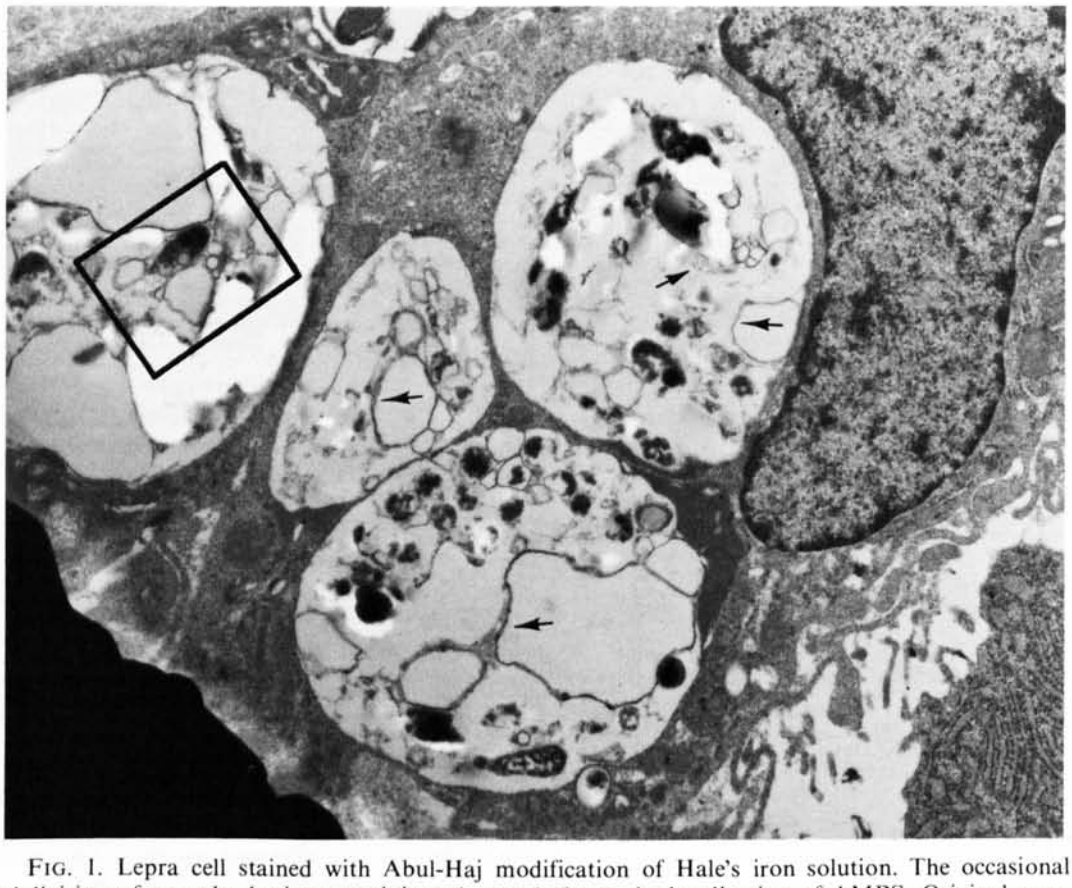

FIG. 1. Lepra cell stained with Abul-Haj modification of Hale's iron solution. The occasional subdivision of vacuoles by iron precipitate (arrows) shows the localization of AMPS. Original magnification: 9,500×. Lepromatous case: S-2699, 64-year-old male, BI = 4.3, MI = 30%, six years after the onset of the disease.

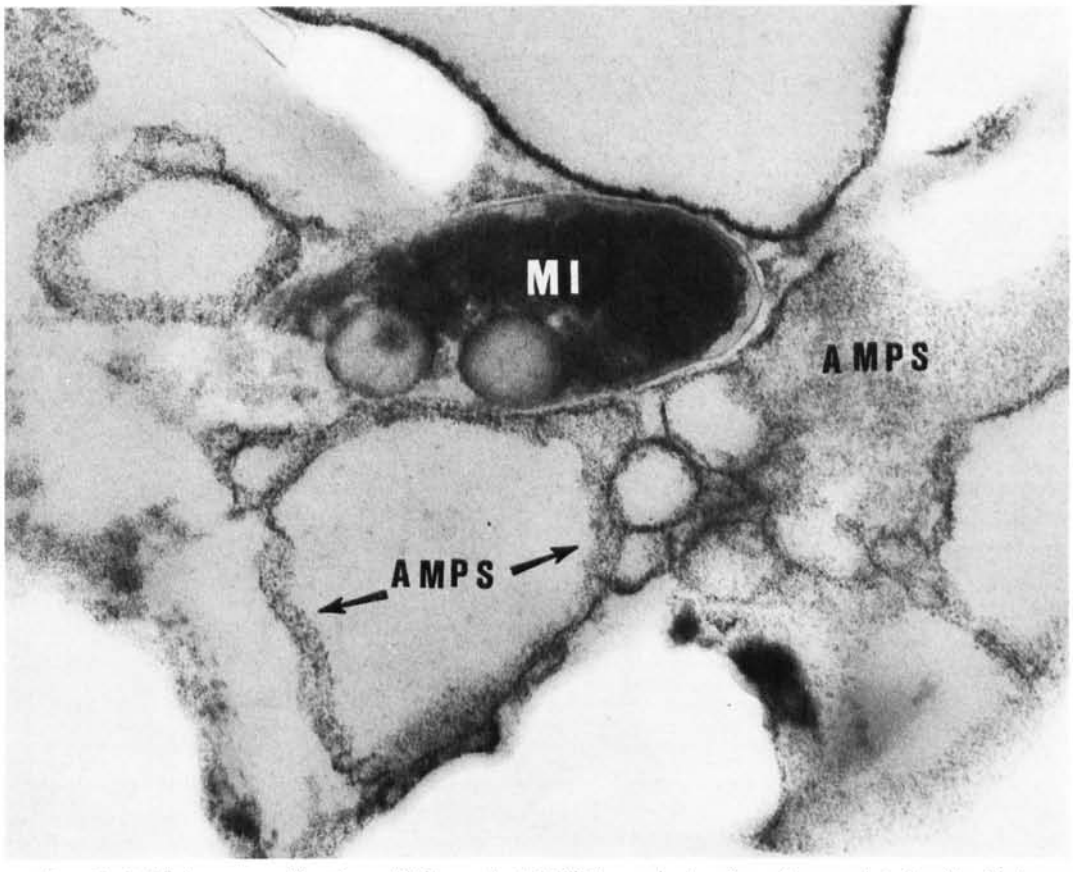

FIG. 2. A higher magnification of Figure 1. AMPS in vesicular form is associated with *M. leprae* (MI). Original magnification: 28,000×.

pathologic diagnosis, as well as the condition of the bacilli and cells participating in the infiltration.

RESULTS

In lepromatous cases (Figs. 1 and 2), AMPS is localized especially around *M. leprae* lying in the vacuoles of lepra cells. The vacuoles are subdivided by various indistinct lines due to the localization of AMPS which form vesicular structures, as shown in Figure 2, and some of the vesicles are attached to bacilli. *M. leprae* attached to heavily stained vesicular structures often seem to be well preserved. In this type of fully developed lepra cell the AMPS is not seen in either the cell membranes or phagolysosomal membranes. However, in some cases with higher Morphologic Indices, AMPS is seen in the membranes of phagolysosomes of lepra cells as shown in Figure 3. In this type of cell, the phagosomes contain more AMPS filling space between bacilli and do not show vesicular structure (Fig. 4). Beta-glucuronidase activity is found in and around *M. leprae* as shown in Figure 5. This enzyme activity is not seen in the lysosomes of lepra cells (Fig. 6). Although by light microscopy the enzyme activity seems to be diffusely present in the cytoplasm of lepra cells, in some lepromatous cases electron microscopy revealed that the localization of the enzyme activity is not in the lysosomes but in and around bacilli. In the majority of the lepromatous cases, the above findings were supported by the localization of β-glucuronidase in vacuoles in lepra cells where bacilli were localized (Figs. 7 and 8). N-acetyl-β-glucosaminidase activity was, however, localized only in the cytoplasm of lepra cells and not in the vacuoles where numerous bacilli were located.

In borderline cases, early epithelioid cells contained a small amount of AMPS in lysosomal membranes (Fig. 9) and occasionally

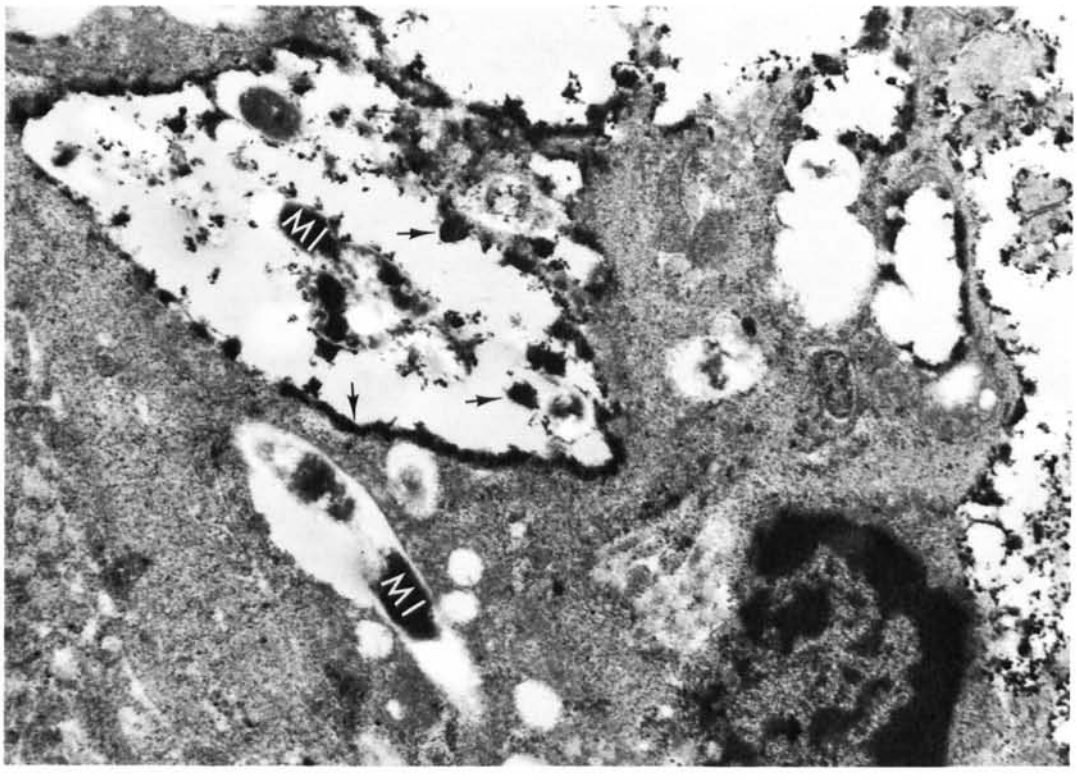

FIG. 3. Lepra cell stained with Abul-Haj modification of Hale's iron solution. AMPS (arrows) is localized as lining the vacuoles. *M. leprae* (MI). Original magnification: 9,500×. Lepromatous case: TSK-17, 60-year-old male, BI = 4.5, MI = 40%, 11 years after the onset of the disease.

trace amounts in cell membranes, but the cell membranes of lymphocytes often contain more AMPS (Fig. 10). Although in lepromatous cases β-glucuronidase seemed to be derived from *M. leprae,* weak activity was also present in the lysosomes of epithelioid cells of borderline cases.

In tuberculoid cases, trace amounts of AMPS were only seen occasionally in the lysosomes of epithelioid cells but the majority of these cells were AMPS free (Fig. 11). These epithelioid cells and Langhans type giant cells often contain β-glucuronidase activity in the lysosomes electron microscopically (Fig. 12). The activity of the enzyme did not seem to be strong but was stronger than that found in cells of other types of leprosy. This enzyme activity as well as that of N-acetyl-β-glucosaminidase was localized light microscopically in the central portions of granulomata where epithelioid and Langhans type giant cells are located.

DISCUSSION

Host immunity to leprosy has been found to comprise a spectrum between the polar lepromatous and tuberculoid types and the disease is classified accordingly ([29, 30]). Figure 13 ([36]) summarizes this concept. Despite varied studies for many years, nothing is known of *M. leprae* growth requirements which might help explain the lesion distribution pattern in the disease, such as the predilection for nerves.

In an earlier study we presented evidence relating HA to the host defense mechanism ([22]). This was suggested by the significant accumulation of HA in active lepromatous lepromas with only a mild accumulation of it in the central portions of early tuberculoid granulomata. However, because lipid storage is also more pronounced in lepromatous leprosy than in other leprosy expression and is thought to be derived from the bacilli ([11, 12, 33, 36]), the question of the origin of HA arose. The results presented in Figure 3 showing stronger localization of HA in the phagolysosomal membranes of lepra cells than around *M. leprae* support the concept that this AMPS is derived from the host.

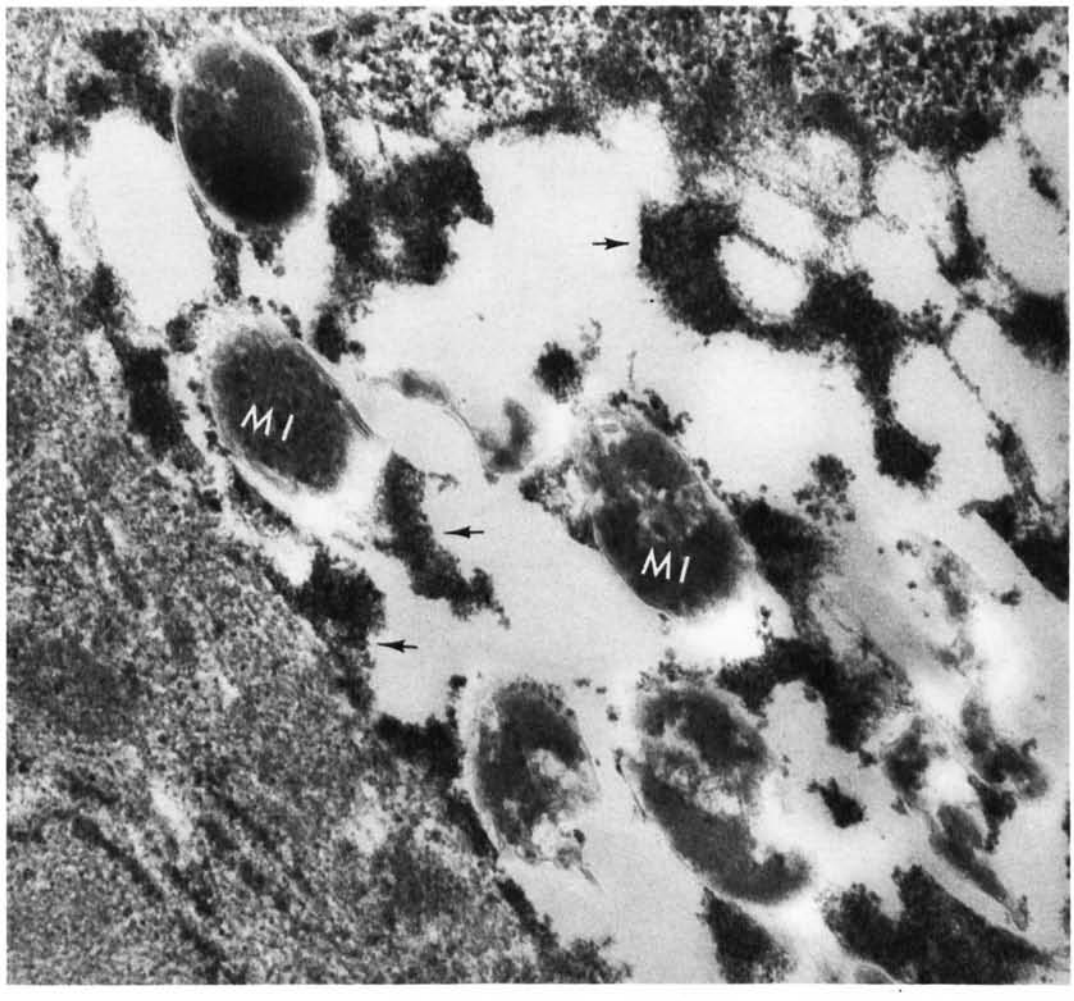

FIG. 4. A vacuolar leprosy inclusion from the same case as Figure 3. AMPS (arrows) lines the vacuole and is stuffed in between bacilli (MI). Original magnification: 28,000×.

Hammond and Dvorak's observation ([10]) that there is antigen-induced stimulation of glucosamine incorporation into the cytoplasm and cell membranes of macrophages of guinea pigs in delayed hypersensitivity may support this concept. Glucosamine is a component of HA ([27]) and it is recognized ([37]) that the antigenic stimulation to humoral antibody production is stronger in lepromatous as compared to tuberculoid leprosy. The localization of β-glucuronidase only in and around *M. leprae* and not in lysosomes independent of bacilli in lepra cells also suggests the possibility that lepra cells are producing HA and that the bacilli are degrading it. Beta-glucuronidase is a lysosomal enzyme according to Barrett ([2]) which plays a role in the degradation of HA as is also shown in Figure 14, adapted from Schütterle and Platt ([35]).

The above findings seem to correlate with the malfunction of macrophages in lepromatous leprosy because macrophages are characterized as having strong β-glucuronidase activity, with a few exceptions such as in sarcoidosis or some types of Hodgkin's disease ([43]).

In tuberculoid granulomas, trace HA was only observed occasionally in lysosomes of epithelioid cells. Beta-glucuronidase activity is, however, often encountered in the lysosomes of epithelioid cells. This seems to parallel the findings by Feher *et al* ([8]) in experimental granuloma, who observed strong β-glucuronidase activity remaining in histiocytes after the disappearance of the AMPS

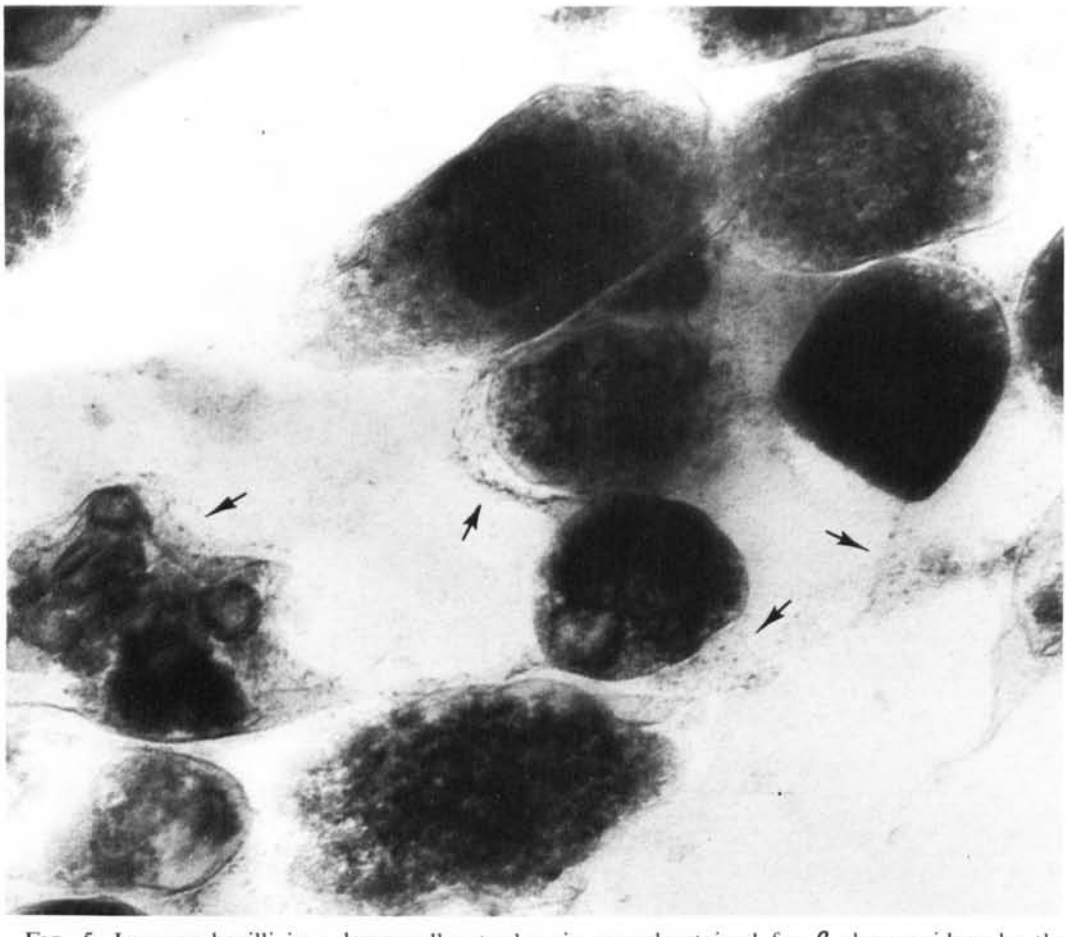

FIG. 5. Leprosy bacilli in a lepra cell cytoplasmic vacuole stained for β-glucuronidase by the method of Smith and Fishman. The enzyme (arrows) is localized around bacilli. Original magnification: 28,000×. Lepromatous case: 64-year-old male, BI = 4.3, MI = 30%, six years after the onset of the disease.

which appeared in the course of inflammation produced by croton oil in rats. Manning and Dipasquale ([21]) also observed temporary increase of β-glucuronidase in the course of wound healing in rats. We have not had the opportunity of studying early tuberculoid cases. However, we determined ([22]) that AMPS diminished with the chronological age of lesions and, therefore, it may be that the enzyme activities that we observed were not at the highest level. Even so, it seems to be characteristic that epithelioid cells and Langhans type giant cells have β-glucuronidase activity in lysosomes and lepra cells do not. This characteristic difference in the two poles of leprosy suggests that HA may be a nutrient for *M. leprae* although HA and hyaluronidase have previously been primarily regarded as spreading factors ([6, 17, 18, 19]). Saccharides with glycosidic linkages have higher value as nutrients than oligosaccharides, according to Day and Pigman ([7]). Although no supporting documentation has been found, it does not seem unreasonable to consider the possibility that these substances may be utilized for energy by microorganisms. *In vivo* and *in vitro* studies probing this possibility are in progress. In this light, the concentration of HA in the peripheral nerves, as shown by Johnson and Helwig ([16]), may relate to the affinity of *M. leprae* for these structures. This possibility is under study.

In borderline cases, although electron microscopy revealed that the activity of β-glucuronidase in early epithelioid cells is mainly associated with bacilli, the enzyme activity

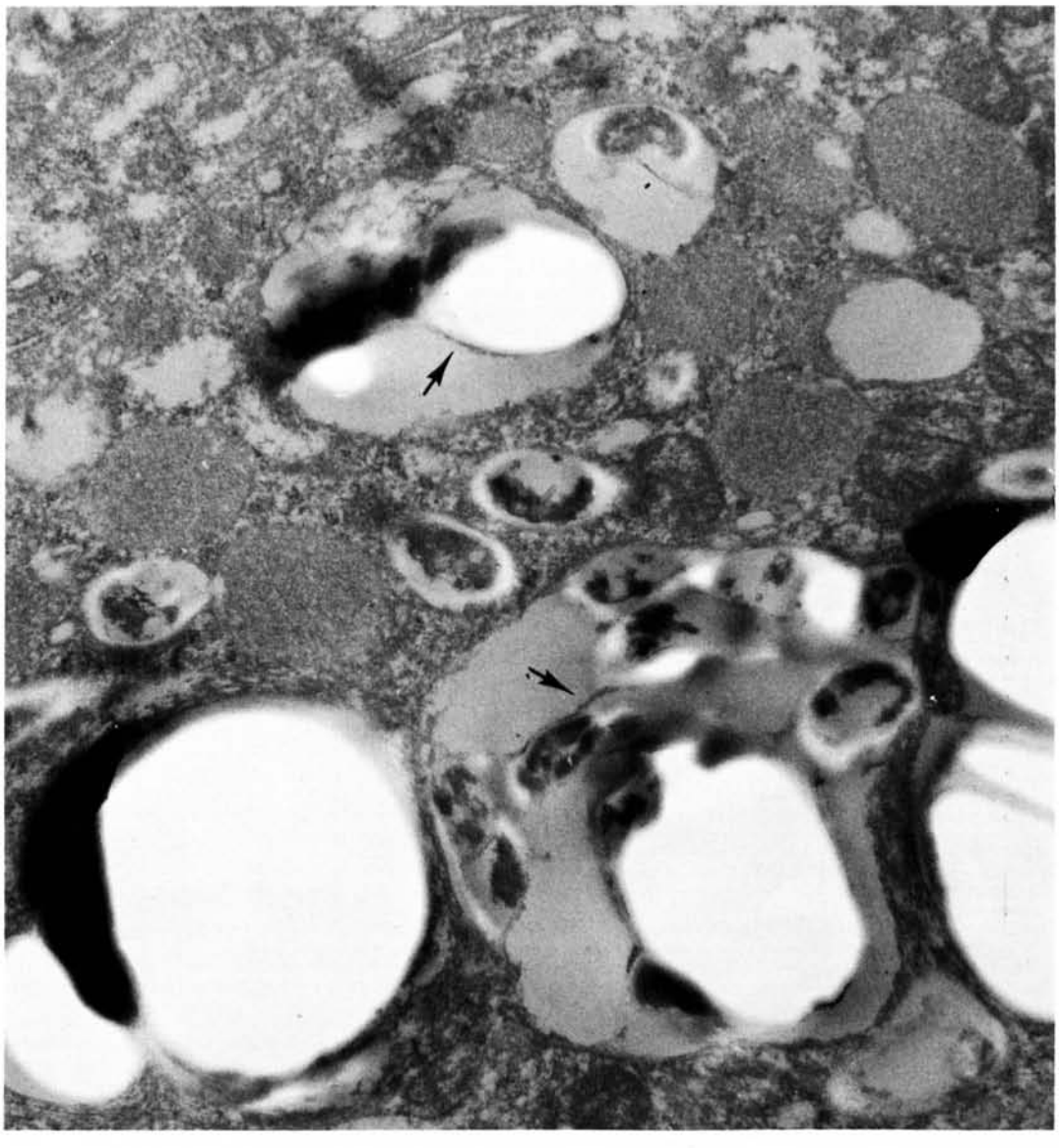

FIG. 6. Lepra cell stained to detect β-glucuronidase. There is absence of β-glucuronidase activity in the lysosomes not containing bacilli and activity (arrows) in and around bacilli. Original magnification: 9,500×. Same case as Figure 3.

is also present in lysosomes independent of bacilli although the activity does not seem to be strong. The light microscopic finding that bacilli were outnumbered by sites of enzyme activity also suggests the presence of the enzyme in the host's cells. Also, in borderline cases, HA was primarily distributed in the cell membranes of lymphocytes although it was also observed in the lysosomal membranes of early epithelioid cells. This might suggest some degree of disability in HA degradation because in tuberculoid cases lymphocytes seemed to have less HA.

N-acetyl-β-glucosaminidase activity seems to show the same pattern of distribution as β-glucuronidase in tuberculoid leprosy but not in lepromatous leprosy. In the latter, the activity was present in lepra cell cytoplasm but not in the vacuoles where bacilli were concentrated. This light microscopic finding has not been confirmed electron microscopically because the methodology has only

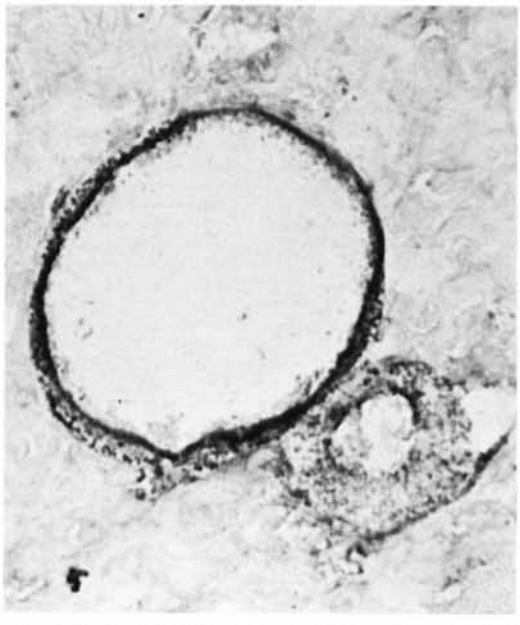

FIG. 7. Light micrograph of leproma stained for β-glucuronidase. Enzyme activity is localized in the periphery of the vacuole. Original magnification: 400×. Same case as that of Figure 3.

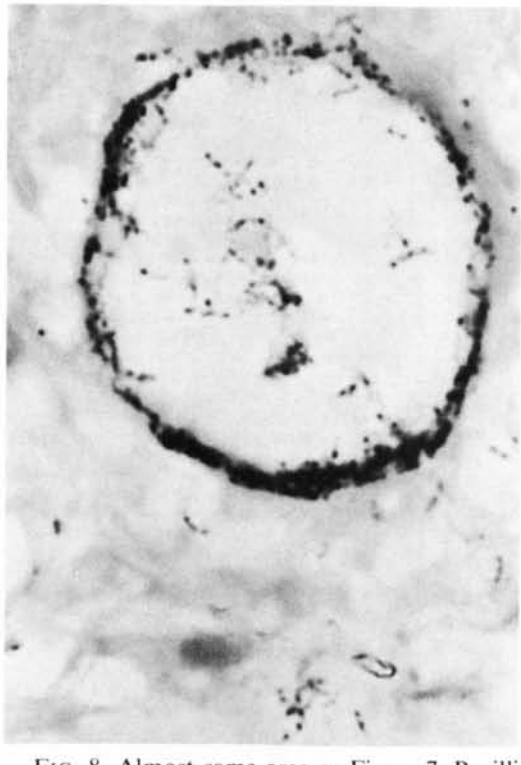

FIG. 8. Almost same area as Figure 7. Bacilli are located on the periphery of the vacuole where β-glucuronidase activity is localized in Figure 7. Original magnification: 1,000×. Acid-fast stain.

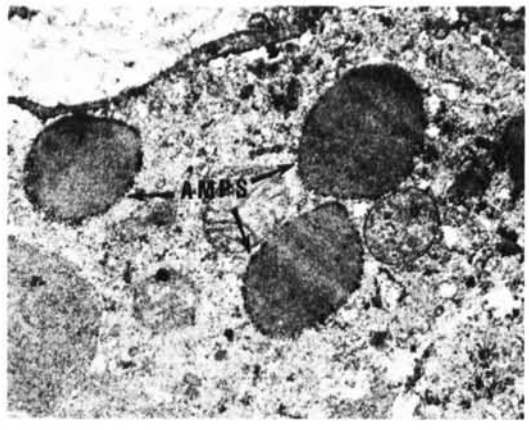

FIG. 9. Lysosomes of dimorphous epithelioid cells stained for AMPS. There are trace amounts of AMPS in the lysosomal membranes. Original magnification: 9,500×. Borderline case; HMT-940, BI = 0.

recently become available. However, according to Okada and O'Brien [26], generalized absence of β-D-N-acetylhexosaminidase (which contains N-acetyl-β-glucosaminidase) occurs in Tay-Sachs disease. As this gangliosidosis is not known to be encountered in the course of any type of leprosy, this enzyme deficiency does not seem to be associated with any possible leprosy cell enzyme deficiency.

Hyaluronidase is also a major enzyme in the degradation of HA, as indicated in Figure 14. Histochemical study of this enzyme in leprosy has been postponed, awaiting the availability of unfixed tissues [24]. There may be such activity, although according to Wells [45] no hyaluronidase is present in the skin. However, he examined normal and burned skin and did not study any type of granulomatous inflammation.

The presented results suggest that the hyaluronic acid is of host origin. Suggestive evidence of the possible role of hyaluronic acid as a nutrient for *M. leprae in vivo* and *in vitro* will be reported separately [23].

SUMMARY

Electron- and light microscopic analyses were conducted on leprosy skin biopsies relative to the origin of hyaluronic acid, which has previously been observed to be distributed inversely in ratio to the degree of cell-mediated immunity. The present study investigated the subcellular localization of hyaluronic acid and its degrading enzyme in

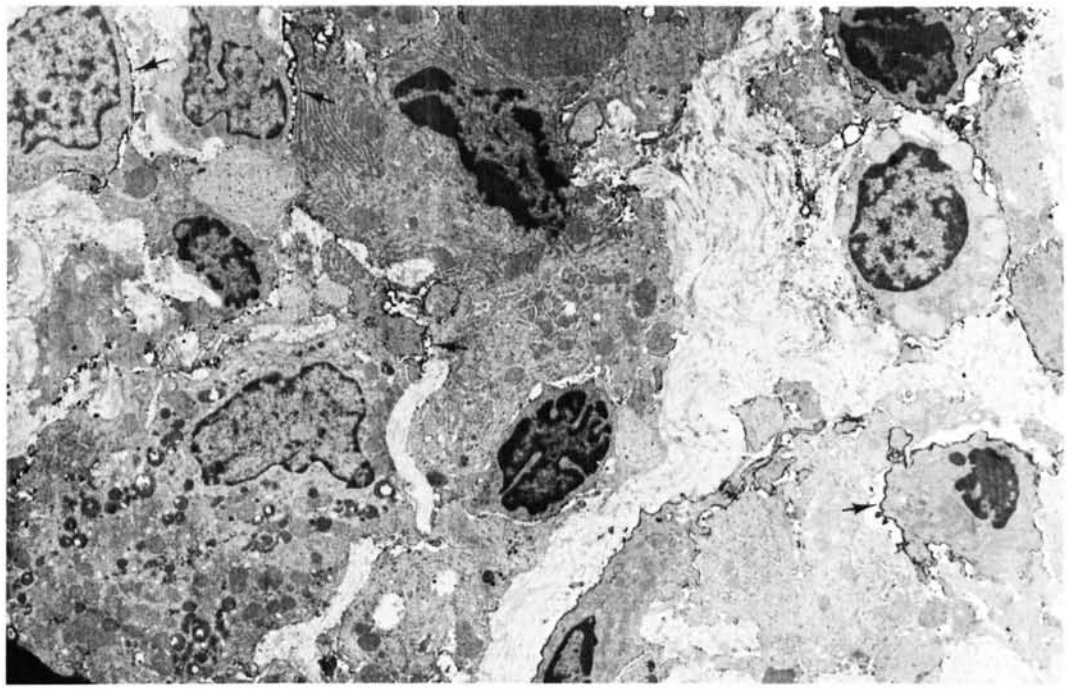

FIG. 10. A lower magnification of the infiltration of the skin which is shown in Figure 9. Strong localization of AMPS (arrows) in the cell membranes of lymphocytes. Original magnification: 1,900X.

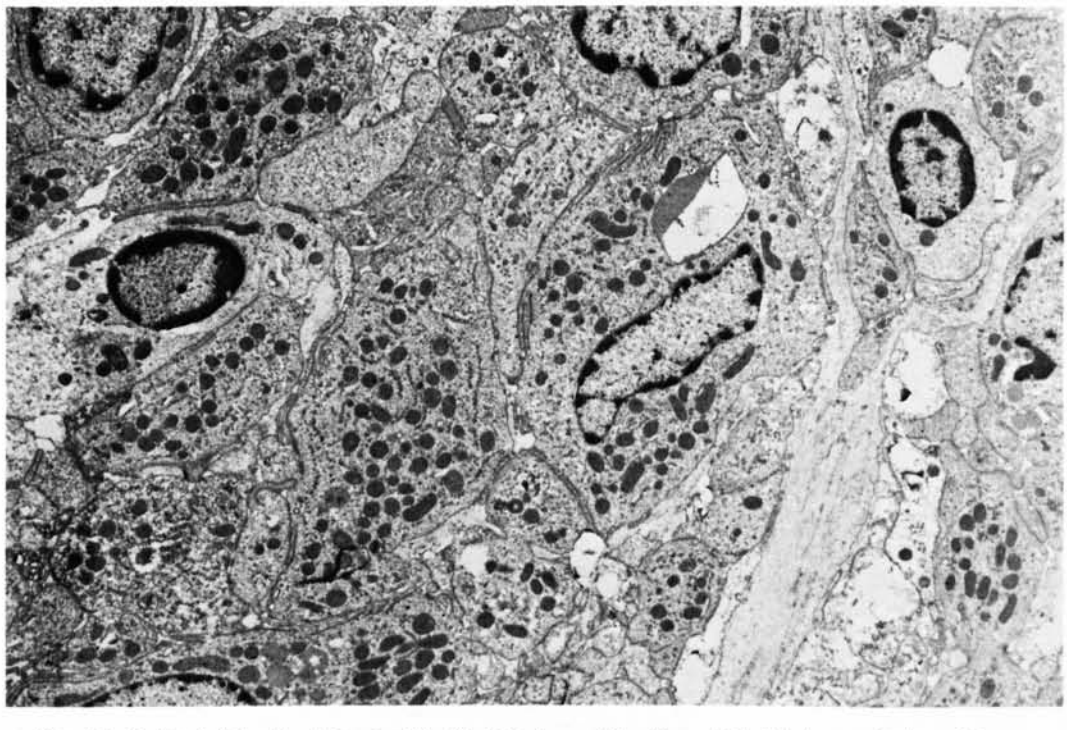

FIG. 11. Epithelioid cells stained with Abul-Haj modification of Hale's iron solution. Absence of AMPS in none of the cells. Original magnification: 1,900X. Tuberculoid case; S-147.

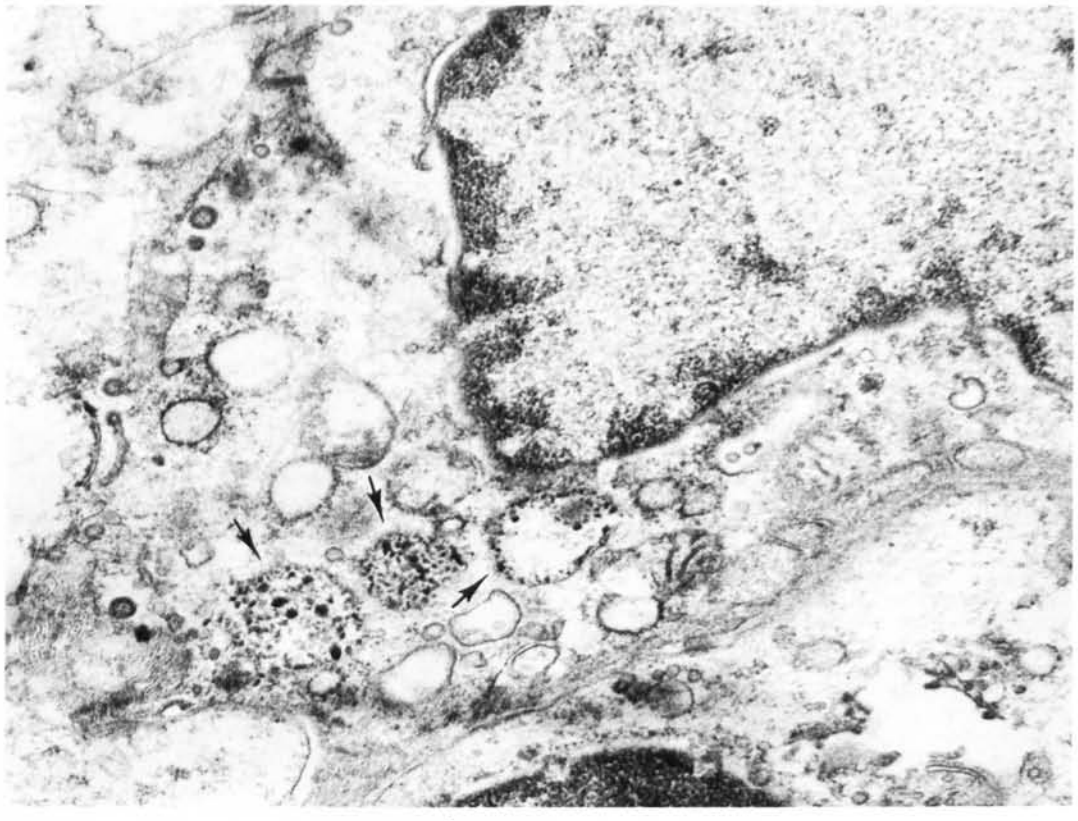

FIG. 12. Epithelioid cell stained for β-glucuronidase. Enzyme activity is in lysosomes (arrows). Tuberculoid case; HMT-1037, 56-year-old male, BI = 0, 10 years after the onset of the disease. Original magnification: 5,500X.

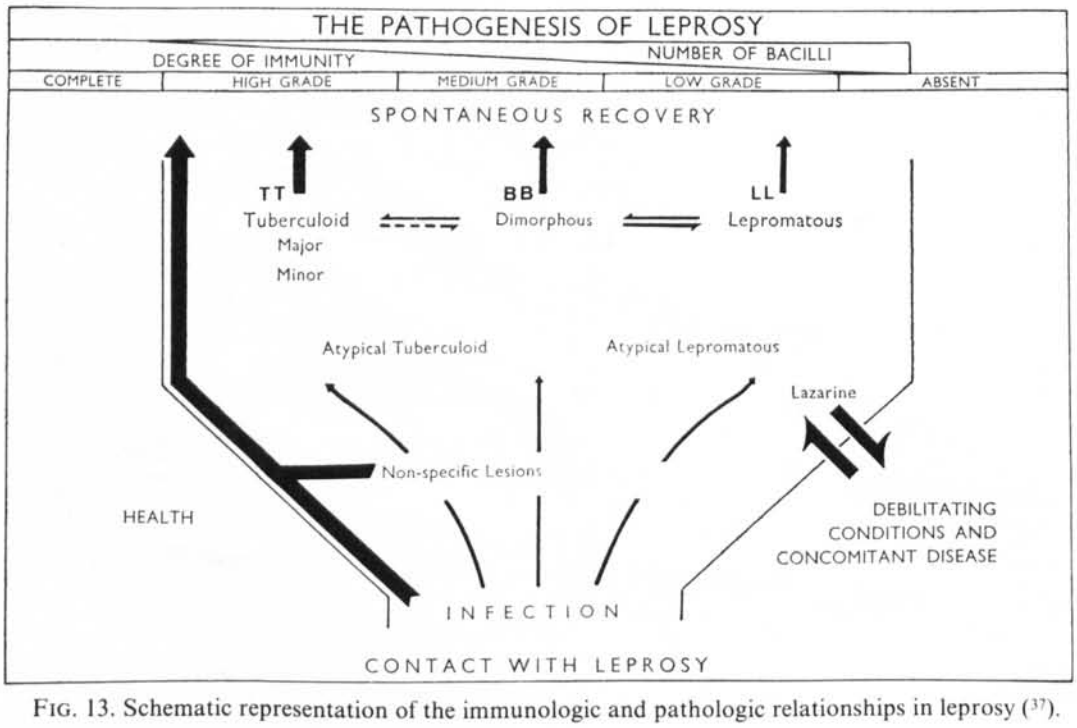

FIG. 13. Schematic representation of the immunologic and pathologic relationships in leprosy ([37]).

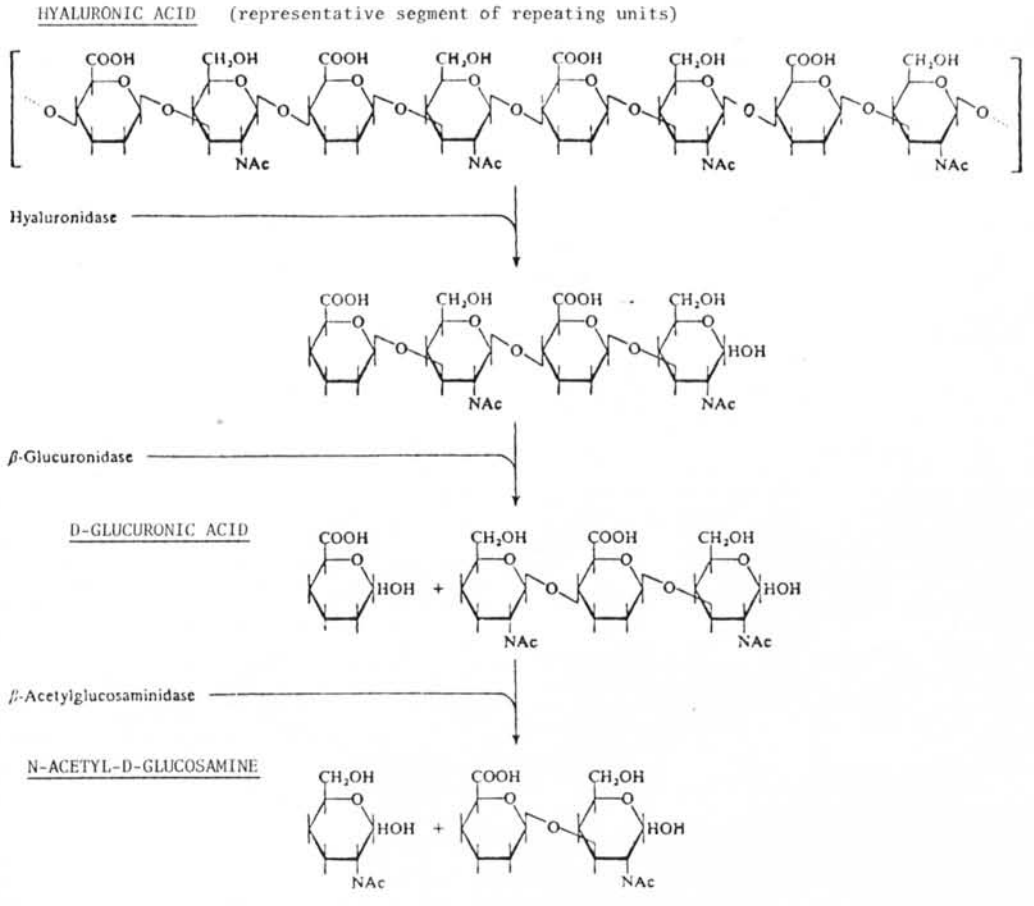

FIG. 14. Schematic representation of the enzymatic degradation of hyaluronic acid ([35]).

various types of leprosy. Hyaluronic acid in some lepromatous leprosy cases was shown to be accumulated in the limiting membranes of the phagosomes of lepra cells and *Mycobacteria leprae* have β-glucuronidase which plays a role in the degradation of hyaluronic acid. Contrariwise, in tuberculoid leprosy, β-glucuronidase was detected in the lysosomes of epithelioid cells and giant cells. This result suggests that the origin of hyaluronic acid is in histiocytes and at the same time it might suggest that *M. leprae* is in competition with enzymes of epithelioid cells for hyaluronic acid, whereas reduced or absent β-glucuronidase in lepra cells enable bacilli to utilize the AMPS as a nutrient.

RESUMEN

Biopsias cutaneas de lepra fueron examinadas mediante la microscopía de luz y electronica en relación con el orijen del ácido hyalurónico, el que en observaciones previas se halló distribuido en relación inversa al grado de inmunidad celular. En el presente estudio la localización celular del ácido hyalurónico y su enzima degradativa fue investigada en varios tipos de lepra. Se demostró que en casos de lepra lepromatosa el ácido hyalurónico se hallaba en las membranas limitantes de los fagosomas de células leprosas y que las *M. leprae* poseían β-glucoronidasa, enzima esta que participa en la degradación del ácido hyalurónico. Por el contrario en la lepra tuberculoide, β-glucoronidasa fue detectada en los lysosomas de células epiteloides y gigantes. Estos resultados sugieren que el ácido hyalurónico se origina en los histiocitos, y también podrían sugerir que las *M. leprae* compiten con las enzimas de células epiteloides por el ácido hyalurónico; mientras que la reducción o ausencia de β-glucoronidasa en células leprosas permiten a los bacilos utilizar AMPS como factor nutricional.

RÉSUMÉ

Au moyen de microscopie optique et électronique on a étudié l'origine de l'acide hyaluronique dans des biopsies cutanées des malades de lèpre.

Un rapport inverse avait déjà été observé entre la présence de l'acide hyaluronique et le degré d'immunité cellulaire. Dans ce travail on a examiné la localisation sous-cellulaire de l'acide hyaluronique et ses ferments dégradants dans les formes différentes de lèpre. La plupart des cas lépromateux ont révélé un entassement de l'acide hyaluronique aux membranes des phagosomes des cellules de Virchow, et en plus on a remarqué aux bacilles de *M. leprae* une activité du ferment β-glucuronidase, qui joue un rôle dans la dégradation de l'acide hyaluronique. Au contraire, chez les malades de lèpre tuberculoïde, la glucuronidase a été trouvée dans les lysosomes des cellules épithélioïdes et cellules géantes. Ces résultats suggèrent que l'acide hyaluronique aux lésions lépreuses est de l'origine histiocytaire et en même temps que *M. leprae* est en concurrence avec les ferments des cellules épithélioïdes pour l'acide hyaluronique, tandis qu'une diminution ou absence de β-glucuronidase dans les cellules de Virchow permet les bacilles d'utiliser le mucopolysaccharide acidique comme aliment.

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