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Studies on Cellular Immunity in Leprosy

I. Lysosomal Enzymes[1, 2]

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Cellular immunity in chronic infections is determined totally or in part by "activation" of macrophages, which then acquire an increased capacity to destroy causative organisms.

In tuberculosis, for instance, immunity is associated with increased levels of certain hydrolytic enzymes in macrophages (6). The appropriate combination of such hydrolases could by itself destroy the tubercle bacillus without the intervention of other factors (7).

In 1947, Hanks (9), found a notable difference in the behavior of human fibroblasts towards *Mycobacterium leprae, in vitro*. The fibroblasts of patients with the tuberculoid form of leprosy were able to destroy *M. leprae*, while those of patients with the lepromatous form were unable to do so. This observation has been confirmed by Yoshie and Sugawara (31). Similar observations were made by Barbieri and Correa (2) in macrophages developed from cultures of blood monocytes.

Macrophages from both lepromatous and tuberculoid patients actively phagocytize dead leprosy bacilli from the first day of incubation. After phagocytosis, a striking difference between the two forms is apparent. The macrophages of tuberculoid patients completely lyse the ingested bacilli, becoming practically free of lipids. However, the macrophages from lepromatous patients are unable to do this, and transform into typical lepra cells, whose cytoplasm contains numerous bacilli and droplets of lipids that are easily stainable by Sudan black.

The lack of lytic activity in the macrophages of lepromatous patients is specific for *M. leprae;* macrophages from lepromatous subjects are able to lyse *M. lepraemurium* as well as *M. tuberculosis* (3).

Moreover, when damaged *M. leprae* are added to cultures of macrophages from lepromatous patients, lysis takes place. This suggests that the inability of these macrophages to lyse *M. leprae* depends on the integrity of the bacterial cell wall (3).

There is a remarkable correlation between the *in vitro* test or the digestion of *M. leprae* by macrophages and the intradermal lepromin test (2). The latter is pos-

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itive in normal persons and in tuberculoid patients, but is negative in cases of lepromatous leprosy.

In recent years, new ideas have been advanced concerning a hereditary factor concerned with individual susceptibility to leprosy ([4, 24]). All this suggests that leprosy occurs when, together with contact with the causative organisms, there is an absence or deficiency of one or more cellular factors responsible for the digestion and ultimate fate of *M. leprae* in human tissues.

Recent studies have shown that biochemical defects of genetic origin are responsible for the marked susceptibility of some persons to certain pyogenic or fungal infections. Examples of these conditions are the chronic granulomatous disease of childhood ([13, 23]), the Ghediak-Higashi syndrome ([20]) and disseminated candidiasis ([16]).

The object of the present investigation is to determine the levels of some enzymes, known as lysosomal, in the different forms of leprosy; to find out if variations in these levels can explain the long presence of *M. leprae* within the lysosomes of lepra cells ([10, 14]), and the inability of these organelles to digest the bacilli in contrast to what is observed in tuberculoid patients and in normal healthy individuals.

MATERIALS AND METHODS

The age of the patients studied varied between 14 and 60 years, and their lesions were of widely different duration. They were all untreated and recently diagnosed, since it has been shown that sulfone treatment modifies the level of lysosomal enzymes ([21]). Criteria for classifying them were; clinical appearance, histology of the lesions, and reaction to the lepromin test.

The controls were volunteer donors of the Blood Bank in Caracas or patients with other noninfectious skin diseases, within the same age range as the leprosy patients and of similar social conditions.

General principles of the biochemical assays. Twenty to 30 ml. of venous blood were taken from fasting patients, and leukocytes were separated by sedimentation in a 2 per cent dextran solution. Contaminating red blood cells were removed by hypotonic lysis with NaCl 0.2 per cent followed in 30 seconds by the same volume of NaCl 1.60 per cent. The separated white cells were subsequently washed with 0.25 M sucrose and centrifuged at 4,900 g/min. The pellet obtained was resuspended in 0.34 M. sucrose containing 100 units of heparin per ml. This treatment lysed the leukocytes completely, as shown by phase contrast microscopy and an increase in the viscosity of the suspension. The suspension was then homogenized in a Potter-Elvehjem homogenizer, at 0°C, with 10 strokes of the Teflon piston. The enzymatic tests were made within 24 hours after the blood samples had been obtained. All biochemical tests were carried out in duplicate, each time with two different volumes of the enzymes tested.

Substrates. β-glycerophosphate (disodium salt), a.q. H_2O_2, sucrose and o-tolidine were obtained from E. Merck A.-G, Darmstadt, Germany; heparin sodium salt, dextran (M.W.204.000), Triton X-100, O-nitrophenyl-β-D-glucosaminide, *Micrococcus lysodeikticus*, phenolphthalein glucuronic acid, bovine albumin and bovine hemoglobin type I were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Enzymatic tests. Myeloperoxidase was assayed by the method of Baggiolini *et al.* ([1]), with slight modifications. The final volume as incubated without detergents was 3.8 ml., and the enzyme was diluted in distilled water.

Acid phosphatase was determined by the method of de Duve *et al.* ([8]) in a final volume of 1 ml. Inorganic phosphorus was determined by the procedure of Chen *et al.* ([5]). Alkaline phosphatase was assayed using the disodium salt of β-glycerophosphate as substrate at a final concentration of 0.1 M in 50 mM carbonate buffer at pH 9.9, in a volume of 1 ml. Inorganic phosphorus was determined as for acid phosphatase.

β-galactosidase was determined in a final volume of 0.8 ml. with 3.1 mM of o-nitrophenyl-β-D-galactopyranoside in 125 mM acetate buffer at pH 5. The o-nitrophenol was measured in the filtrate at 420 mμ using glycine-carbonate buffer at pH 10.5.

The determination of β-N-acetylglucos-

aminidase (β-H-acetylaminodeoxyglucosidase) was carried out in a total volume of 1 ml. of 50 mM acetate buffer at pH 5.0 with 1 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. The reaction was stopped by the addition of 3 ml. of a solution containing glycine (0.4 M), Na_2CO_3 (0.25 M) and NaCl (0.2 M) buffered to pH 10.5 and the liberated p-nitrophenol measured at 400 mμ-glucoronidase was measured in a final volume of 1 ml. of 50 mM acetate buffered to pH 5.0 with 1 mM phenolphthalein glucuronidate as substrate. The next steps of the test were as for the preceeding enzyme. Readings were done at 540 mμ.

Lysozyme was assayed with *M. lysodeikticus* as substrate, according to the method of Prasad and Litwack ([22]), in a final volume of 3.4 ml.

The protein analysis was made according to the method of Lowry *et al.* ([17]) using bovine serum albumin as standard.

The enzymatic tests were carried out at 37°C for all the enzymes, except for lysozyme and myeloperoxidase which were done at 25°C.

In our experiments, the results represent total activity, for which Triton X-100 was added at 0.1 per cent to the incubation mixture of all the enzymes, except for myeloperoxidase activity. It was proved that under these conditions the activity, as measured, was proportional to the concentration of the enzyme and to the time of incubation.

RESULTS

The enzymatic levels in controls and in patients with different clinical forms of leprosy are given in Table I. Statistical analyses were carried out by the "t" test; values of $P>0.05$ were considered to be significant. It will be seen that there is no statistically significant difference in the levels of enzymes under investigation, either in the values of the controls as compared with those of the leprosy patients, or in the values observed in the patients with different forms of leprosy.

The enzymes studied included a nonlysosomal hydrolase, namely alkaline phosphatase, which has been shown recently to be localized in PMN leukocytes inside particles which are also rich in lysozyme ([1]). High alkaline phosphatase activity has been found in patients with various infectious diseases ([27, 28]) or with tuberculosis ([29]) which is also a chronic mycobacterial disease. We have found no such variations in leprosy patients.

A particularly interesting feature is the great dispersion of the enzymatic values as regards β-galactosidase, β-glucuronidase and N-acetyl-β-glucosaminidase in patients with different forms of leprosy in comparison with the controls. It has not been possible for us to ascertain the causes of this dispersion, although the patients studied varied greatly as to the duration and evolution of the disease. It was impossible for us to obtain exact information as to when the initial lesion appeared.

DISCUSSION

The practicability of using leukocytes from peripheral blood in the investigation of diseases characterized by the absence or inadequacy of one or more of the enzymes of the lysosomal system has been demonstrated in many papers ([11, 15, 25]).

The long periods during which *M. leprae* remains inside vacoules, rich in acid phosphatase or lysosomes ([10, 14]) and the specificity of such defect, points strongly to an enzymatic aberration in the macrophages which should normally digest the bacillus. This hypothesis gains strength when the defective lysis of the bacillus by fibroblasts and macrophages of lepromatous leprosy patients is considered in contrast with those of the tuberculoid form and normal individuals.

This paper has dealt with the enzymes that are considered as key factors in intracellular defense against infection, namely myeloperoxidase ([12, 18]), and lysozyme ([30]) as well as other enzymes important for the digestion of major macromolecular constituents of the bacterial cell wall ([19, 26, 30]). However, no statistically significant differences were found, either between controls and leprosy patients, or among the leprosy patients themselves. These results do not preclude the possibility that the deficiency may be due to the lack of some other lysosomal enzymes or substances that act as defensive factors.

TABLE 1. *Enzymatic activities in human leukocytes.*

	Acid β-glycero-phosphatase[a]	Lysozyme[b]	Acid myelo-peroxidase[c]	Acid β-galacto-sidase[a]	N-acetyl-β-glucos-aminidase	β-glucu-ronidase[a]	Alkaline β-glycero-phosphatase[a]
Control	(20)	(14)	(11)	(13)	(6)	(6)	(8)
Average	0.688	2.19	157	0.081	0.223	0.118	0.213
± S.D.	0.321	0.83	78	0.010	0.156	0.011	0.144
Range	0.210–1.39	1.05–3.40	92–369	0.057–0.103	0.205–0.277	0.102–0.134	0.074–0.491
Lepromatous leprosy	(14)	(14)	(12)	(15)	(7)	(7)	(10)
Average	0.525	1.84	224	0.083	0.165	0.108	0.145
± S.D.	0.238	0.93	175	0.036	0.047	0.041	0.087
Range	0.148–0.941	0.61–3.47	67–784	0.034–0.168	0.112–0.228	0.063–0.167	0.068–0.356
Tuberculoid leprosy	(8)	(8)	(9)	(7)	(1)	(1)	(7)
Average	0.715	1.86	131	0.058	0.221	0.135	0.151
± S.D.	0.230	0.68	65	0.016			0.137
Range	0.336–1.100	1.42–2.90	66–216	0.037–0.081			0.062–0.450

[a] Enzymatic activity expressed as μ Mole/mg./hr.
[b] Enzymatic activity expressed as μgr. of lysozyme of egg white/mg./min.
[c] Enzymatic activity expressed as Δ OD/mg./min. The numbers in parentheses refer to the number of patients studied. $P > 0.05$ for all the values between themselves.

SUMMARY

The activity of some lysosomal enzymes has been measured in peripheral blood leukocytes, obtained from normal human subjects and 36 untreated patients with tuberculoid and lepromatous leprosy.

There is no statistically significant difference in the levels of acid-β-glycerophosphatase, β-glucuronidase, acid myeloperoxidase, β-galactosidase, N-acetyl-β-glucosaminidase, lysozyme and alkaline-β-glycerophosphatase either between leprosy patients and controls, or between lepromatous and tuberculoid patients.

RESUMEN

Se midió la actividad de algunas enzimas lisosomiales en leucocitos de sangre periférica obtenida de sujetos normales y 36 pacientes con lepra lepromatosa y tuberculoide, no tratados.

No se encontraron diferencias estadísticamente significativas en los niveles de beta-glicerofosfatasa ácida, betaglucoronidasa, mieloperoxidasa ácida, beta-galactosidasa, N-acetil-beta-glucosaminidasa, lisosima y beta-glicerofosfatasa alcalina, ya sea entre los enfermos de lepra y los controles o entre los enfermos con lepra lepromatosa y los con lepra tuberculoide.

RÉSUMÉ

L'activité de quelques enzymes lysosomiques a été mesurée dans les leucocytes du sang périphérique obtenues chez des sujets humains normaux et chez 36 malades atteints de lèpre tuberculoíde et de lèpre lépromateuse et non traités.

On n'a pas relevé de différence significative, chez les malades atteints de lèpre et chez les témoins, ou entre les patients lépromateux et les patients tuberculoïdes, dans les taux des enzymes suivants: la β-glycérophosphatase acide, la β-glucuronidase, la myéloperoxydase acide, la β-galactactosidase, la N-acetyl-β-glucosaminidase, le lysozyme et la β-glycerophosphatase alcaline.

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