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Phagocytosis in Leprosy
2. Production of Superoxide by Circulating Blood Leukocytes from Lepromatous Patients [1]

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Superoxide anion, hydrogen peroxide, hydroxyl radicals, singlet oxygen and the myeloperoxidase-hydrogen peroxide-halide system ([2,7]) are the main oxidative bactericidal intermediaries of phagocytic cells, and their appropriate levels and activities are responsible for their adequate function. In the extreme case of the clinical condition known as chronic granulomatous disease (X-linked), the polymorphonuclear leukocytes (PMN) are deficient in their overall ability to undergo the phagocytosis induced oxidative changes that characterize their normal phagocytic function ([10]), the result being an enhanced host susceptibility to severe infections by germs that under normal conditions are mildly or nonpathogenic ([5,11]). In relation to leprosy, in a previous paper ([8]) we reported that circulating leukocytes from lepromatous patients do not differ from those of normal (healthy) subjects with respect to their levels of β-glucuronidase, β-galactosidase, acid and alkaline phosphatase and lipase activities, nor was there a significant difference in the "diaphorase" activity of both lepromatous and normal groups as determined by the nitro-blue tetrazolium (NBT) test. Complementary results have been obtained by other groups with respect to lysosomal enzymes ([1]) and NBT reduction ([9,12]). This communication presents our findings as related to the superoxide (O_2) anion production by phagocytizing peripheral blood leukocytes from lepromatous and normal individuals.

MATERIALS AND METHODS

Subjects. Seventeen normal individuals and 19 patients with either diffuse or nodular lepromatous leprosy were studied. The patients have been under medical control at the Centro Dermatológico Pascua of Mexico City, and represented a heterogeneous group with respect to age, sex, duration of disease and clinical status at the time of the study. Although most patients were under conventional treatment (DDS, 25-50 mg daily) all of them presented a still active form of the disease. Several cases were complicated by some form of leprosy reaction (*erythema nodosum leprosum* or Lucio's phenomenon).

Cell preparations. A 20 ml sample of peripheral blood was collected in plastic disposable syringes containing 20 IU per ml of heparin. Each syringe was inverted and the red cells were sedimented at 37°C until the leucocyte enriched supernatant plasma was collected through an 18 gauge needle, bent to an angle of 90°, into siliconized 50 ml glass centrifuge tubes. Then the tubes were centrifuged at 1,200 rpm for 5 minutes at 4°C. The sediment was washed once with Krebs-Henseleit-Bicarbonate (KHB) buffer, pH 7.1-7.4 ([6]), containing 0.2% glucose, resuspended in distilled water for 30 seconds to lyse erythrocytes and washed two more times with KHB buffer (1,200 rpm/5 min/4°C). Finally, the pellet was resuspended, counted and diluted with KHB buffer to obtain 5-10 × 10^6 cells per ml. No viability tests were performed.

Superoxide determination, modified from Babior *et al* ([3]). The following were added to duplicate 10 ml conical centrifuge tubes kept in an ice bath: 3 ml of the leukocyte suspension (15-30 × 10^6 cells), 0.1 ml of oxidized cytochrome-C (Sigma C-7752, type VI) solution in distilled water (180 nM) and 0.1 ml of latex particles (Difco, 3102-65) previously dialyzed against water and diluted in KHB so as to have about ten latex spherules per cell. After shaking, 1.6 ml of each reaction mixture were incubated for 20 minutes at 37°C with occasional shaking. The re-

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maining 1.6 ml were preserved at 0°C for use as a blank. Additional blanks included mixtures without latex, cytochrome-C or leukocytes. Reactions were terminated by placing the vessels in melting ice. After centrifugation at 3,000 rpm (radius = 15 cm) for 30 minutes at 4°C, the absorbances of the incubated supernatants at 418, 519 and 550 nm were determined using the unincubated blank supernatants as reference.

In this paper, the superoxide activity is expressed as the optical density change (incubated minus unincubated) at 550 nm produced by 5×10^6 PMN, under the above described conditions and the results are the average of at least duplicate determinations.

RESULTS

Incubation of leukocytes both from lepromatous and normal subjects with oxidized cytochrome-C and latex spherules was found to lead to reduction of cytochrome-C. The omission of latex particles in the reaction mixture did not result in the reduction of the cytochrome by either normal or lepromatous leukocytes. The complete reaction mixtures kept in the ice bath, to be used as blanks, showed a negligible reduction of cytochrome as determined by reading the supernatant's absorbance at 550 nm against a blank supernatant from a reaction mixture without latex particles.

Although the results reported are calculated on the basis of the 550 nm absorption peak, an absorption spectrum from 400 nm to 600 nm was ascertained for most determinations. Typical normal and lepromatous spectra are depicted in Figure 1. In both cases, the appearance of peaks at 418, 519 and 550 nm clearly indicates the reduction in cytochrome-C as a result of the O_2^- production by PMN. This particular result was obtained with cell suspensions having 6.7×10^6 (normal) and 8.7×10^6 (leprous) PMN per ml. After corrections, the superoxide activities were, respectively, 0.070 and 0.050 per 5×10^6 PMN.

Table 1 records the superoxide (O_2^-) activities found in the studied groups (mean ± standard deviation and range). No statistical analysis of results was needed as they were very similar regardless of the group. Of the 19 lepromatous patients studied, 7 were nodular (NLL) cases without leprosy reactions, 2 were diffuse lepromatous (DLL), one with Lucio's phenomenon and the other with *erythema nodosum leprosum* (ENL), 2 were NLL with *erythema multiforme,* and 8 were NLL with ENL. No differences in O_2^- activity were observed among patients when they were grouped into NLL without reaction and NLL with leprosy reactions.

In Figure 2, the superoxide (O_2^-) results

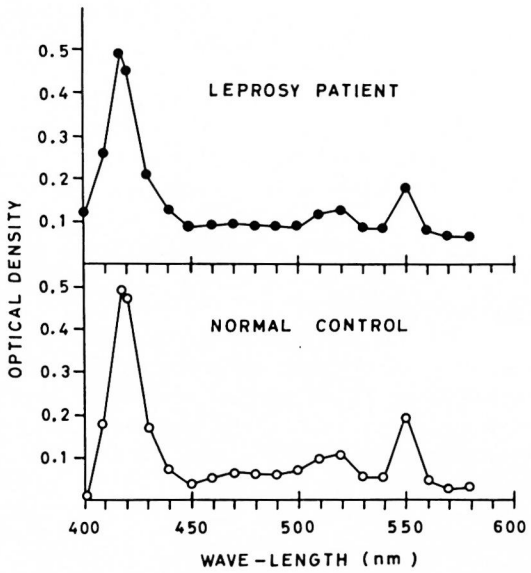

FIG. 1. Typical absorption spectra of cytochrome-C reduced by superoxide generated by phagocytizing normal or lepromatous leukocytes. Real O.D. values are shown and each point is the average reading of duplicate determinations.

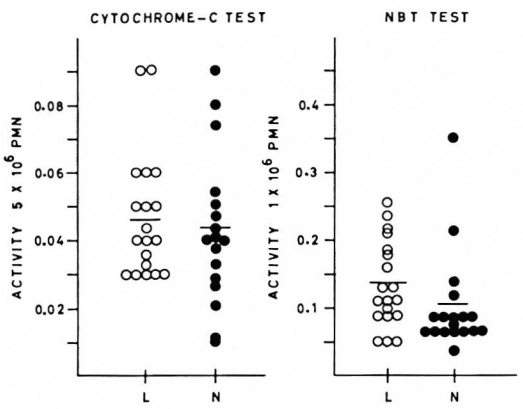

FIG. 2. Comparative cytochrome-C and NBT reduction tests by normal (N) and lepromatous (L) individuals. NBT results were previously reported in detail (8). Horizontal lines are the mean values.

TABLE 1. *Superoxide production by latex-phagocytizing leukocytes from normal and lepromatous individuals.*

Group	Number	Superoxide activity[a] (mean ± s.d.)	Range
I. Normal controls	17	0.043 ± 0.022	0.010 - 0.090
II. Patients without L.R.[b]	7	0.043 ± 0.022	0.030 - 0.090
III. Patients with L.R.	12	0.049 ± 0.017	0.030 - 0.090
IV. All patients (II + III)	19	0.047 ± 0.018	0.030 - 0.090

[a] Activity is given as the average optical density change at 550 nm produced by 5×10^6 PMN under the conditions described in Materials and Methods.

[b] L. R. stands for leprosy reactions, several types were included: Lucio's phenomenon, *erythema nodosum leprosum, erythema multiforme.*

are shown and compared with the NBT results obtained in similar lepromatous and normal groups ([8]). Both tests gave similar results. Again, as with the NBT test, the mean O_2^- activity in the lepromatous group ($0.047/5 \times 10^6$ PMN) is slightly higher than in normal controls ($0.043/5 \times 10^6$ PMN) but this difference, obviously, does not have statistical significance.

In general terms, the results obtained showed good reproducibility, the ranges were not broad and were similar for both groups (0.010 to 0.090 in normals and 0.030 to 0.090 in patients). The individual duplicate results were always over 90% comparable, and there was a fairly good correlation between the number of cells assayed and the observed degree of cytochrome-C reduction. We always worked with cell suspensions having no more than 10×10^6 PMN per ml, as O.D. readings are proportional to the cytochrome-C reducing activity of O_2^- at low rather than high number of cells.

DISCUSSION

The fact that the O_2^- production by phagocytizing PMN from lepromatous patients was not different from its production by PMN from normal individuals, correlates well with our previous results ([8]) regarding the ability of these cells to reduce the NBT dye while exhibiting phagocytosis. This result had to be expected if we accept the diagram in Figure 3 as the mechanism that correlates the O_2^- production with the NBT reduction. According to this mechanism the same reducing system that transforms the O_2^- into O_2^-, namely an oxidase and NADH and/or NADPH cofactors, is involved in the NBT reduction. Alternatively, the dye is reduced through its chemical reaction with O_2^- ([4]).

That the O_2^- is produced by PMN rather than by contaminant lymphocytes is deduced from the fact that in a mixture of PMN and lymphocytes, O_2^- is not appreciably produced unless the cell mixture is induced to phagocytose, and there is no evidence to date of the lymphocytes' ability to endocytose latex spherules. Phagocytosis is, on the other hand, a key function of PMN (and monocytes).

Although the idea that the O_2^- produced during phagocytosis might be a potential microbicidal agent by itself has fallen into disfavor ([2]), the O_2^- anion is still an intermediary and a precursor of the bactericidal

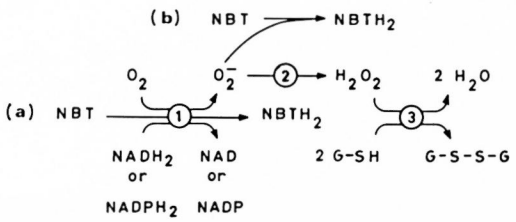

FIG 3. Initial phagocytosis-induced metabolic changes include an increased oxygen consumption. The O_2 is immediately reduced to superoxide (O_2^-) by an oxidase ① that uses NADH ([14]) or NADPH ([13]) cofactors. The O_2^- is then converted into H_2O_2, either spontaneously, or by means of a superoxide dismutase ② , and the H_2O_2 is decomposed into O_2 and H_2O by catalase or reduced to H_2O by glutathione peroxidase ③ . Oxidized NBT is reduced a) by the same system that reduces O_2 to O_2^-, or b) as a consequence of its chemical reaction with O_2^-, the latter mechanism being the most probable ([4]).

components better represented by the H_2O_2, the H_2O_2-myeloperoxidase-halide system, the OH' radicals, and the singlet oxygen. The possibility remains, however, that at high concentrations, as those possibly found in the vicinity of the phagosome, O_2^- by itself might be a useful microbicidal agent ([2]).

This and other reports ([9,12]) suggest that the metabolic changes induced by *in vitro* phagocytosis by PMN cells from lepromatous patients, do not differ from those found in normal controls, at least on the basis of the NBT and cytochrome-C reduction tests performed. Goihman-Yahr *et al* ([9]) found that patients with any type of leprosy, except the reactional (RLL) lepromatous leprosy, had normal numbers of NBT reducing cells. In patients with RLL, the proportion of reducing cells was significantly raised. We did not find a significant increase in the O_2^- levels produced by PMN from patients with RLL when compared with lepromatous patients without reaction.

Although the levels of the few lysosomal enzymes so far studied ([1,8]): β-galactosidase, β-glucuronidase, acid and alkaline phosphatases, lipase, acid myeloperoxidase, lysozyme and N-acetyl-β-glucosaminidase, support the idea that PMN from lepromatous patients behave normally, these results are by no means conclusive, and other metabolic and enzymatic activities have to be studied before a definitive statement can be made regarding the functional state of PMN (and MN) in leprosy.

SUMMARY

The ability of polymorphonuclear leukocytes from lepromatous patients to produce superoxide (O_2^-) anion while phagocytizing latex particles was studied. The results were compared with those obtained in a group composed of normal individuals. No differences were found between lepromatous and normal groups. No differences were found either when the comparison was made between patients showing any form of leprosy reaction and patients without leprosy complications at the time of the study. Together with other results previously reported, our findings suggest that PMN from lepromatous patients are not different from PMN from healthy individuals with respect to their ability to generate and show the metabolic oxidative changes (as measured by the NBT- and the O_2^--tests) induced by *in vitro* phagocytosis of latex particles. These metabolic activities remain normal even when PMN may show alterations at other levels of the phagocytic process.

RESUMEN

Se estudió la capacidad de los leucocitos polimorfonucleares obtenidos de un grupo de pacientes con lepra lepromatosa para generar superoxido (O_2^-) durante la fagocitosis estimulada con partículas de látex. Los resultados se compararon con los obtenidos en un grupo de personas sanas. No se encontraron diferencias entre los grupos normal y lepromatoso estudiados. Tampoco hubieron diferencias entre los niveles de O_2^- producido por los pacientes con algun tipo de reacción leprosa y aquellos producidos por los pacientes sin complicaciones en el momento del estudio. Junto con otros datos publicados previamente, los resultados sugieren que los leucocitos PMN de los pacientes con lepra lepromatosa no difieren de los PMN obtenidos de personas sanas en cuanto a su capacidad para mostrar los cambios metabólicos oxidativos (medidos por las pruebas del NBT y del O_2^-) inducidos *in vitro* por fagocitósis de partículas de látex.

Las funciones metabólicas antes mencionadas permanecen normales aún cuando los PMN puedan presentar alteraciones a otros niveles del proceso fagocítico.

RÉSUMÉ

On a étudié la capacité qu'ont les leucocytes polymorphonucléaires provenant de malades lépromateux à produire un anion superoxide (O_2^-) lorsqu'ils phagocytent des particules de latex. Les résultats ont été comparés avec ceux obtenus dans un groupe composé d'individus normaux. Aucune différence n'a été notée entre les malades lépromateux et le groupe de sujets normaux. Aucune différence n'a davantage été observée lorque la comparaison a été effectuée entre des malades présentant une forme quelconque de réaction lépreuse, et les malades ne souffrant pas de complications lépreuses au moment de l'étude. Mises en rapport avec d'autres résultats obtenus antérieurement, ces observations suggèrent que le PMN de malades lépromateux n'est pas différent du PMN de sujets sains en ce qui concerne leur capacité à développer et à témoigner de modifications métaboliques oxydatives induites par la phagocytose *in vitro* de particules de latex, mesurées par les tests NBT et O_2^-. Ces activités métaboliques restent normales même lorsque le PMN montre des altérations à d'autres étapes du processus phagocytaire.

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