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Phagocytosis in Leprosy. III. Defective Adhesive and Endocytic Abilities of Circulating Leukocytes in Lepromatous Leprosy[1]

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The role of phagocytic cells is of known importance for the control or progress of most infectious diseases. In leprosy, this is particularly important as *M. leprae* parasitizes the phagocytic cells themselves and uses them for its own survival. In lepromatous leprosy, it has been seen that macrophages are less efficient than those from tuberculoid or healthy people in regard to their capacity to digest *M. leprae* ([2,3,12]), but this deficiency is better explained on the basis of a defective cell-mediated immunity at the level of *M. leprae*-reactive T-lymphocytes ([5,8,9]). Others ([6,14]) have been unable to find macrophage deficiencies in lepromatous or tuberculoid leprosy.

Polymorphonuclear leukocytes (PMN), on the other hand, do not seem to be defective when compared with PMN from normal controls. These cells show normal levels and activities of a variety of hydrolytic enzymes ([1,7]) and an adequate bactericidal function ([6]). They also show the normal metabolic changes induced during phagocytosis ([10,11,13]). In spite of these data, lepromatous patients show a progressively devastating disease and a depressed inflammatory response to several skin-testing antigens that is more generalized and pronounced in patients with long lasting or untreated lepromatous leprosy.

In this paper, evidence is presented that the defective inflammatory response in lepromatous leprosy may in part be explained on the basis of the presence of soluble serum factors that interfere with parts of the phagocytic function (namely endocytosis and polystyrene adherence). Other factors in leprosy that block the chemotactic response of blood leukocytes have been described by others ([17,19]).

MATERIALS AND METHODS

Subjects. Patients with nodular (NLL) or diffuse (DLL) lepromatous leprosy were both ambulatory persons who attended the Centro Dermatológico Pascua (México City) for medical care and permanent inhabitants of the leprosarium "Dr. Pedro López" in the state of Zoquiapan, México. The patients were 28 adults of either sex, heterogeneous in regard to age, time of onset of leprosy, and length and type of treatment. All of them, however, had leprosy which was still active, and several were experiencing a reactional episode at the time of the study. Normal controls were adult personnel of our Department of Immunology and healthy volunteers who had no relation with leprosy (n = 18).

Cell preparations. For the first part of the study, about 20 ml of blood was withdrawn in a 20 cc disposable syringe containing 500 IU heparin. After sedimentation by gravity of the red cells (between 1 and 2 hr in normals and around 30 min in patients), the leukocyte-rich plasmas were collected through a 20 gauge needle bent to a 90° angle into sterile screw capped tubes and centrifuged at 400 × *g* for 3 min at 4°C. The

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supernatants were separated and saved for further studies, and each sediment was gently suspended in Alsever's solution. After one more wash with Alsever's, the cell pellet was treated for 30 sec with a 0.2% NaCl solution and then with 1.6% NaCl to lyse the erythrocytes. This hypotonic treatment was repeated once when required. Two additional washes with Alsever's solution (250 × *g*, 3 min, 4°C) were performed before the cells were suspended in plain TC Medium 199 (Difco) and adjusted to 50 million leukocytes per ml of medium.

Short-term cultures. Five million washed leukocytes were added to Leighton tubes (Bellco, window size: 40 × 12 mm), each containing a glass coverslip (18 × 9 mm) and 1.0 ml of TC Medium 199 without supplements. Cultures were set up in duplicate and incubated for 3 hr at 37°C in a 7% CO_2 atmosphere to allow attachment of the leukocytes to the glass coverslip. Then, after the nonadherent cells were washed out, 1.0 ml of fresh medium and 12 × 10^6 *Mycobacterium lepraemurium* (Hawaii strain) in 20 μl of normal or lepromatous serum were added and the cultures incubated for another 3 hr period. Bacilli were isolated from infected mice according to Ueno, *et al.* ([18]) and opsonized with fresh normal or lepromatous serum for 30 min at 37°C. The nonphagocytized bacilli were washed out, and 1.0 ml of Medium 199 containing 20% pooled normal serum plus penicillin (100 IU per ml) and streptomycin (100 μg per ml) was added to each culture. After 12 hr of incubation, the coverslips were removed, washed with normal saline, fixed with absolute methanol for 2 min, stained by the Ziehl-Neelsen method, mounted with resin, and observed under the microscope for endocytosis.

Hematologic studies. In the second part of the study, originally designed to seek possible changes in total leukocyte counts, differential counts, and leukocyte viability along an 8 hr incubation period *in vitro* at room temperature (27°C), 31 leprosy patients (21 lepromatous and 10 indeterminate) and 21 normal controls were included. Conventional hematologic methods were employed: a) total leukocyte counts were performed with the aid of a Neubauer chamber using Turck's fluid with 0.01% gentian violet as diluent; b) differential counts were made from smears stained with buffered Wright's stain, pH 7.0; and c) leukocyte viability was assessed by incubating one part of undiluted leukocyte rich plasma with 19 parts of 0.2% Trypan blue in 0.01 M phosphate buffered saline, pH 7.4, for 15 min at 37°C, and counting the percent of viable (dye excluding) and dead (stained) leukocytes.

From 8 ml heparinized blood samples, about 2 ml were placed in sterile disposable 12 × 75 mm plastic tubes (Falcon 2054). The remaining 6 ml of blood were kept undisturbed for about 30 min to allow erythrocyte sedimentation after which the leukocyte rich plasmas were collected and placed in plastic tubes as before. Whole blood was used for total and differential counts and the leukocyte rich plasma for viability determinations after 0 and 8 hr of incubation at 27°C.

Statistical analysis. The Student's *t* test for small samples and the Student's *t* test for paired samples were applied. Differences with a $p \leq 0.05$ were considered as statistically significant.

RESULTS

Since the subjects were studied on different days, every time lepromatous blood samples (1 to 3) were analyzed, a normal control was included. The percent endocytosis varied somewhat from day to day among controls (and patients). In order to normalize results, the endocytosis values for patients are given as a percent of the value for the normal control that was processed simultaneously. This value was taken as 100% endocytosis, and because of this no standard deviations are shown when the control values are depicted. It was found that the phagocytic cells from lepromatous patients, both mononuclears (MN) and polymorphonuclears (PMN), ingest bacilli less frequently than these cells from healthy controls under similar circumstances whether they are incubated in the presence of normal serum or lepromatous serum during phagocytosis (Fig. 1). In normal serum (NS) the average endocytic activity, compared with controls, was 65% for MN and 58% for PMN while in lepromatous serum (LS) it was 65% for PMN and 72% for MN. This last value is not statistically different from that for the control

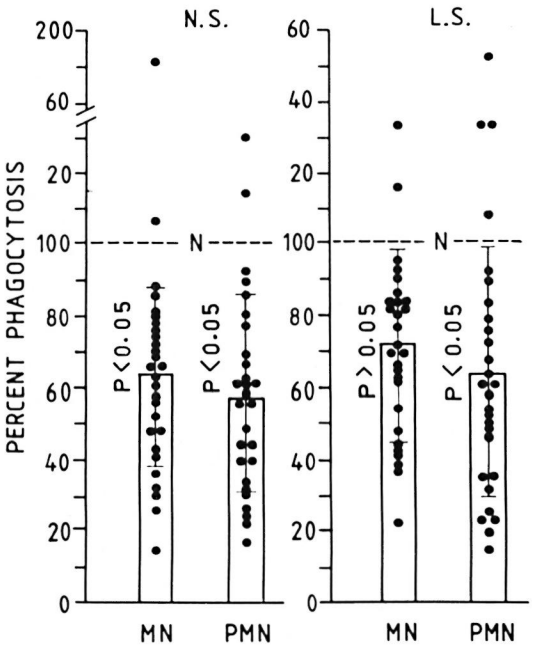

FIG. 1. Endocytosis of *M. lepraemurium* by lepromatous monocytes (MN) and polymorphonuclears (PMN) in the presence of normal (NS) or lepromatous (LS) serum. The results are referred to those obtained with normal phagocytes (N) in the presence of NS or LS. To facilitate the interpretation, normal values were taken as 100% endocytosis. Mean endocytosis values and standard deviations for the lepromatous phagocytes are shown.

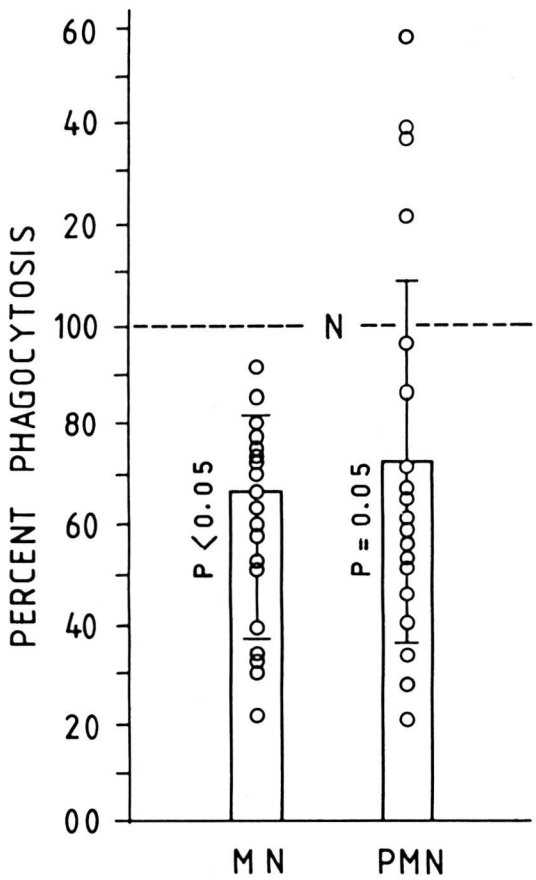

FIG. 2. Effect of lepromatous serum on the endocytosis of *M. lepraemurium* by normal monocytes (MN) and polymorphonuclears (PMN). Vertical bars are the mean endocytosis values and standard deviations for normal phagocytes in the presence of lepromatous serum. The horizontal line represents endocytosis in the presence of normal (N) serum (taken as 100%).

group, but this can be explained by the fact that LS affects the endocytic activity of normal phagocytes, reducing the difference between normal and lepromatous phagocytes (see below).

The endocytic ability of normal cells in the presence of LS, compared with their endocytic ability in the presence of NS, was 65% for MN and 73% for PMN. In the presence of LS, normal cells behaved like lepromatous cells in regard to their endocytic ability whether the latter cells were incubated in the presence of NS or in the presence of LS (Fig. 2). We therefore deduced that lepromatous patients have a serum factor or factors that interfere with the endocytic ability of normal cells, lowering their efficiency. In our system, normal serum does not correct the low rate of endocytosis shown by lepromatous phagocytes (Fig. 1, endocytosis in the presence of normal serum).

Figure 3 shows the cell distribution in normal controls according to the number of bacilli per cell. It can be seen that nearly 60% of the ingesting cells (both MN and PMN) ingested only 1 bacillus, about 20% ingested 2 bacilli, 10% ingested 3 bacilli, and so on. A thoroughly similar distribution was observed in the lepromatous phagocytes. From this, there do not seem to be subpopulations within the MN and PMN cells of either group (normal or lepromatous) regarding their endocytic ability. It should be pointed out, however, that the bacilli to cell ratio (about 2:1) was small and hence may have been inadequate to dem-

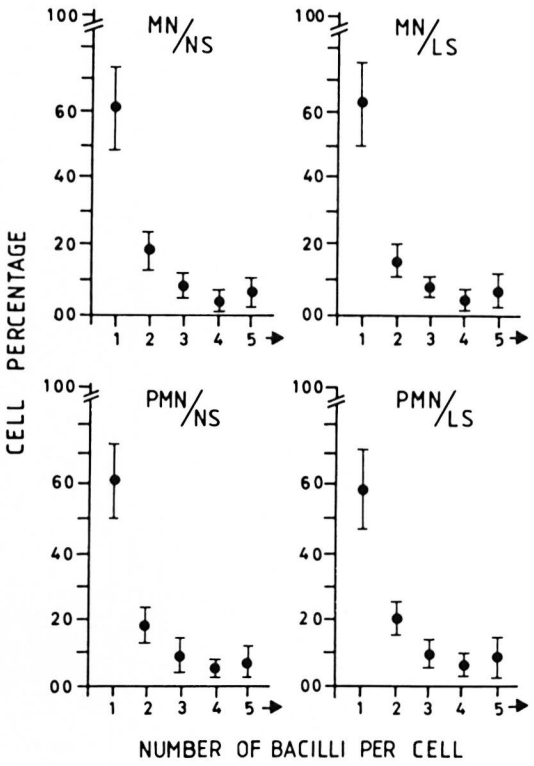

FIG. 3. Distribution of normal phagocytes according to their ability to endocytose an inoculum of *M. lepraemurium*. MN/NS depicts the behavior of monocytes in the presence of normal serum, MN/LS corresponds to monocytes in the presence of lepromatous serum, PMN/NS and PMN/LS describe the behavior of PMN in the presence of normal or lepromatous serum, respectively.

onstrate endocytic subpopulations among circulating MN or PMN phagocytes.

Regarding the second part of our study, the observed changes in both normal and leprosy PMN and lymphocyte (Lc) counts after 8 hr incubation as related to 0 hr counts are shown in Table 1. An increase or decrease in the cell counts represents a deviation from the 0 hr count of more than 10%. Variations equal to or lower than 10% were considered as no change. It can be seen that the great majority of controls (81%) showed a decrease in the proportion of PMN; most LL patients (43%) showed no change, and most of the indeterminate (IL) patients (60%) behaved as the controls, showing a decreased proportion of PMN.

The proportions of Lc reflected the changes in PMN proportions. Most of the normals (71.4%) and IL patients (60%) showed an increase in the proportion of Lc relative to the 0 hr counts, and most of the LL patients (52%) showed no change.

Within the LL group, 28.5% of the patients showed changes in PMN and Lc proportions similar to those of the majority of controls (a decrease in the proportion of PMN and an increase in the proportion of Lc). A variable, and small, number of individuals showed an increase in the percentage of PMN and a proportional decrease in the percentage of Lc after the 8 hr incubation period. These results are discussed below.

The average PMN and Lc proportions for normals, LL, and IL groups at 0 hr and 8 hr are shown in Fig. 4. A similar pattern was displayed by the normal and IL groups, and a different one by LL leukocytes. Zero hr versus 8 hr PMN percentages for normal, LL, and IL groups were respectively: 59.2 ± 11.3 vs 43.6 ± 12.5, 55.4 ± 20.0 vs 54.3 ± 13.3, and 59.7 ± 9.3 vs 47.5 ± 16.8. Zero hr versus 8 hr Lc percentages for the same groups were respectively: 36.8 ±

TABLE 1. *Effect of* in vitro *incubation for 8 hr at 27°C on the proportion of blood leukocytes from normal individuals, lepromatous, and indeterminate patients.*[a]

	Normals	Lepromatous	Indeterminate
Polymorphonuclears			
Decrease	17/21 (81.0%)	6/21 (28.5%)	6/10 (60.0%)
No change	3/21 (14.3%)	9/21 (43.0%)	3/10 (30.0%)
Increase	1/21 (4.7%)	6/21 (28.5%)	1/10 (10.0%)
Lymphocytes			
Decrease	1/21 (4.7%)	4/21 (19.0%)	1/10 (10.0%)
No change	5/21 (24.0%)	11/21 (52.0%)	3/10 (30.0%)
Increase	15/21 (71.4%)	6/21 (28.5%)	6/10 (60.0%)

[a] Deviations of more than 10% from the 0 hr counts are considered to be changes.

11.5 vs 52.7 ± 12.3, 41.2 ± 21.0 vs 43.3 ± 14.0, and 37.1 ± 9.0 vs 48.1 ± 16.5.

The data in Table 2 suggest that the above changes in the leukocyte population were not due to a different initial cell distribution in the groups of individuals that were studied. While the total number of leukocytes was higher in the LL patients, the proportions of the individual leukocyte classes were similar in all groups. The changes were also independent of differences in the viability of the leukocytes since the viability after 8 hr at 27°C was about 98% of that at 0 hr for all groups (normal, LL, and IL).

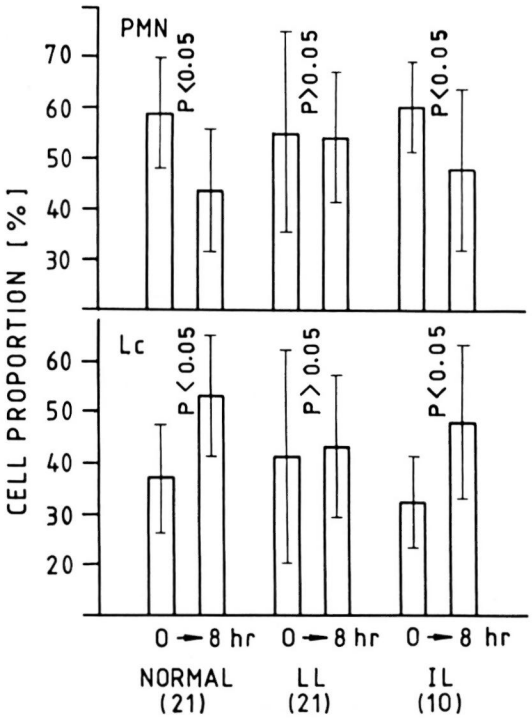

FIG. 4. Changes in the proportions of polymorphonuclears (PMN) and lymphocytes (Lc) after 8 hr of incubation at 27°C. Values at 8 hr are compared with values at 0 hr for each group: normal, lepromatous (LL) and indeterminate (IL). The mean proportions and one standard deviation of each cell population are depicted.

DISCUSSION

Our results show a defective endocytic ability of phagocytes of lepromatous patients. An important point in this study is that such phagocytic function was measured shortly (3 hr) after the blood was drawn. The monocytes were not allowed to transform into macrophages before they were exposed to *M. lepraemurium*. This probably accounts for our low values for endocytosis (14.4% ± 8.0% for normal PMN and 16.6% ± 7.7% for normal MN in the presence of normal serum) compared with those reported in other studies. Other studies have usually involved not MN (nor PMN as these are rapidly eliminated from the cultures due to their short life-span) but macrophages, generated during the culture conditions in a rather "activated state." Activation is induced by protein or other constituents of supplements added to the culture medium. The most common supplement is blood serum from a variety of sources, and such sera are rich in activating components.

We conclude from the present observations that even if lepromatous phagocytes are intrinsically normal, their endocytic ability is depressed by "factors" present in lepromatous serum. Ward, *et al.* ([19]) have

TABLE 2. *Total and differential leukocyte counts in blood from normal individuals, lepromatous (LL) and indeterminate (IL) leprosy patients.*

Cells	Normals (21)[a]	LL (21)	IL (10)
Total leukocytes per mm³	7.19 ± 1.47[b]	11.04 ± 4.70	9.40 ± 4.21
Lymphocytes (%)	36.80 ± 11.55	41.20 ± 20.80	37.10 ± 8.80
Monocytes (%)	3.85 ± 2.77	3.38 ± 2.50	2.90 ± 1.85
Neutrophils (%)	57.76 ± 11.56	53.29 ± 20.61	57.80 ± 10.60
Eosinophils (%)	1.05 ± 1.07	1.76 ± 2.16	1.80 ± 3.12
Basophils (%)	0.48 ± 0.68	0.52 ± 0.98	0.40 ± 0.69

[a] Figures in parenthesis are the number of individuals in each group.
[b] Total leukocyte counts are given in thousands. The mean values ± one standard deviation are shown.

reported a high incidence of a leukotaxis inhibitor in lepromatous sera. This inhibitor acts directly on leukotactic factors (bacterial chemotactic factors, C3 and C5 fragments) and makes them irreversibly inactive. The presence of this chemotactic factor inactivator (CFI) correlated well with poor skin reactivity to several skin-testing antigens: lepromin, PPD, trychophytin, candidin, and mumps. They concluded that this CFI might be responsible, at least in part, for some of the defects in the cellular inflammatory responses in lepromatous leprosy.

In the present study, the inability of normal serum to correct the endocytic defect shown by lepromatous phagocytes could be due to the serum factor(s) which inhibit endocytosis remaining specifically or nonspecifically attached to the cell surface and continuing to block its normal membrane function. Whether or not this is the case, and, if so, the length of time that this attachment persists, remain to be elucidated.

The decreases in the proportions of PMN, with concomitant increases in the proportion of Lc with incubation *in vitro,* are due to the PMN adhering to the plastic surfaces. The present studies also suggest that lepromatous plasma but not normal or indeterminate leprosy plasmas can decrease the plastic adhesivity of autologous PMN leukocytes.

We do not have a definite explanation for the small and variable percentage of individuals in all groups that showed a decrease in the proportions of Lc after incubation *in vitro.* It is interesting that most of the individuals that showed these changes belonged to the LL group (4/21). The control group showed this pattern least frequently (1/21), and the IL group behaved in an intermediate manner. Possible explanations for this pattern of a decrease in the proportion of Lc present after *in vitro* incubation would include the adhesivity of B lymphocytes (B-Lc). B-Lc are generally increased in active lepromatous leprosy while T-Lc are decreased. An additional explanation might be that, as judged by conventional staining techniques, at least 20% of peripheral blood cells counted as lymphocytes are actually cells of the monocyte series ([20]). These are adherent cells and may be increased in patients with lepromatous leprosy ([16]).

These *in vitro* observations correlate well with the defect *in vivo* of lepromatous patients in regard to their ability to mobilize inflammatory cells (largely PMN) to skin windows ([4]) and with those observations of Saúl ([15]) regarding the early histopathologic changes following the intradermal injection of integral lepromin in patients with diverse types of leprosy. In the work of Bullock, *et al.* ([4]), lepromatous patients showed, on the average, a 50% reduction in their capacity to accumulate inflammatory cells (PMN and MN) at skin windows when compared with normal or tuberculoid controls. In Saúl's work ([15]), the number of acid-fast bacilli found in biopsies taken from a 4 hr lepromin reaction varied depending on the type of leprosy. In tuberculoid cases, bacilli were eliminated as early as 4 hr after the injection while in lepromatous cases bacilli were found intact with the exception of a few patients under treatment. These results might suggest also an impaired leukocytic mobility or activity.

The defective phagocytic function in lepromatous leprosy, as deduced from the above findings, can be caused by soluble factors and can explain, at least in part, the diminished inflammatory response of these patients to a variety of antigenic stimuli. The nature of these soluble factors remains to be elucidated, but soluble immune complexes or soluble mycobacterial substances may be involved.

Finally, we want to underline the fact that the patients studied, although all had active leprosy, were heterogeneous in regard to age, time of onset of disease, and length and type of treatment. Several patients had a "controlled" but not necessarily cured leprosy while others had an acute or insidious disease. It is possible that if these studies had been done in patients grouped according to their "degree of disease," sharper differences would have been found.

SUMMARY

Two aspects of the phagocytic process in normal and lepromatous polymorphonuclears (PMN) and monocytes (MN) were studied. These aspects were endocytosis and adherence to surfaces. These 2 phenom-

ena are closely related to each other and to the whole phagocytic process as they depend on membrane activity.

It was found that both lepromatous phagocytes (PMN and MN) show, on the average, a depressed ability to endocytose opsonized *Mycobacterium lepraemurium* when compared to normal phagocytes. This impairment, however, does not seem to be related to intrinsic defects in the phagocytic cells themselves but to the presence in lepromatous serum of factors that interfere with the endocytic function. This conclusion was drawn from the fact that lepromatous serum causes a similar diminution in the endocytic ability of normal phagocytes. On the other hand, lepromatous plasma but not indeterminate leprosy nor normal plasmas interfere with the capacity of PMN leukocytes to adhere *in vitro* to plastic surfaces.

These impairments in the phagocytic function might help to better understand the depressed overall inflammatory responses observed *in vivo* in lepromatous patients with a long-lasting disease.

RESÚMEN

Se estudiaron dos aspectos del proceso fagocítico en los polimorfonucleares (PMN) y en los monocitos (MN) derivados de personas sanas y de pacientes con lepra lepromatosa. Estos dos aspectos fueron la endocitosis y la capacidad de adherencia a superficies. Los dos fenómenos estan íntimamente relacionados entre sí y con el proceso fagocítico general, ya que dependen de la actividad de la membrana celular.

Se encontró que, en comparación con los normales, los fagocitos PMN y MN de los pacientes lepromatosos mostraron una capacidad (promedio) deprimida para endocitar un inóculo de *M. lepraemurium*. Esta depresión, sin embargo, no pareció estar relacionada con defectos intrínsicos de las células fagocíticas sino con la presencia de factores en el suero lepromatoso que interfieren con la función endocítica. Esto se concluyó en base a la observación de que el suero lepromatoso causó una disminución similar en la capacidad endocítica de los fagocitos normales. Por otro lado, el plasma de los pacientes lepromatosos (pero no el plasma normal ni el de pacientes indeterminados) interfirió con la capacidad de los PMN para adherirse *in vitro* a superficies de plástico.

Estas alteraciones en la función fagocítica podrían ayudar a comprender mejor la deprimida respuesta inflamatoria general observada en los pacientes con una infección leprosa (lepromatosa) de muy larga duración.

RÉSUMÉ

Deux aspects du procès de la phagocytose des polynucléaires (PMN) et monocytes (MN) provenant de patients lépromateux ont été étudiés. Ces aspects sont endocytose et adhérence à une surface. Ces deux phénomènes sont liés entre eux et avec le phénomène de la phagocytose en général parcequ'ils dependent de l'activité de la membranne.

Les phagocytes lépromateux PMN et MN ont révélé une diminution dans leur habelité d'endocyter au *Mycobacterium lepraemurium*, si on le compare avec les phagocytes normaux. Cette dépression ne semble pas être liée avec une défaut intrinsèque de les cellules phagocytaires, sinon avec la présence de facteurs qui se trouvent dans le sérum de lépromateux et qui interférent avec la fonction endocytaire. On aboutit à cette conclusion, après avoir demontré que le sérum des lépromateux produit une diminution dans la capacité endocytaire des phagocytes normaux. D'autre part, le plasma des malades lépromateux interfère avec la capacité d'adhérence des polynucléaires à une surface de plastic *in vitro*.

Ces anormalités dans la fonction des phagocytes peuvent nous aider à comprendre la diminution de la réponse inflammatoire observée *in vivo* chez les patients lépromateux.

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