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In vitro Activation of Neutrophils by Suspensions of *Mycobacterium leprae*[1]

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In the standard form of lepromatous leprosy (LL), multiplication of *Mycobacterium leprae* inside phagocytic cells occurs without obvious inflammatory phenomena. *M. leprae* also circulate in the blood, free or inside phagocytic cells ([2,9]). During the evolution of their disease, some patients show inflammatory phenomena in the skin, peripheral nerves, joints, and eyes. This is called reactional lepromatous leprosy (RLL). Spontaneous neutrophil activation (defined here as an increase in the proportion of circulating neutrophils [PMNs] capable of spontaneously reducing nitroblue-tetrazolium [NBT] *in vitro* takes place in many bacterial infections. Its magnitude may be estimated by determining the proportion of neutrophils that show blue formazan precipitates in their cytoplasm after incubation with NBT (Formazan-Positive [FP] cells). Activation may be induced *in vitro* by incubating PMNs with endotoxin, *Staphylococci*, or latex particles. In diseases such as granulomatous disease of childhood, activation cannot be induced.

We have shown previously ([3,5]) that in RLL, but not in LL, there is spontaneous neutrophil activation. PMNs from patients with any type or group of leprosy were equally well activated by *in vitro* incubation with endotoxin ([5]). The question remained as to the reason why spontaneous activation does not occur in PMNs from patients with LL. We wished to rule out the following possibilities: a) that *Mycobacterium leprae* by itself would be unable to induce neutrophil activation *in vitro*; and b) that PMNs from patients with LL would have a specific inability to be activated *in vitro* by *M. leprae*. We were able to show that: a) *M. leprae* suspensions are able to activate neutrophils *in vitro* and b) that PMNs from lepromatous patients behave in a comparable way to neutrophils from other individuals vis-à-vis *M. leprae* insofar as activation is concerned.

MATERIALS AND METHODS

Individuals tested. Three groups of persons were explored. Healthy controls were volunteers recruited from personnel of the Instituto Nacional de Dermatológia. Unrelated disease controls included individuals with diseases unrelated to leprosy. They were hospitalized in our wards for a variety of conditions (scleroderma, cutaneous leishmaniasis, chronic stasis ulcer, and paracoccidiodomycosis). Patients with leprosy were from our out-patient clinics or from our wards. They were subdivided according to the Madrid classification ([11]). Diagnosis was established by clinical examination, slit-skin smears for bacilli, Mitsuda reaction, and biopsy. The presence of reactional phenomena was established by clinical criteria and biopsy. Patients with overt systemic bacterial or viral infection were not included, but the presence of mild upper respiratory infection, foci of infection in teeth or skin, and, in one case, an infected circumscribed thermal burn, were no cause for exclusion. The patients had not received corticosteroids or cytotoxic

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drugs for at least one year prior to the tests. Therapy with sulfones (dapsone, DDS) or thalidomide was not withdrawn. Reactional patients were not tested at the beginning of their reactional epidode, and in most the intensity of the reaction was waning. Table 1 summarizes additional features of the experimental group.

***Mycobacterium leprae* suspensions.** They were prepared from subcutaneous nodules and lymph nodes of experimentally inoculated armadillos (*Dasypus sabanicola*). Tissues were initially put in phosphate buffered saline pH 7.2 (PBS) and autoclaved. Further processing was done under sterile conditions. "Centrifuged lepromin" was prepared as follows: a) autoclaved tissues were minced with scissors and ground by means of a Ten-Broeck apparatus until a fine suspension was obtained; b) the suspension was centrifuged at low speed (500 rpm for ten minutes in a Sorvall RC-3 General Purpose Centrifuge with a radius of 14.6 cm); c) the supernatant was saved and the sediment ground and centrifuged again. The process was repeated three times; d) pooled supernatants were then centrifuged at high speed (20,000 rpm in a Sorvall RC-2B refrigerated centrifuge and SS-34 rotor with a radius of 10.8 cm) for two hours; e) sedimented bacilli were resuspended in PBS to a concentration of 8.2×10^8 bacilli per ml. To obtain "trypsinized lepromin," tissues were processed as described above, but after initial grinding, a) the suspension was mixed with an equal volume of trypsin (trypsin 1:250 [Difco Laboratories, Detroit, Michigan, U.S.A.] 1% in water mixed with an equal volume of PBS pH 7.8); b) the tissue-trypsin mixture was kept at 37°C for four hours under continuous agitation; c) the suspension was then centrifuged, ground three times, and bacilli harvested by high speed centrifugation as described above. This bacillary suspension was also adjusted to a concentration of 8.2×10^8 bacilli per ml. No preservatives were added to any of the lepromins. Standard tests to rule out bacterial contamination were done at the end of the processing and again before use.

***Limulus* lysate assays.** These were done as recommended by Difco Laboratories ([1]). In this test, *Limulus* amebocyte lysates firmly gel after adding 5 ng and 0.5 ng of endotoxin (positive controls). Lepromin samples were run simultaneously with the two positive controls and a negative control (pyrogen-free water). Two concentrations of each lepromin (full strength and 1:9 v:v) were tested.

TABLE 1. *Main features of experimental groups.*

Group	Number of individuals	Age (years)	Sex
Healthy controls	6	30.4[a] (25–36)[b]	2 M[c]/ 4 F[d]
Unrelated disease control	6	35.0 (15–54)	1 M/ 5 F
Lepromatous leprosy	11	34.2 (16–57)	8 M/ 3 F
Reactional lepromatous leprosy	3	19.0[e] (16–22)	2 M/ 1 F
Borderline leprosy	9	45.6 (23–62)	5 M/ 4 F
Tuberculoid leprosy	8	30.3 (15–55)	3 M/ 5 F

[a] Figures indicate mean age (years) rounded to one decimal.
[b] Figures in parentheses indicate the range.
[c] Male.
[d] Female.
[e] In one patient the age was not recorded.

Reduction of NBT *in vitro*. This was estimated employing a modification of the technique of Matula and Patterson ([8]) as reported by us ([3,4,5]). Briefly, 1 ml of fasting blood was mixed with 100 U of sodium heparin (Eli Lilly and Company, Indianapolis, Indiana, U.S.A.) in siliconized glass tubes. One tenth ml aliquots were mixed with the same volume of NBT solution (a 0.28% frozen sterile stock solution of p-nitrobluetetrazolium [Calbiochem-Behring Corp., San Diego, California, U.S.A.] in normal saline) and was thawed and diluted at the time of testing with an equal volume of PBS pH 7.2. The blood-NBT mixture was incubated at 37°C for 25 minutes in siliconized excavated glass slides (VDRL Boerner test slides) placed in a moist chamber. After this, coverslip smears were done and stained with Wright-Giemsa. Simultaneously, *in vitro* activation was performed. The same basic method was used, but the NBT stock solution was diluted in PBS which contained 200 μg per ml of endotoxin (Li-

TABLE 2. *Activation in neutrophils from leprosy patients and control individuals.*

Group	NBT/ saline	NBT/ endotoxin[b]	NBT/"centrifuged" lepromin F.S.[b]	NBT/"centrifuged" lepromin 1:9[c]	NBT "trypsinized" lepromin F.S.[b]	NBT "trypsinized" lepromin 1:9[c]
Healthy controls	26.2 ± 5.1[a]	69.5 ± 10.6	62.3 ± 10.3	45.5 ± 9.7	61.5 ± 8.4	61.5 ± 16.4
Unrelated disease controls	22.2 ± 7.3	60.0 ± 9.9	52.8 ± 6.4	32.5 ± 6.8	53.7 ± 5.1	54.7 ± 10.5
Lepromatous leprosy	19.0 ± 4.0	70.6 ± 5.3	58.3 ± 5.8	36.2 ± 6.0	64.2 ± 5.4	63.4 ± 5.2
Reactional lepromatous leprosy	34.3 ± 9.2	64.0 ± 9.7	58.0 ± 11.7	64.3 ± 9.5	66.7 ± 10.9	37.0 ± 18.1
Borderline leprosy	16.1 ± 5.5	53.0 ± 7.6	43.9 ± 8.9	31.4 ± 7.6	40.3 ± 6.1	38.4 ± 7.9
Tuberculoid leprosy	19.9 ± 6.7	65.4 ± 5.8	52.3 ± 7.0	40.3 ± 6.2	59.1 ± 6.9	57.8 ± 6.5

[a] Figures show mean percentage of FP cells ± standard error of the mean. N's given in Table 1.
[b] F.S. = full strength, 8.2×10^8 *M. leprae* per ml.
[c] 1:9 = 0.91×10^8 *M. leprae* per ml.

popolysaccharide W, *E. coli* 026: B6, [Difco Laboratories, Detroit, Michigan, U.S.A.]), or else NBT stock solution was diluted in each of the lepromins at full strength and 1:9 concentrations. Coverslips were mounted and at least 100 neutrophils were counted. Only intact cells were taken into consideration. Results were expressed as percentage of FP cells.

Statistical significance. This was estimated by the t test for small independent samples or the t test for small paired samples as required.

RESULTS

Table 2 summarizes the results. As previously reported ([4]), PMNs from patients with all forms of leprosy (except RLL) do not show spontaneous activation above that of control groups (baseline levels were somewhat higher than those previously reported because criteria for inclusion were less stringent for all groups). PMNs from patients with all forms of leprosy were activated *in vitro* by incubation with endotoxin (in the current series, patients with borderline leprosy had a lower percentage of FP cells than other groups when activated with endotoxin; this difference was not statistically significant; $p > 0.2$). Suspensions of *M. leprae* were able to activate *in vitro* PMNs from individuals of all groups. The percentage of FP cells in samples incubated with such suspensions was significantly higher than that of samples incubated with NBT-saline for all groups and all dilutions of lepromin ($p < 0.01$ or $p < 0.001$). There were no significant differences between the groups except that patients with borderline leprosy had a lesser degree of activation than PMNs from lepromatous individuals ($0.02 > p > 0.01$ for full strength trypsinized lepromin). Trypsinized lepromin 1:9 was more activating than centrifuged lepromin 1:9 ($p < 0.001$ in the case of lepromatous leprosy).

Both undiluted lepromins gelled *Limulus* lysates. Trypsinized lepromin, however, did not induce gelification when diluted 1:9. Centrifuged lepromin induced a soft gel at this dilution (much softer than that induced by 0.5 ng of endotoxin).

DISCUSSION

Our results demonstrate that there is no intrinsic anergy of PMNs from LL patients vis-à-vis *M. leprae* suspensions. *M. leprae* is able to induce activation in neutrophils from individuals of all groups.

Suspensions of *M. leprae* were capable of gelling *Limulus* lysates. It may be argued whether this should be attributed to contamination of these suspensions by endotoxin or whether a high concentration of *M. leprae* by itself was capable of inducing gelification. The latter is likely because not only endotoxin but also peptidoglycans from Gram-positive bacteria and synthetic N-acetylmuramylpeptides with adjuvant activities are pyrogenic and induce gelification of *Limulus* and *Tachypleus* amebocyte lysates ([6, 7, 13]). Concentrations needed are much higher than active concentrations of Gram-negative endotoxin. In any event, lepromin-induced PMN activation in our study cannot be explained by endotoxin contamination. Even if it existed, the levels in trypsinized lepromin 1:9 would have been less than 0.5 ng. In centrifuged lepromin, the levels would not have exceeded that amount by much. We have found optimum levels for endotoxin-induced activation to be 5000 ng (final concentration), and activation drops when concentration is lowered. Park and Good ([10]) used 10,000 ng and Matula and Patterson ([8]) could not detect *in vitro* activation induced by endotoxin with concentrations lower than 500 ng/ml. Thus, putative endotoxin contamination of our preparations would have been of a very much lower magnitude than that required to produce *in vitro* induced activation.

Only a small number of patients with RLL was studied. We were currently more interested in their PMNs' basic ability to be activated *in vitro* by *M. leprae* than in spontaneously occurring activation. Since patients were chosen when the acme of their reactional state had passed, their level of spontaneous activation was somewhat lower than that previously reported ([3,5]).

Treatment with DDS or thalidomide was not discontinued. We had found thalidomide unable to affect activation either *in vivo* or *in vitro* ([4]). DDS has been reported not to affect oxidative metabolism of neutrophils linked to random movement, chemotaxis, or phagocytic uptake of yeast cells ([12]). Our current results showed no inhibitory effect attributable to these medications.

Leukocytes from patients with borderline leprosy were less reactive to lepromin. We have not found them to be less reactive to endotoxin in the past ([5]). More work will be necessary before it could be asserted that such leukocytes are more sluggish vis-à-vis *M. leprae* than those from patients of other groups. In the current study, patients with borderline leprosy were older than patients with other forms of leprosy or control individuals (Table 1).

Activation was induced *in vitro* using final concentrations of 2.5 × 10^8 and 0.28 × 10^8 *M. leprae* per ml. These are much higher numbers than those estimated to be present in circulating blood from lepromatous patients ([2,9]). It has been also reported that patients with RLL have a lower number of circulating bacilli than patients with LL ([9]). Activation in RLL should owe to an indirect mechanism, presumably immunologic in nature, and not to the simple presence of *M. leprae*.

SUMMARY

Activation, defined as an increase in the proportion of cells that reduce nitrobluetetrazolium *in vitro*, is present in neutrophils from patients with reactional lepromatous leprosy but not in neutrophils from patients with non-reactional lepromatous leprosy. Neutrophils from patients with all forms of leprosy are equally well activated by endotoxin *in vitro*. We have now shown that *in vitro* activation induced by *Mycobacterium leprae* suspensions is of comparable magnitude in neutrophils from patients with all forms of leprosy (including lepromatous and reactional lepromatous leprosy). There is no intrinsic neutrophil anergy in patients with lepromatous leprosy vis-à-vis *M. leprae* as pertains to activation. Spontaneous activation in reactional lepromatous leprosy is likely due to an indirect mechanism, probably of immunologic nature, and not simply to the presence of circulating *Mycobacterium leprae* in the blood.

RESUMEN

Se define activación como un aumento en la proporción de celulas que reducen al nitroazul de tetrazolio *in vitro*. Demostramos anteriormente que los neutrófilos circulantes de pacientes con lepra lepromatosa reaccional se encuentran activados. No así aquellos de pacientes con lepra lepromatosa usual. No obstante, los neutrófilos de pacientes con todos los tipos y grupos de lepra pueden ser activados *in vitro* por la endotoxina. Demostramos ahora que suspensiones de *Mycobacterium leprae* pueden inducir activación *in vitro*, aunque se necesitan concentraciones relativamente elevadas. La activación resultante es en conjunto, de una magnitud similar en los neutrófilos de personas normales, de pacientes con diversas afecciones no relacionadas con disfunciones neutrofílicas generales conocidas y de pacientes con los diferentes grupos y tipos de lepra (incluso lepra lepromatosa y lepra lepromatosa reaccional). No hay por lo tanto, una anergia específica de los neutrófilos lepromatosos frente al *M. leprae*, al menos en lo referente a la activación.

La activación espontánea presente sólo en la lepra lepromatosa reaccional, se debe probablemente a algún mecanismo indirecto posiblemente de naturaleza immunológica.

RÉSUMÉ

L'activation peut être définie comme l'augmentation dans la proportion de céllules qui reduissent le nitro-

bluetetrazolium *in vitro*. Nous avons préalablement demontré que dans la lèpre seulement les neutrophiles du sang des malades de lèpre lepromateuse reactionnelle étaient spontannément activés, mais pas les neutrophiles des malades de lèpre lépromateuse commune. Les neutrophiles des malades de toutes les formes et types de lèpre pourraient s'activer *in vitro* par l'endotoxine. Nous démontrons maintenant que des suspensions de *Mycobacterium leprae* peuvent induire l'activation des neutrophiles *in vitro*. La magnitude de cette activation est comparable dans des leucocytes des gens normales, des gens avec des maladies variés et des gens avec les types et groupes divers de lèpre (en inclouant la lèpre lépromateuse). Il n'ya pas donc de l'anergie spécifique des neutrophiles lépromateux vis-à-vis *M. leprae*, au moins en ce qui concerne l'activation.

L'activation spontanée présente seulement dans la lèpre lépromateuse réactionnelle est produite par un mechanisme indirect, probablement de nature immunologique.

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