CORRESPONDENCE

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Macrophage-Induced Procoagulation Assay for the Detection of Defective Macrophage Activation in Leprosy Patients

TO THE EDITOR:

In investigations of immune responses in leprosy patients, it is often of interest to have a relatively simple method to measure the activity of the macrophages. The cellular immune response in lepromatous leprosy patients is generally low and that of tuberculoid patients generally high. Several groups have reported deficient production of lymphokines by lymphocytes of leprosy patients, including interleukin-2, gamma-interferon and macrophage-activating factors ([6, 7, 8]). The latter deficiency resulted in a reduced capacity of macrophages to phagocytose and kill microorganisms ([14]). More such studies could be carried out in endemic areas if there were tests to detect macrophage activation which did not necessitate, for example, the use of radioactive materials. A candidate for such a test is the demonstrated ability of macrophages to secrete factors promoting blood coagulation ([3, 9]). The products released by sonication of macrophages can shorten the recalcification time of platelet-poor plasma (procoagulation), and activated macrophages show an increase in this procoagulant activity ([4, 11, 13, 14, 15]). Therefore, a measurement of the spontaneous clotting time of platelet-poor plasma in the presence of calcium and macrophage sonicate is a way to estimate macrophage activation and the effect on it of different factors.

In this study, we have used a clotting assay for human plasma, in the presence or absence of sonicates of mouse macrophages, treated or not treated with supernatants of normal lymphocyte cultures or those from leprosy patients, in order to investigate lymphokine production by leprosy patients' lymphocytes.

The patients, from the Bach Mai Hospital in Hanoi, Vietnam, included 2 with the LL form (bacterial index [BI] 6+) who had been treated with a multidrug regime for less than 4 months, 8 with the borderline form (BI 1–3+) who had not yet been treated, and 1 with TT leprosy, bacteriologically negative and not yet treated. For production of the macrophage-activating factors (MAF)-containing supernatants, lymphocytes separated from whole blood over Ficoll-Isopaque were cultured with or without phytohemagglutinin (PHA, Sigma type IV, 100 μg/ml) in RPMI 1640 medium plus 10% fetal calf serum, sodium pyruvate (1 mM), 2-mercaptoethanol (5×10^{-5} M), and gentamicin (Squibb, 50 μg/ml) at a concentration of 1×10^6 per ml in tissue culture tubes. After 48 hr at 37°C in 5% CO_2, the tubes were centrifuged and the supernatants were removed and stored at 4°C until tested for MAF activity. After collection of the supernatants, PHA was added to those collected from cultures not already treated with the mitogen.

The mouse peritoneal cells (PEC) were collected into cold siliconized tubes, pooling cells from 15–20 BALB/c mice, and adjusted to 10×10^6 cells/ml in RPMI 1640 plus supplements as above. Normally, these

suspensions contained at least 80% macrophages; erythrocytes were removed by hypotonic shock and suspensions with many granulocytes or lymphocytes were discarded. To prepare the cells for the asssay, 0.3 ml of macrophage cell suspension was mixed with 2.7 ml of lymphocyte culture supernatant. After incubation at 37°C in 5% CO_2 for a further 2 hr, the macrophages in each tube were sonicated (Soniprep, minimum power, 15 sec × 4) to release the factors produced.

To assay the procoagulant activity, platelet-poor plasma was prepared by mixing 9 volumes of fresh blood with 1 volume of sodium citrate buffer, 3.8% in 0.9% sodium chloride solution. This was centrifuged at 1800 *g* × 30 min and the plasma collected. The normal recalcification time (plasma clotting time) was determined by mixing 0.1 ml plasma with 0.1 ml PHA-containing RPMI 1640 medium; after incubation for 1 min at 37°C, 0.1 ml of 25 mM $CaCl_2$ was added and the time in seconds from the addition of the $CaCl_2$ to the appearance of the clot was recorded. Plasma samples with recalcification times of less 150 sec or more than 200 sec were not used in the determination of macrophage procoagulant activity. For this determination, 0.1 ml of sonicated macrophage (Mø) suspension was added to 0.1 ml of plasma, again 1 min before the addition of $CaCl_2$. The percentage decrease in the recalcification time (RT) was calculated as:

$$\frac{\text{RT without Mø sonicate} - \text{RT with Mø sonicate}}{\text{RT without Mø sonicate}} \times 100\%.$$

The recalcification time of normal human plasma was 178 ± 29.3 sec (The Table). The sonicate of the control mouse macrophages shortened this time by an average 18.6 ± 4.2 sec. Supernatants from normal human lymphocyte cultures, with or without PHA treatment, also decreased the recalcification time, by 12 ± 5 and 10.6 ± 6.4 sec, respectively. This slight procoagulant activity in lymphocyte culture supernatants was observed to be somewhat higher in supernatants from the cell cultures of leprosy patients, but not significantly so. It is clear from the data presented in The Table that the products of mouse macrophages exposed for 2 hr to supernatants from PHA-treated control cultures were able to decrease significantly the recalcification time of human plasma, a decrease of nearly 61%. Interestingly, the supernatants from untreated lymphocytes also induced macrophages to produce procoagulant factors, although to a lesser extent (a decrease in recalcification time of 45%). When the same experiment was done using cells from leprosy patients (LL and B results were pooled), the results were comparable in the controls without PHA but, in the cultures treated with the mitogen, there was more procoagulant activity in the lymphocyte culture supernatants (a 24% reduction in recalcification time compared to 12% by controls), and there was less generation of procoagulant activity in the mouse macrophages treated with these supernatants (46% reduction in recalcification time compared to 61% by macrophages treated with control supernatants).

These results demonstrate that the activation of macrophages to produce factors promoting coagulation of platelet-poor plasma (procoagulant activity) can be used to measure the deficiency of lymphokine production by lymphoid cells from leprosy patients. It has been shown that there are at least two blood coagulation factors produced by human monocytes: tissue thromboplastin and factor X ([2, 9, 11–13]); macrophages can also produce prothrombinase ([16]). The procoagulant activity of macrophages increases when they are activated by endotoxin ([13, 14]), antigen-antibody complexes ([11, 15]), complement components ([10]), adherence to glass ([18]), mitogens ([11]), antigens ([12]) or lymphokines ([4]). In this study, PHA was added to the supernatants collected from unstimulated lymphocytes to account for the contribution made by the mitogen to the stimulation of mouse macrophages from PHA-treated lymphocytes. Geczy and coworkers ([4, 5]) have demonstrated that the enhancement of macrophage procoagulant activity in PPD-immunized guinea pigs was due to macrophage-lymphocyte interaction and the production of lymphokines, and that this increase was parallel to increased delayed-type hypersensitivity. They reported that the supernatants from the lymphocyte cultures themselves had no procoagulant activity, but our finding of low levels of such

THE TABLE. *Recalcification times of human platelet-poor plasma in the presence or absence of procoagulant factors.*

Added	Controls (N = 10)		Patients			
			LL, B (N = 10)		TT (N = 1)	
	RT[a]	D%[b]	RT	D%	RT	D%
Medium	178 ± 29.3		178.5 ± 11.8		180	
Sonicate, untreated macrophages	144.5 ± 22	18.6 ± 4.2	140.5 ± 10.9	21.2 ± 5.2	150	16.6
Supernatant, cultures without mitogen	158 ± 20.5	10.6 ± 6.4	144 ± 21.4	19.3 ± 13.1	155	5.5
Supernatant, cultures with mitogen	155.5 ± 21.8	12.2 ± 5.0	136 ± 25.7	23.9[c] ± 15.2	160	11.1
Sonicate, treated macrophages (supernatant from control cultures)	94.8 ± 15.4	45.4 ± 11.8	102.5 ± 12.5	41.2 ± 7.2	100	44.4
Sonicate, treated macrophages (supernatant from PHA-treated cultures)	68 ± 19.4	60.9 ± 12.9	95.4 ± 12.2	45.9[d] ± 8.0	80	55.5

[a] Recalcification time in seconds ± standard deviation.
[b] Percentage decrease in RT ± standard deviation.
[c] Significantly different from control, $p < 0.05$.
[d] Significantly different from control, $p < 0.01$.

activity in the supernatants of PHA-treated lymphocyte cultures is in accordance with the observation of Coeugniet and Bendixen ([1]) that human lymphocytes treated with concanavalin A were able to produce procoagulant effects.

There is some discussion in the literature as to whether the defect in cellular immune response in lepromatous leprosy is due to defective lymphokine production ([7]) or to defective macrophage activity in producing monokines (e.g., interleukin-1 [19]). We show here that lymphocytes from lepromatous or borderline leprosy patients were able to induce less activation of macrophages to produce PCA than were controls; our one tuberculoid patient's cells behaved as healthy control cells in this respect. This is, therefore, evidence for the deficient production of at least some lymphokines in leprosy patients, in this case all recently diagnosed cases having had little or no treatment. There may be, however, more than one weak point in the chain of reactions which leads to protective responses, and the weak point may be different in different patients, even at one pole of the spectrum of leprosy symptoms. With this relatively simple test, it may be possible to investigate many more patients in countries where the disease is endemic in order to obtain more information on this point.

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