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Established Macrophagelike Cell Lines Synthesize Interleukin-1 in Response to Toxic Shock Syndrome Toxin

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Toxic shock syndrome toxin is already known to induce the production of interleukin-1 (IL-1) by preparations of monocytes and macrophages that are presumably contaminated with other types of cells. The response is enhanced by increasing the density of such monocytes, suggesting that the monocyte's response to toxic shock syndrome toxin may be augmented by its interaction with some other cell. Nevertheless, we now show that several human and murine macrophagelike cell lines (U937, J774, P388D_1, and WEHI 3) produce IL-1 when exposed to toxic shock syndrome toxin, and therefore the basic response does not require the presence of cells of other lineages. The cultured cells generally produce less IL-1 than do monocytes, but considerably more IL-1 is induced from cells that have undergone a terminal differentiation as a result of exposure to 1α,25-dihydroxyvitamin D_3. High concentrations of cultured cells suppress the production of IL-1; this effect is apparently not due to long-lived inhibitors of IL-1 production or of IL-1 activity, but may be due to a short-lived inhibitor of production.

Toxic shock syndrome, an acute illness characterized by fever, hypotension, a desquamating rash, diarrhea, vomiting, and other abnormalities, is associated with certain strains of *Staphylococcus aureus* that produce an extracellular pyrogenic protein known as toxic shock syndrome toxin 1 (TSST) (15, 16). Ikejima et al. (7) showed that this toxin, either crude or pure, would induce human blood monocytes to produce interleukin-1 (IL-1), which in turn may be responsible for the fever and possibly other signs of the syndrome. We are therefore interested in studying the intermediate steps between a cell's first interaction with TSST and its production of IL-1. An analysis of these steps may be complicated by effects of the other cell types that almost inevitably contaminate monocytes obtained from blood. Indeed we describe in this paper that a maximal response may require the cooperation of two cell types in monocyte preparations. Accordingly, we decided to evaluate the response of macrophagelike cell lines to determine (i) whether homogenous cells indeed produce any IL-1 on their own and (ii) how best to use cultured cells to study the biochemical basis of the toxin's action. We find that in various degrees all four macrophagelike lines chosen for investigation (one human and three murine) respond to TSST with the production of IL-1 and that the human line U-937, which we studied the most, can have a varied sensitivity according to its cell density and exposure to exogenous agents including mercaptoethanol and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$].

MATERIALS AND METHODS

Materials. Cell culture media were obtained from GIBCO Laboratories, Grand Island, N.Y.; Hypaque-M was obtained from Winthrop Laboratories, New York, N.Y.; fetal calf serum was obtained from Hyclone Sterile Systems, Logan, Utah; and penicillin, streptomycin, and glutamine were obtained from Irvine Scientific, Santa Ana, Calif. Rabbit antiserum against human IL-1 and normal rabbit serum were kind gifts from Charles A. Dinarello, Tufts University School of Medicine. 1α,25$(OH)_2D_3$ was generously given by Milan Uskokovic, Hoffmann-La Roche Inc., Nutley, N.J.

TSST. For most experiments we used a culture filtrate of the toxic shock syndrome-associated *Staphylococcus aureus* strain, FRI-1169, which we have characterized previously (7). Toxin purified by the method of Schlievert et al. (16) was a gift from Roger Stone of the Procter and Gamble Co., Cincinnatti, Ohio.

Cells. J774 is a macrophage line derived from a tumor that arose in a female BALB/c/NIH mouse in 1968 (14). A 5′-nucleotidase-positive variant of J774 was provided by Miguel Stadecker, Department of Pathology, Tufts University School of Medicine. P388D_1 is a macrophage line derived from a lymphoid neoplasm (P388) of a DBA/2 mouse (8). WEHI 3 is a macrophage line derived from a myelomonocytic leukemia in a BALB/c mouse (19). WEHI 3 and P388D_1 were the gifts of Daniel Gold, also of the Department of Pathology, Tufts University School of Medicine. These three lines were grown in Dulbecco modified Eagle medium containing 10% heat-inactivated fetal calf serum, 2 mM additional glutamine, penicillin G (100 U/ml), and streptomycin (100 μg/ml). The U937 line was established in 1975 from a human patient with "true" histiocytic lymphoma (17). It was provided by Peter Knudsen, Department of Medicine, Harvard Medical School. U937 cells have a monocytoid morphology and cytochemical profile. They were maintained in suspension culture in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 10 mM additional HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer (pH 7.4), penicillin G (100 U/ml), and streptomycin (100 μg/ml).

Cells were seeded at concentrations ranging from 10^4 to 10^6 per ml into the wells of 96-well flat-bottom microtiter plates (NUNC, Roskilde, Denmark) in a total volume of 0.2 ml of RPMI 1640 containing 10% heat-inactivated fetal calf serum, 20 μM indomethacin, and 5 μg of polymyxin B per ml. They were incubated with various concentrations of crude or pure toxin at 37°C for 24 h in an atmosphere of 5% CO_2. Their supernates were clarified by centrifugation at

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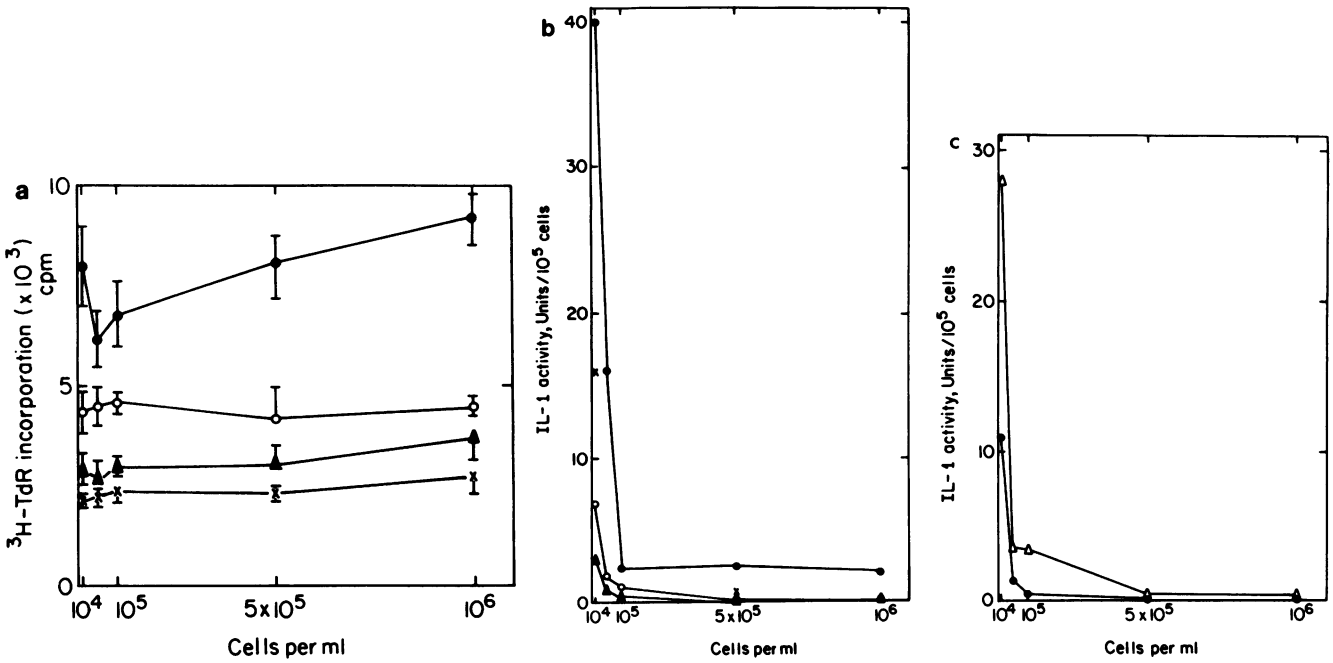

FIG. 1. Dependence of IL-1 production on the toxin concentration and cell number. (a) U937 cells were incubated with crude toxin at final concentrations of 1% (●), 0.1% (○), 0.01% (▲), and 0 (×). The raw counts obtained from the LAF assay are recorded. TdR, Thymidine. (b) The data from (a) normalized for cell number and expressed as units of IL-1 as described in Materials and Methods. Data for J774 cells and 1% toxin are also given (×). (c) As for (b), but the toxin used was purified: U937 cells were incubated with TSST at 10 ng/ml (●) and 100 ng/ml (△).

1,000 × *g* for 10 min and assayed for IL-1 content as described below.

Human monocytes. Mononuclear cells from the buffy coats of normal donors were purified by gradient centrifugation in Ficoll-Hypaque (3), washed three times, and suspended at various concentrations in minimal essential medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), polymyxin B (5 μg/ml), HEPES buffer (pH 7.4; 10 mM), and glutamine (2 mM). As a source of protective protein we included 1% heat-inactivated human AB serum that contained no detectable antitoxin or anti-IL-1. The monocyte preparations were incubated with various concentrations of toxin in the same conditions as the cultured cells.

IL-1 determination by a lymphocyte activation (LAF) assay. Macrophage and monocyte cell-free supernatants were assayed for their ability to stimulate the proliferation of thymocytes from 4- to 8-week-old C57B6/6J mice. Single-cell suspensions of the thymocytes were obtained by gently grinding the thymus between two frosted glass slides into RPMI 1640. The cells were filtered through a nylon mesh, washed three times by centrifugation (300 × *g*, 10 min) in RPMI 1640, and suspended to a density of 5 × 10^6 cells per ml in RPMI 1640 containing 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (pH 7.4, 10 mM), and beta-mercaptoethanol (50 μM). The thymocytes were cultured for 72 h at 5 × 10^5 cells per 0.2 ml in a well in the presence of 1 μg of phytohemagglutinin (Wellcome Research Laboratories, Beckenham, England) per ml and the following dilutions of the samples to be assayed: 20-, 40-, 80-, and 160-fold. For the final 24 h of incubation, the cultures were pulsed with 1 μCi of tritiated thymidine (6.7 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.). The cells were collected on glass fiber filters with a PHD cell harvester (Cambridge Technology Inc., Cambridge, Mass.) and counted by liquid scintillation. As controls, toxin was added to similar monocyte or macrophage cultures after their incubation and immediately before harvesting the culture supernatants. These controls measured an apparent direct effect of the toxin (explained below) and any potential interference from material in the culture medium. For Fig. 3 to 5, these control counts were subtracted before calculating the IL-1 units.

Units of IL-1 activity. Unknowns containing IL-1 were compared with a standard preparation of IL-1 by probit analysis as described by Gillis et al. (5). A stock solution of IL-1 produced by U937 at 10^4 cells per ml stimulated by 1% crude toxin was used as a standard. It was arbitrarily assigned an activity of 1 U/ml. Serial twofold dilutions of the standard and unknown were assayed by the LAF assay, and the results were plotted on probit paper. The activity of the unknown was then calculated from the relative dilutions of the standard and unknown that gave a response that was 50% of the maximum.

RESULTS

We have measured IL-1 by the LAF assay, that is, by the stimulation of proliferation of mouse thymocytes in the presence of a subthreshold concentration of phytohemagglutinin (Fig. 1a). To normalize the data, we transformed the counts to constant units of IL-1 activity that we defined by reference to a standard preparation of IL-1 as described in Materials and Methods.

Using this assay we found that TSST induced the release of IL-1 from four lines of cultured cells with macrophagelike properties (Table 1). As others have reported for short incubations of each of these lines (9, 13), none of them released a detectable amount of IL-1 without a stimulus.

The analysis was somewhat complicated by the fact that TSST causes a degree of proliferation when it is added directly to a preparation of mouse thymocytes (the data

TABLE 1. IL-1 production by macrophagelike cell lines incubated with TSST[a]

Toxin	Concn	% Increase in toxin incorporation by strain:						
		U937				WEHI 3 (7,360)	$P388D_1$ (603)	J774 (4,430)
		(2,668)[b]	(6,854)[b]	(4,916)[b]	(2,090)			
Crude	0.001%	55	14 (0)[c]		25 (0)	16		
Crude	0.01%	184	63 (14)		39 (0)	167	63	53
Crude	0.1%	428	200 (109)		110 (35)	254	190	92
Crude	1%	952	440 (223)		280 (129)	271 (115)	333	188 (107)
Pure	1 ng/ml			36 (0)				
Pure	10 ng/ml			122 (62)				
Pure	100 ng/ml			210 (59)				
Pure	1,000 ng/ml			330 (103)				

[a] Results of seven separate experiments. Since the absolute counts vary so much between different batches of thymocytes, results are expressed in relation to the appropriate backgrounds (given in counts per minute within parentheses after strain designations). The data for U937 cells show the extent of variation within one cell line. In all cases, cell concentrations were 10^4/ml.

[b] Supernatants were assayed immediately after formation; other supernatants were stored frozen for 1 or 2 weeks before addition to thymocytes.

[c] Numbers within parentheses after data represent control values for the apparent direct effect of toxin in which the toxin was added to the monocyte medium after incubation and then added directly to the mouse thymocytes.

given in parentheses in Table 1). This is not in fact an immediate effect of the toxin upon thymocytes, but seems to be mediated by IL-1 produced by the few mouse macrophages that inevitably contaminate such thymocytes. Two of the observations that lead to this conclusion are as follows: (i) the "direct" effect is blocked by an antibody against mouse IL-1, and (ii) in the absence of macrophages a cloned T-cell line (D 10) does not respond to TSST. Full details will be reported elsewhere (T. Ikejima, M. Minami, D. M. Gill, and C. A. Dinarello, manuscript in preparation). Appropriate controls to allow for this direct effect were included in every experiment, and its contribution has been subtracted for Fig. 3 to 5 (see Materials and Methods).

Since anti-human IL-1 does not neutralize mouse IL-1, it could be used to confirm that the active substance induced by the toxin in the human-derived U937 cells was indeed IL-1. When the antiserum was present during the thymocyte incubation, it blocked all proliferation mediated by U937 supernatants (presumably via human IL-1), but not the direct effect of the toxin via mouse IL-1. The U937 supernatants that contained toxin were then no more effective than toxin alone (Table 2).

Maximal IL-1 induction at low cell density. When the macrophagelike cells were grown in flat-bottom wells, the LAF activity per cell was strikingly increased, at least by 20-fold and sometimes by as much as 500-fold, by lowering the cell density from 10^6 to 10^4 cells per ml. As would be expected, the direct effect of the toxin in the supernatants was not affected by the macrophagelike cell density. The enhanced production at low cell density was demonstrated for all four cell lines, although not to the same degree. The data for U937 and J774 are given in Fig. 1b and c where, respectively, crude culture supernatant and purified toxin were used. To investigate the basis of this effect we chose to work just on the U937 line, because it is one of the more sensitive lines and because it is of human origin and most likely to be relevant to the toxic shock syndrome.

We asked first whether the lack of activity at high cell concentrations could be attributed to the presence of some substance in the supernatant that inhibited the action of IL-1. Amento and co-workers found that IL-1 could be detected in U937 supernatants, stimulated with a T-lymphocyte product, only after the supernatant had been passed over a gel filtration column to remove some ill-defined suppressor (2). In case a similar problem applied to our situation, we fractionated on Ultrogel AcA54 the cell culture supernatant derived from U937 cells stimulated at 10^6 cells per ml. LAF-positive material was detected as a composite peak in the molecular weight range of 11,000 to 30,000 (Fig. 2). Anti-IL-1 inhibited the activity in the molecular size range 11,000 to 17,000 which is the size expected for IL-1 (2). The material at about 20,000 M_r which is not inhibited by anti-IL-1 is probably TSST-1 itself or a derivative. There is some additional active material in the column eluate at about 26,000 M_r which is nonspecifically inhibited by sera; normal serum as well as anti-IL-1. We do not know what this represents. To quantitate recoveries, we compared the activity that was specifically inhibited by anti-IL-1 in a pool of all the active fractions from the column and in an unfractionated sample diluted to the same volume. Far from being increased by gel filtration, the IL-1 activity was reduced by two-thirds, which makes it quite unlikely that our supernatant had contained a removable inhibitor of IL-1 activity of the sort described by Amento et al. (2).

As a second approach to understanding the mechanism of the suppressive effect of high cell concentrations, we grew U937 cells in separate wells at 10^6 and 10^4 cells per ml, exchanged their culture supernatants after 24 h, then added toxin, and continued to incubate for another 24 h. The exchange of the supernatants did not affect the IL-1 production (Table 3). We deduce that no long-lived stimulator of

TABLE 2. Inhibition of LAF activity by anti-human IL-1[a]

Prepn	Serum	IL-1 equivalent (U/ml)		% of control
		IL-1 + toxin	Toxin	
Toxin + U937	None	2.5		100
	Anti-IL-1	0.7		29
	Normal	2.0		81
Toxin alone	None		0.7	29
	Anti-IL-1		0.6	23
	Normal		0.6	25

[a] U937 cells (10^6/ml) were stimulated with 0.1% crude toxin for 24 h. The culture supernatant, still containing toxin, or the original concentration of toxin alone, was incubated with normal rabbit serum or with rabbit anti-human IL-1 serum for 2 h at 37°C and were then assayed for their ability to stimulate thymocyte proliferation. Equivalent units of IL-1 activity were calculated as described in the text, but without first subtracting the contribution of the direct effect. This "direct" stimulatory activity of the toxin is expressed on the same scale, for the values probably represent mouse IL-1.

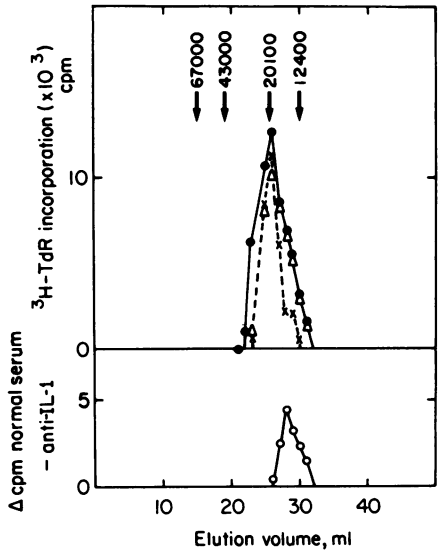

FIG. 2. Ultrogel AcA54 column chromatography of a concentrated supernatant from a culture of U937 cells at 10^6 cells per ml stimulated with 0.1% crude toxin. Fractions were assayed for LAF activity after incubation with anti-IL-1 serum (×), normal rabbit serum (△), or no serum (●). Each point in the lower panel represents the difference in counts per minute between the assay conducted with anti-IL-1 serum and that with normal serum. This difference is a complex function of the IL-1 content.

IL-1 production exists in the low-density cell supernatant and that no long-lived inhibitor of its production exists in the high-density cell supernatant. Further, the suppressive effect of high cell densities cannot be attributed to the lack in the medium of some nutrient or to the presence of something that neutralizes or inactivates the toxin.

Third, we grew U937 cells in round-bottom microtiter wells, instead of the previous flat-bottom wells, so that the cell densities would be high and relatively independent of the actual cell numbers. This maneuver much diminished the effect of cell number (Fig. 3). It greatly reduced IL-1 production at low cell numbers while having little effect on the already low production at high cell numbers. It is clear, therefore, that cell proximity is required for the suppression of IL-1 production.

Finally we compared the production of IL-1 by cells that were static and by cells that were grown in glass bottles in a water bath and shaken constantly (Table 4). We anticipated that the shaking would cause the cells to remain separate from each other, but it had no effect on the IL-1 production at high or low cell densities. Thus it would seem that the suppressive effect of high cell densities is less likely to be mediated by actual contact between cells than by a labile soluble mediator.

TABLE 3. Exchanging the U937 culture medium has no effect on IL-1 production[a]

Cell concn (per ml)		IL-1 (U/ml)	
Original culture	Added culture	0.1% TSST	1% TSST
10^6	10^6	0.115	1.84
	10^4	0.115	1.60
10^4	10^6	0.100	4.52
	10^4	0.103	4.52

[a] U937 cells were cultured for 24 h at 10^6 and 10^4 cells per ml. The medium was removed from each culture and added back to the same or crossed cell sample as shown in the second column. Crude TSST was added to the concentration shown, and the cells were cultured for 24 h more. The final samples of culture medium were then assayed for LAF activity.

Comparison with the response of human monocytes. The cultured cells were ordinarily less responsive than human peripheral monocytes. Whereas the monocytes respond to about 0.3 ng of a stock *S. aureus* supernatant per ml (7), U937 and WEHI 3 cells required about 3 ng/ml for a detectable response and the J774 and P388D_1 lines required substantially more still. The lesser sensitivity seems to result from a combination of at least the suppression considered above, the absence of augmentation from other cell types (as considered below), and a failure to express differentiated functions, which can be rectified by providing a derivative of vitamin D_3 (as described below).

IL-1 production by human monocytes at different densities. Ficoll-Hypaque-purified human monocytes respond to an increase in cell concentration with an increased production of IL-1 (Fig. 4), the opposite of the cultured cells. In some cases the slope of the line relating log IL-1 produced to log cell number was approximately 2. This was the case for crude toxin in Fig. 5, and we obtained similar data with purified toxin. The result suggests that the maximum response to TSST involves the interaction of at least two types of cells present in the monocyte preparation.

Enhancement of response by 1α,25-dihydroxyvitamin D_3. Amento et al. (1) demonstrated that the active form of vitamin D_3, 1α,25$(OH)_2D_3$, induces a terminal differentiation of U937 cells into a form with a greater IL-1 production in response to a certain lymphokine. Based on their finding, we looked for, and in fact found, a similar enhancement of the response of U937 cells to all concentrations of TSST. When U937 cells were preexposed to the 1α,25$(OH)_2D_3$ for 3 to 5 days, they produced several hundredfold more IL-1. The concentration of 1α,25$(OH)_2D_3$ that stimulated the IL-1 response to TSST-1 was the same as that required to induce maturation, as measured by cessation of proliferation, namely, 10^{-8} M (Table 5).

Among agents tested which failed to show such an effect

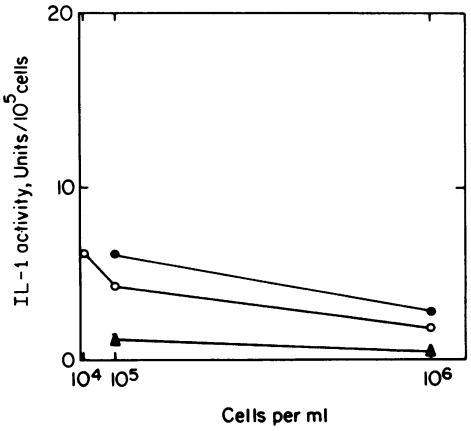

FIG. 3. IL-1 production by U937 cells incubated in U-shaped wells. The data have been corrected for the direct effect of the toxin upon thymocyte preparations. Other conditions were the same as in Fig. 1.

were retinoic acid (10^{-6} M), tuftsin (10 μg/ml), and γ-interferon (10,000 U/ml for 3 days).

Suppression of IL-1 production by mercaptoethanol. Beta-mercaptoethanol (50 μM) made the production of IL-1 by cell lines independent of the cell concentration. It suppressed the production at low concentrations while hardly changing the amount produced at a high cell density (10^6/ml). We noticed that cells ceased to be evenly distributed but tended to cluster at the margins of wells when mercaptoethanol was present. This change is certainly consistent with the effect of round-bottom wells described earlier, but it seems to us unlikely that mercaptoethanol acts solely by raising the local cell density.

DISCUSSION

Ikejima et al. established that peripheral blood monocytes released IL-1 when they were incubated with the culture supernatants of *S. aureus* from toxic shock patients or with the toxin purified from these supernatants (7). The IL-1 could be assayed either by the induction of fever in rabbits or by the enhancement of mouse thymocyte proliferation (the LAF assay). The monocytes used by Ikejima et al. were not pure, and the possibility remained that some cell other than a monocyte-macrophage was also required for IL-1 release. Although cell-to-cell effects may alter the size of the response, it is now clear that cells of the monocyte lineage have the capacity to respond to TSST on their own, for several established cell lines produce IL-1 when they are incubated with TSST. Such cells can thus be used to probe the mechanism of IL-1 induction without the influence of other cell types. It is particularly relevant to use the human line U937, since toxic shock syndrome is a human disease.

We found two ways of improving the cellular response. One is to preexpose the cells to $1\alpha,25(OH)_2D_3$, whose effect is probably a consequence of an induced maturation. The other method, which was a surprise, is to maintain a low cell density. We have shown that long-lived inhibitors or activators of IL-1 production cannot be responsible for the density dependence and that it is not possible to detect inhibitors of IL-1 activity in the high-density cell supernatants. The most reasonable interpretations of the effect are that IL-1 production is reduced by contact between cells, in a manner akin to the contact inhibition of cell growth and movement (6), or that there is a labile mediator that signals the cell density. It is not easy to distinguish rigorously between these two alternatives, but the results of the shaking experiment (Table 3) tend to support the existence of a labile mediator. If such a mediator exists, it is unlikely to be a prostaglandin since all of the experiments were conducted in the presence of indomethacin. The suppression can be mimicked by beta-mercaptoethanol, an agent that can enhance immune responses in vitro by modifying a serum stimulator of T lymphocytes (reviewed in reference 12) and can promote the growth of many cells, including lymphoma cell lines (14). Unfortunately, the biochemical basis of the action of mercaptoethanol is not known (4).

The cell density-dependent inhibition is not evident in the case of Ficoll-Hypaque-purified human monocytes. Rather

TABLE 4. Agitation does not alter IL-1 production[a]

Cell concn (per ml)	IL-1 (U/ml)	
	With shaking	Static
10^4	0.97	0.97
10^5	1.19	1.19
10^6	1.23	1.19

[a] U937 cells were cultured with 0.1% toxin for 24 h at 10^4, 10^5, and 10^6, cells per ml in static plastic dishes or in 100-ml glass bottles containing 20 ml of medium, which was shaken continuously to keep all cells in suspension. The culture supernatants were then assayed for IL-1 by the LAF assay.

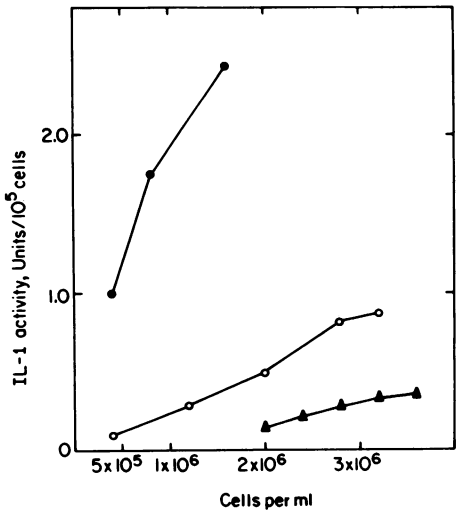

FIG. 4. Production of IL-1 by various concentrations of human peripheral monocytes. The monocyte preparation was incubated with crude toxin at final concentrations of 0.1% (●), 0.01% (○), and 0.001% (▲) for 24 h at the cell concentrations shown. Note the difference in scale between this figure and Fig. 1 and 3.

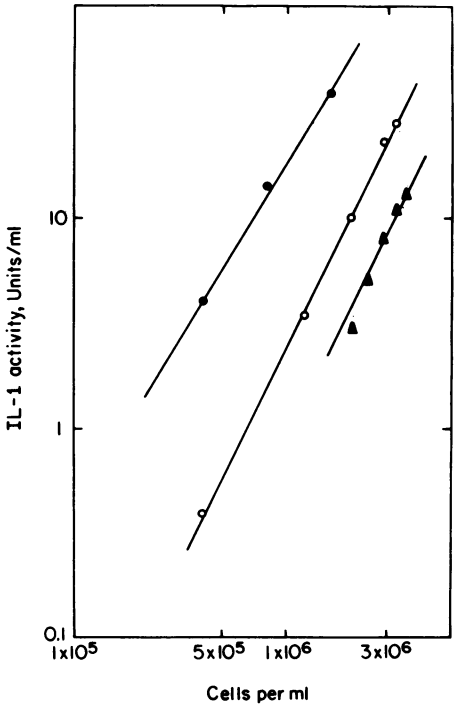

FIG. 5. Response of human monocytes to TSST increases with cell density. Data are from Fig. 5 with log response plotted against log cell number. Human monocytes were incubated with toxin at 0.1% (●), 0.01% (○), and 0.001% (▲).

TABLE 5. Changes in U937 cells in response to $1\alpha,25(OH)_2D_3$[a]

$1\alpha,25(OH)_2D_3$ concn (M)	Final no. of cells per well	IL-1 (U/10^5 cells)
0	13×10^5	1.6
10^{-10}	11×10^5	1.6
10^{-9}	8.9×10^5	6.3
10^{-8}	1.0×10^5	300

[a] Samples of 10^5 cells were exposed for 5 days to the stated concentrations of $1\alpha,25(OH)_2D_3$ and were then challenged with a maximally effective dose of TSST (1% of bacterial supernatant).

we obtained evidence that cell-to-cell interactions could boost IL-1 induction, for we found that the production by monocyte preparations increased disproportionately rapidly with cell concentration. When the logarithms of the resulting responses were plotted against the logarithms of cell numbers, straight lines were obtained (Fig. 5) whose slopes (approximately 2) indicate that a maximum of two cells interact directly or indirectly to give the maximum response (11, 18). Whether the second cell is another monocyte, a contaminating thymocyte (10), or another sort of contaminant is not presently known. However, future experiments involving the controlled addition of various cells to U937 cultures may reveal exactly what cell types are involved and also provide a better way of maximizing the TSST-induced production of IL-1.

ACKNOWLEDGMENTS

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