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Lymphokine-Induced Production and Release of Lysosomal Enzymes by Macrophages

(delayed hypersensitivity/lysosome/cell mediated immunity)

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ABSTRACT **Macrophages are associated with most chronic inflammatory lesions, and these cells contain enzymes that are able to destroy connective tissue constituents. Normal lymphoid cells responding to a mitogen, phytohemagglutinin-P, release factor(s) that cause a marked increase in the size and enzyme content of mononuclear phagocytes maintained in culture. The stimulated macrophages, which by several criteria remain otherwise viable and healthy, selectively release large quantities of hydrolytic enzymes to the culture medium.**

The stimulation of cultured lymphocytes by mitogen or previously sensitized lymphocytes by antigen results in the release of lymphokines, some of which affect the activity of mononuclear phagocytes (1–5). The phagocytes enlarge and they exhibit decreased mobility (6), a greater tendency to adhere to culture vessels (7), increased levels of oxidative activity and protein synthesis (8, 9), and enhanced capacity to phagocytose and kill microorganisms (10–12). The effect of lymphokines on the levels and distribution of various cellular and lysosomal enzymes of mononuclear phagocytes has not been determined. This is an important question because mononuclear phagocytes contain numerous hydrolytic enzymes that are able to alter connective tissue constituents (13–17), and these cells play an important role in delayed hypersensitivity reactions and other chronic inflammatory lesions (18). We have found that stimulated human peripheral blood lymphocytes release products that not only induce in cultured macrophages the effects described above, but also cause the cells, which are otherwise viable and healthy, to produce and selectively release large quantities of lysosomal enzymes into the extracellular culture environment.

EXPERIMENTAL METHODS

Human peripheral blood leukocytes were prepared and cultured at a concentration of 1.5×10^6 cells per ml of Waymouth's medium with 15% autologous plasma in Falcon 3033 culture tubes as described by Cooperband and Green (19). The cells were stimulated by the addition to the experimental cultures of 5 μg of phytohemagglutinin-P (Difco Laboratories, Detroit, Mich.) per ml at the beginning of a 72-hr incubation period. Control cultures were treated in an identical manner, except that phytohemagglutinin was added at the termination of the incubation period.

Abbreviations: CLS, control lymphocyte supernatant: material obtained by dialysis and freeze-drying of the medium from leukocyte cultures to which mitogen was added at the termination of the incubation period; SLS, stimulated lymphocyte supernatant: material obtained by dialysis and freeze-drying of the medium from leukocyte cultures to which mitogen was added at the beginning of the incubation period.

The culture medium was harvested after centrifugation, dialyzed against distilled water, and freeze-dried. In order to measure the extent of blast transformation, 1 μCi of [^{3}H]thymidine per ml was added to some of the cultures at 72 hr, the incubation continued for 4 additional hours, and the uptake of label into DNA measured by the method of Valentine (20). Phytohemagglutinin-stimulated cultures prepared in this way routinely incorporated 500- to 800-fold more [^{3}H]thymidine than control cultures. In five separate experiments, the amount of freeze-dried supernatant material obtained per 1.5×10^6 cells was 4.7 ($\pm$0.9 SD) mg from the stimulated cells and 4.8 ($\pm$0.7) mg from the unstimulated control cells.

Mononuclear phagocytes were collected from Sprague–Dawley mice (strain HA/IC) by peritoneal lavage and cultured in medium 199 containing 10% newborn calf serum as described by Davies *et al.* (21) without modification. Aliquots of 5 ml of lavage fluid containing approximately 10^6 mononuclear cells per ml were placed in 60 mm plastic petri dishes and incubated for 2 hr at 37° in an atmosphere of 5% carbon dioxide and 95% air. Nonadherent cells were removed by washing the plates four times with warm phosphate-buffered saline. Fresh medium containing control (CLS) or stimulated lymphocyte supernatant (SLS) was then added. After an additional 48 hr of incubation, the medium was harvested, the cell layer was washed once with warm phosphate-buffered saline, and the cells were released by the addition of saline containing 0.1% Triton X-100 (v/v). The protein content of the cell-containing fraction was measured by the method of Lowry *et al.* (22), and enzyme activity in the medium and cell-containing fractions was assayed as described (15, 21). In all experiments, quadruplicate cultures were done and the biochemical data are expressed as the mean $\pm$ (SD).

RESULTS

Within 30 min after the addition to macrophage cultures of the material (SLS) from leukocyte cultures stimulated with phytohemagglutinin, the cells began to spread and to extend numerous cytoplasmic processes. After 48 hr the cells exhibited a marked increase in the quantities of cytoplasmic vacuoles and phase-dense granules and enhanced ruffle membrane activity; they had increased approximately 4-fold in size (Fig. 1). These changes did not occur in cells exposed to equal concentrations of material from control leukocyte cultures (CLS) to which phytohemagglutinin was added at the end of the incubation period nor in the cells to which phytohemagglutinin alone had been added. The increase in cell size was reflected by an elevation in cellular protein levels (Table 1). Macrophages, cultured for 48 hr in the presence of SLS, ex-

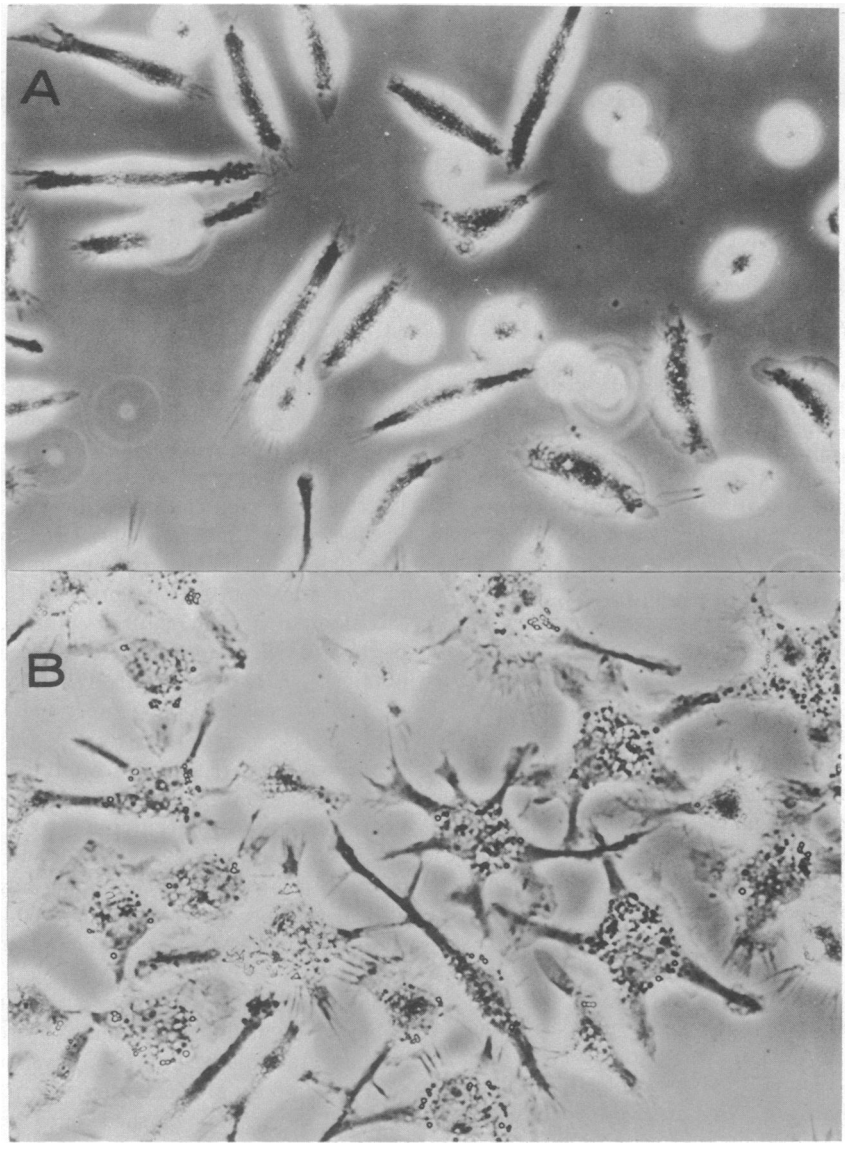

FIG. 1. Phase contrast photomicrographs, at an original magnification of 500-fold, of living macrophage cultures after 48 hr of incubation in medium 199 containing 10% newborn calf serum and (A) 1.5 mg/ml of control lymphocyte supernatant material (CLS) or (B) 1.5 mg/ml of stimulated lymphocyte supernatant material (SLS).

hibited a statistically significant increase ($P < 0.005$) in protein content when compared to cells exposed to an equal amount of CLS.

The changes in cell morphology and protein content were accompanied by a marked elevation in the levels of activity of various lysosomal and other cellular enzymes in the cultures. Cellular levels of lactate dehydrogenase (EC 1.1.1.27) and leucine-2-naphthylamidase increased approximately 2- and 3-fold, respectively, in the SLS-containing cultures during a 48-hr incubation period (Tables 2 and 3). In addition, statistically significant increases ($P < 0.001$) in activity were also observed in the stimulated cultures for β-glucuronidase

TABLE 1. *Protein content of mononuclear phagocytes exposed to 1.5 mg/ml of SLS or CLS for 0 and 48 hr*

No. of hr	CLS	SLS
0	35.3(1.8)*	33.7(1.5)
48	33.8(2.0)	39.3(1.6)

* Measured as described by Lowry *et al.* (22) and reported as μg per plate ± (SD).

TABLE 2. *Lactate dehydrogenase activity*, at various times during incubation, in the cells and media of macrophage cultures containing 1.5 mg/ml of CLS or SLS*

	CLS		SLS	
No. of hr	Cells	Media	Cells	Media
0	4.5(0.2)†	0.6(0.1)	4.6(0.1)	0.7(0.1)
48	4.8(0.2)	0.5(0.1)	8.5(0.4)	0.7(0.2)

* Lactate dehydrogenase activity was measured as described by Davies *et al.* (21).

† Reported as milliUnits/μg of macrophage protein ± (SD).

TABLE 3. *Leucine-2-naphthylamidase activity, at various times during incubation, in the cells and pellet of macrophage cultures exposed to 1.5 mg/ml of CLS or SLS*

	CLS		SLS	
No. of hr	Cells	Pellet†	Cells	Pellet
0	24.3(1.5)*	2.9(2.3)	26.1(1.3)	5.6(2.2)
48	25.5(2.4)	3.1(0.3)	72.1(3.8)	3.5(1.7)

* Leucine-2-naphthylamidase activity was determined according to the methods of Davies *et al.* (21) and reported as nmol of product/μg of macrophage protein per hr ± (SD).

† At the end of the incubation period, nonadherent cells were collected from the culture medium by centrifugation and the leucine-2-naphthylamidase activity in the cell pellet was measured.

(EC 3.2.1.31), acid phosphatase (EC 3.1.3.2), β-galactosidase, (EC 3.2.1.23) and β-*N*-acetylglucosaminidase (EC 3.2.1.30), while these enzyme levels did not change significantly in the CLS-containing cultures (Table 4). The elevated enzyme levels were not a result of increased cell numbers. The possibility that SLS stimulates macrophage mitosis *in vitro* was examined by carrying out incubations in the presence of tritiated thymidine (1 μCi/ml) for 72 hr and measurement of cell utilization of radioactivity by liquid scintillation counting. There were no significant differences in thymidine uptake between cultures containing CLS or SLS.

In the SLS-containing cultures, a statistically significant ($P < 0.001$) portion of the acid hydrolase activity was released from the cells to the culture medium (Table 4). The magnitude of this release for β-glucuronidase, β-galactosidase, and β-*N*-acetylglucosaminidase was in the range of 70–80%, and for acid phosphatase about 40% of the total enzyme present. The release appeared to be selective since lactate dehydrogenase, a soluble cytoplasmic enzyme, and leucine-2-naphthylaminidase, a predominantly membrane-bound nonlysosomal enzyme, remained confined to the cells (Tables 2 and 3). Previous studies demonstrated that maximal lysosomal enzyme production and release occurs in cultures exposed to 1.5 mg of SLS per ml; an amount obtained from approximately 0.5×10^6 lymphoid cells (not shown). The presence of equal amounts of CLS failed to induce enzyme production and release. Thus, stimulated lymphoid cells appear to release factors that induce macrophages to produce lysosomal and other cellular enzymes and to release the lysosomal enzymes to the culture medium.

DISCUSSION

Several different lines of evidence support our view that the lysosomal enzymes appearing in the culture medium are released from otherwise healthy and viable cells and do not derive from disrupted cells or from dead nonadherent cells released to the culture medium. Levels of lactate dehydrogenase, a soluble cytoplasmic enzyme which leaks rapidly from damaged cells, increased ($P < 0.001$) in cells exposed to SLS, and levels in the culture medium remained constant (Table 2). The levels of this enzyme within the cells and in the culture medium of cells exposed to CLS remained unchanged. The SLS-exposed cells excluded trypan blue dye equally as well as the CLS-exposed cells throughout the 48-hr incubation period. Both the CLS- and SLS-exposed cells can be maintained in culture for at least 1–2 weeks, and the SLS-exposed

TABLE 4. *Acid hydrolase activity at various times during incubation in the cells and media of macrophage cultures containing 1.5 mg/ml of CLS or SLS*

	CLS		SLS	
	Cells	Media	Cells	Media
β-Glucuronidase				
0 hr	2.4(0.1)*	0.3(0.2)	2.3(0.1)	0.3(0.1)
48 hr	2.3(0.2)	0.3(0.1)	1.1(0.1)	3.6(0.1)
Acid phosphatase				
0 hr	14.7(0.7)	0.7(0.6)	16.1(1.3)	0.5(0.1)
48 hr	16.3(1.3)	1.5(0.4)	24.6(1.1)	16.1(2.3)
β-Galactosidase				
0 hr	1.9(0.1)	0.1(0.1)	1.6(0.2)	0.2(0.1)
48 hr	2.0(0.3)	0.1(0.1)	1.0(0.3)	2.6(0.2)
β-*N*-Acetylglucosaminidase				
0 hr	20.2(0.8)	2.7(0.3)	19.3(0.9)	2.4(0.8)
48 hr	20.1(1.0)	2.6(1.5)	10.3(2.3)	38.3(2.6)

* Measured as described by Davies *et al.* (21) and reported as nmol of product/μg of macrophage protein per hr ± (SD).

cells can be cultured in the absence of serum. In cultured macrophages, leucine-2-naphthylamidase is a membrane-bound enzyme that is not released from cells losing their adherence properties and appearing in the culture medium. In order to evaluate the number of cells released into the culture medium, we collected the nonadherent cells by centrifugation and measured the leucine-2-naphthylamidase activity in the pellet (21). The observed activity in the pellets ranged from 2.9 ± (2.3) to 5.6 ± (2.2) nmol of product per μg of protein per hour, and there were no significant differences observed between the CLS- and SLS-exposed cultures (Table 3).

The observations we describe show that stimulated lymphoid cells release products that not only arrest macrophage mobility and lead to cell enlargement, but also induce the production of lysosomal and other cellular enzymes and the release of the hydrolytic enzymes into the extracellular environment. By very similar procedures, collagenase production and release by macrophages have been induced (16). The releasing cells appear to be otherwise viable and healthy by several criteria. The released enzymes have the capacity to cause extensive pathologic alteration to tissues (13–18). A similar response can be induced in mononuclear phagocytes exposed to a variety of bacterial and other substances associated with chronic inflammatory lesions (14, 15, 21). Mononuclear phagocytes are considered to be partly responsible for the tissue damage associated with cell-mediated hypersensitivity reactions (18) and other lesions of the chronic inflammatory type (14). The long-term production and release of various hydrolytic enzymes by macrophages stimulated directly by various bacterial substances or indirectly by mediators released by stimulated sensitized lymphoid cells may account, at least in part, for the observed tissue destruction.

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