Lysozyme Enhances Monocyte-Mediated Tumoricidal Activity: A Potential Amplifying Mechanism of Tumor Killing

By Paul LeMarbre, John J. Rinehart, Neil E. Kay, Robert Vesella, and Harry S. Jacob

The mononuclear phagocyte is well established as an in vitro cytotoxic effector cell for certain human tumors. The mechanism(s) for this action remains unclear. Increased levels of lysozyme, a cationic enzyme synthesized in large amounts by mononuclear phagocytes, are associated with increased resistance to transplantable animal tumors. In this study, we provide evidence that human lysozyme, isolated from the urine of leukemic patients, has marked potentiating effects on human monocyte-tumor-cell cytotoxic activity. In addition, lysozyme-exposed monocytes incorporate increased quantities of leucine, suggesting a significant increase in serum lysozyme levels, while such levels remained unaltered, or actually decreased, in nonresponders.

Stimulated by this apparent association between heightened LZM activity and resistance to neoplasms in both humans and animals, we have examined the effect of this phagocyte constituent as a modifier of monocyte-tumor-cell interaction in vitro. Our results indicate that LZM, partially by virtue of its marked positive charge, potentiates monocyte-tumor-cell interaction and associated tumor cell death.

MATERIALS AND METHODS

Monocyte Preparation

Blood was collected from healthy, consenting donors, anticoagulated with EDTA, and separated by Ficoll-Hypaque gradient centrifugation. Purified monocyte preparations were formed as previously described. Briefly, interface cells were washed in Seligman's balanced salt solution (SBSS); after differential and total cell counts were performed, the cells were suspended (5 x 10⁶ monocytes/ml) in RPMI with 20% autologous serum; 0.1 ml aliquots were added to individual 6-mm microwells (Linbro, New Haven, Conn.) and incubated 1 hr at 37°C in humidified air with 5% CO₂; after 1 hr the wells were washed with cold Hank's balanced salt solution (HBSS). To the monolayers thus formed, which contained 0.8-1.2 x 10⁶ cells (95% of which were monocytes), was added 0.1 ml of final medium (Leibowitz or Waymouths, containing 20% AB sera, 100 µg/ml penicillin, 100 µg/ml streptomycin, 0.3 mg/ml glutamine, and 0.075% NaHCO₃).

Tumor Cells

Two human tumor cell lines were maintained in culture as previously described: Malme-3M, a melanoma cell line, and T-24, a transitional cell carcinoma of the bladder.

Assays of Tumor Cell Damage

Three to seven days after culture plating, the tumor cells were prelabeled with 2.5 µCi/ml tritiated thymidine (New England
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Nuclear, Boston, Mass.) 24 hr before addition to microwells. The cells were harvested with 0.03% Trypsin (Gibco, Grand Island, N.Y.), washed by centrifugation, and suspended in final medium to achieve specific effector:target cell ratios; 0.1-ml aliquots were then added to quadruplicate control wells containing 0.1 ml media or to monocyte monolayers. The plates were incubated at 37°C with 5% CO₂, then emptied, washed 5 times with cold SBSS, and the contents of each well were removed with cotton swabs as previously described. The swabs were allowed to dry for 24 hr, and the cotton tips were then counted in liquid scintillation fluid. The percentage cytoidal activity was determined by the following formula:

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\text{Cytoidal activity} = 1 - \frac{\text{cpm target cell plus effector cells}}{\text{cpm target cells alone}}
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Metabolic Studies

Two metabolic functions of blood monocytes, isolated as described above, were assayed as follows:

1. For leucine, uridine, and thymidine uptake: 10⁶ monocytes were added to microtitre wells with or without various concentrations of LZF, and then exposed to 0.5 μCi ¹³C-thymidine, 0.5 μCi ³H-leucine, or 5 μCi ³H-uridine (New England Nuclear, Boston, Mass.). Triplicate samples were incubated at 37°C for 24 hr, spun down, and then lysed with 5% TCA and harvested with a MASH II harvester. The filters were air dried and counted in a Beckman scintillation counter (Beckman Instruments, Palo Alto, Calif.)

2. For evaluation of hexose monophosphate shunt metabolism, 2 x 10⁶ peripheral blood monocytes were added to 25 ml Erlenmeyer flasks, permitted to adhere, and then incubated with 100 μCi of 1-¹⁴C-labeled glucose (New England Nuclear, Boston, Mass.) either with or without methylene blue to maximally stimulate hexose monophosphate shunt metabolism; various concentrations of human LZF were added to the monocytes at the initiation of culture. After incubation for 2 hr at 37°C in a shaking water bath, perchloric acid was added, and evolved ¹⁴C-labeled CO₂ was trapped in 0.3 ml of hyamine (Rohm and Hass, Philadelphia, Pa) in plastic center wells suspended over the cell suspension; after solubilization of hyamine in 10 ml of scintillation fluid, radioactivity was measured in a Beckman scintillation counter.

Reagents

Lysozyme. Human lysozyme (HLZF) was isolated from the urine of patients with monocytic or myelomonocytic leukemia either by absorption to bentonite and elution with pyridine: sulfuric acid alone, or by this procedure followed by ion exchange chromatography on carboxymethyl cellulose. The HLZF used in these experiments exhibited a single component when separated by PAGE-SDS gel electrophoresis and was endotoxin free by the limulus assay. The enzyme was provided most generously by Dr. E. Osserman, Department of Medicine and Cancer Research Center, Columbia University, New York, N.Y.

Protamine sulfate (crystalline). This was obtained from Sigma Chemical Corp. (St. Louis, Mo.).

Heparin. Heparin was obtained from Abbott Laboratories, North Chicago, Ill.

Tri-N-acetylglucosamine (TNAG). The trisaccharide was kindly provided by Dr. G. Lienhard, Dartmouth Medical School, Hanover, N.H.

Statistical Analysis

The Student’s t test was employed to estimate the significance of difference between means.

RESULTS

Preliminary dose–response studies of HLZF effects on monocyte–tumor-cell interaction were carried out at LZF concentrations ranging from 4 μg/ml to 250 μg/ml. Enhancement of tumor cell killing was apparent at each concentration, and an intermediate concentration (60 μg/ml) was selected for further experiments, since no particular concentration appeared to show a peak effect. HLZF at 60 μg/ml caused reproducible enhancement (4 of 4 experiments) in 2 different cytotoxicity assays.

Utilizing our cytoidal assay in which tumor cells were prelabeled with tritiated thymidine, we observed monocyte-induced antineoplastic activity against a variety of human tumor cell lines (data not shown). We chose to study in most detail Malme-3M (melanoma) and T-24 (bladder carcinoma) cells; when HLZF (60 μg/ml) was added to the cultures of monocytes plus tumor cells, and to control wells with tumor cells alone, a 3-4 fold increase in cytoidal activity was observed with both tumor cell types (Fig. 1). The enhancement was not due to the presence of endotoxin in HLZF, since limulus assay demonstrated no endotoxin in the HLZF preparation. HLZF was not
directly toxic for tumor cells, having no effect on counts in control wells containing only tumor cells.

When HLZM was added at the initiation of coculture, its capacity to increase monocyte-mediated tumor cytocidal activity was first apparent after approximately 8 hr and became increasingly so over the next 40 hr (Fig. 2); an approximate threefold increase in cytotoxic activity was induced after 48 hr of incubation.

At least a portion of HLZM's stimulatory effect on monocyte-mediated cytocidal activity may be derived from its ability to enhance monocyte protein anabolism. Thus, uptake of radiolabeled leucine (but not thymidine or uridine) markedly increases in lysozyme (60 µg/ml) exposed monocytes (Fig. 3). This increase in leucine incorporation, which was easily perceived at 48 hr, was still evident after 7 days; thymidine and uridine incorporation remained virtually the same in HLZM-treated and control incubates (data not shown).

A less striking effect of HLZM on oxidative metabolism of monocytes was detected by examining hexose monophosphate shunt activity. Thus, high concentrations of HLZM (250 µg/ml) but not intermediate levels (60 µg/ml) approximately doubled monocyte 14CO2 evolution from 14C-1-glucose (2278 ± 421 versus 1080 ± 171 [mean ± sem] in untreated monocytes).

Tri-N-acetyl glucosamine competitively inhibits HLZM enzymatic activity17 and has been used in numerous past studies to validate the specificity of various actions of HLZM. The trisaccharide at relevant concentration (100 µg/ml) decreased by approximately 60% the stimulatory effect of HLZM on monocyte-mediated cytocidal activity for T-24 tumor cells (Fig. 4). The inhibitor alone (in the absence of HLZM) had no effect on tumor cell viability either in the presence or absence of monocytes.

Since HLZM is highly positively charged, its enhancing effect on monocyte–tumor-cell interaction might to some degree reflect abrogation of mutually repulsive negative charges on the two cell types. In partial support of this prediction, another positively charged molecule, protamine sulfate (50 µg/ml), also enhanced monocyte-mediated tumor cytocidal activity (Fig. 5). Heparin (a negatively charged molecule) negated the protamine enhancement when added simultaneously (Fig. 5), while having no effect on protamine-free incubates (not shown).

**DISCUSSION**

These studies demonstrate that HLZM enhances monocyte cytotoxicity towards human tumor cell lines in vitro. Since HLZM is elaborated continuously by monocytes (at least in culture),8 and since elevations of serum HLZM have been noted in a variety of neoplastic diseases, our findings may be relevant in systems of host resistance to tumors. Our data suggest several underlying mechanisms of HLZM activity in this system: (A) HLZM enzymatic modification of the monocyte membrane (or tumor cell membrane, or
Fig. 4. The addition of the lysozyme inhibitor (tri-N-acetyl glucosamine) (right bar) to lysozyme-exposed human monocytes inhibits their cytocidal activity toward T-24 cells. Data are presented as mean ± SEM.

Fig. 5. The positively charged substances protamine sulfate (second bar, \( p < 0.05 \)) and lysozyme (fourth bar) potentiate monocyte-mediated cytocidal activity against Malme cells. Addition of negatively charged heparin (third bar) abrogates the protamine effect. Data are presented as mean ± SEM.

An appreciation for the range of HLZM's biologic functions has only recently been achieved. The enzyme was discovered by Fleming in 1922, and for many years attention was focused on its antibacterial action. Lysozyme, found primarily in granulocytes and mononuclear phagocytes was specifically noted to lyse bonds between N-acetylmuramic acid and N-acetylglucosamine, both being substituents of certain bacterial cell walls. Evidence has now accumulated indicating LZM effects in other cellular systems: modification of membrane receptors, changes in the microscopic appearance of membranes exposed to HLZM and apparent reacquisition of contact inhibition in transformed cells; and modulation of neutrophil function. Lysozyme may modify the expression of certain membrane receptors; a particular receptor that binds Shigella enterotoxin has been identified, and this receptor can be blocked by HLZM treatment. In the presence of TNAG (trisaccharide employed in other studies to competitively block the enzymatic action of HLZM since it combines with HLZM's active site), HLZM loses its blocking effect. Exogenous HLZM caused visible flocculation of liver cell suspensions; with the use of electron microscopy, mitochondrial clumping was observed and was partially reversed with exposure to TNAG. Addition of HLZM to transformed mouse fibroblasts resulted in a more centralized growth pattern, suggesting a possible reacquisition of contact inhibition. Critical to these activities may be HLZM's capacity to hydrolyze \( \beta1-4 \) linkages between two N-acetyl glucosamines, which are present in a number of membrane and plasma glycoproteins.

When monocytes were exposed to a HLZM concentration of 60 \( \mu g/ml \), the resultant cytocidal values for both Malme-3M (\( p < 0.01 \)) and T-24 (\( p < 0.001 \)) increased 3–4-fold (Fig. 1). Although enhancement of monocyte cytocidal and cytostatic activity was observed against four human cell lines, Malme-3M and T-24 were used in these experiments, since positive but low baseline values for cytocidal activity were observed at a monocyte:tumor-cell ratio of 4:1.

Levels of HLZM in inflammatory sites have been reported as high as 300 \( \mu g/ml \); despite the fact that the inherent level of HLZM produced in our cocultures has not been determined, it is likely (based on previous secretory studies), that the level is inconsequential in comparison to the concentration of 60 \( \mu g/ml \) due to added HLZM.
When TNAG was added simultaneously with HLZM to the cocultures, inhibition of cytocidal enhancement was evident (Fig. 4). The possibility that HLZM exerts its cytocidal enhancement via an enzymatic mechanism is suggested by the decreased effect in the presence of TNAG, and by the inherent historical nature of HLZM itself. Of note is the observation that neither HLZM nor the trisaccharide appeared to be overtly cytocidal for tumor cells in the absence of monocytes.

An increase in leucine incorporation and hexose monophosphate shunt activity (at higher concentrations) was observed in HLZM exposed monocytes. Biochemical criteria for mononuclear phagocyte “activation” have recently been summarized; increased protein and glucose metabolism may be associated with monocyte-macrophage activation. Thus, monocyte activation may be a mechanism of HLZM action in this system. Potential cytocidal mechanisms could be related to increased protein synthesis and hexose monophosphate shunt activity; HLZM may increase monocyte protease synthesis or secretion, and increased hexose monophosphate shunt activity may be associated with increased O₂ radical production that may have cytocidal effects.

Lysozyme is not only a bacteriolytic enzyme but also a cationic protein. In order to evaluate the role of positive charge in the enhancement of monocyte cytotoxicity, we utilized another positively charge molecule, protamine sulfate, at a similar in vitro concentration as HLZM. We found protamine (Fig. 5) to be approximately as effective as HLZM in promoting tumor cytocidal activity (p < 0.05). Evidence that charge itself is important in this enhancement is that negatively charged heparin abrogates the protamine effect, while heparin alone exhibits no direct effect on cell growth (data not shown). Weiss suggests that a decrease in negative charge density on the surface of monocytes may be associated with closer contact and enhanced engulfment of negatively charged particles. In addition, although of questionable applicability, protamine has been administered in a therapeutic trial in breast cancer patients with favorable results.

In conclusion, HLZM, a protein previously recognized for antibacterial action, may well have an important role in augmenting the capabilities of human monocytes to deal with tumor cells. We suspect the potential benefit of HLZM in promoting monocyte-tumor-cell interaction may ultimately have therapeutic implications.

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