Lysozyme-Induced Inhibition of the Lymphocyte Response to Mitogenic Lectins[1] (42829)

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Abstract. **Both human lysozyme (HL) and hen egg white lysozyme (HEWL) inhibited the proliferative response of peripheral blood lymphocytes to T cell mitogens such as the lectins phytohemagglutinin and concanavalin A. This inhibition was observed both when HL or HEWL was added to the lymphocyte cultures in combination with phytohemagglutinin or concanavalin A and when lymphocytes were pretreated with either lysozyme and extensively washed prior to culture with mitogens. Under both conditions, the effects were strictly dose dependent; the lysozyme concentrations yielding maximal inhibitory effect were 5 μg/ml for HL and 1 μg/ml for HEWL, while both lower and higher concentrations were less effective. Specific antilysozyme rabbit sera completely prevented the inhibitory effects of both HL and HEWL on the proliferative response of lymphocytes to phytohemagglutin or concanavalin A. Chitotriose (a lysozyme inhibitor) caused a strong reduction in the inhibitory effects of the two lysozymes on the lymphocyte response to either lectin. HL and HEWL also were found to markedly inhibit the polyclonal B cell proliferation and differentiation induced by pokeweed mitogen and T cells. A less marked inhibition was also obtained when T cells, but not B cells, were pretreated with HL or HEWL. Again, as in the experiments with T cell mitogens, the effects were dose dependent and 5 μg/ml HL and 1 μg/ml HEWL proved to be the most effective concentrations. The possible mechanisms by which lysozyme inhibits the lymphocyte response to mitogenic lectins are considered and discussed. The enzymatic activity seemed to perform an essential function, as shown by the loss of effect when the heat- or trypsin-inactivated lysozymes were used and by the fact that only the enzymatically active compound, among certain semisynthetic derivatives of HEWL, inhibited the lymphocyte response to the mitogens. However, the cationic properties of the lysozyme molecule appeared to be essential too, since enzymes with a similar specificity of action showed effects similar to those observed with HL or HEWL only when they carried a strong positive charge. It is suggested that lysozyme, which is naturally secreted by monocytes and macrophages, might interact with lymphocyte surface receptor sites and participate in the complex mononuclear phagocyte-lymphocyte interactions and in the modulation of lymphocyte activation.**

[P.S.E.B.M. 1989, Vol 190]

Lysozyme is a ubiquitous cationic enzyme (isoelectric point about 11) capable of degrading bacterial cell walls through its muramidase activity (1, 2). Although a wealth of information is available about the physical, chemical, and enzymatic properties of lysozyme, its biologic role remains an issue still poorly understood (3). The antibacterial function conventionally attributed to lysozyme in vertebrates on the basis of its bacteriolytic activity does not appear to be consistent with its paradoxically high concentrations in certain sites and the actual nonsusceptibility of most pathogenic or saprophytic bacteria to its lytic action. Therefore many investigators no longer favor the idea that this is its sole or major role in higher organisms. On the other hand, several effects of lysozyme on vertebrate cells, suggesting that it may interact with nonbacterial substrates, have been described (4–12). Although lysozyme is considered to be a constitutent of primitive nonspecific defense mechanisms associated with the mononuclear phagocyte system of cells (phy-

[1] This work was supported in part by Grant 8500885-52 from the Consiglio Nazionale delle Ricerche.

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Received March 14, 1988. [P.S.E.B.M. 1989, Vol 190]
Accepted September 15, 1988.

0037-9727/89/1901-0054$2.00/0

logenetically older than the lymphocyte-plasma cell-immunoglobulin system), certain findings indicate that lysozyme may also be involved in specific immune defense mechanisms (12–14). We have previously suggested that this wide range of biologic functions attributed to lysozyme could be framed into a general role in the regulation of cellular and tissular processes of morphogenesis and differentiation (10, 15).

To investigate further such lysozyme functions relating to the regulation of the differentiation processes, human lymphocytes undergoing mitogen-induced dedifferentiation and differentiation *in vitro* were employed as the experimental models in this work. Mitogens used included the lectins phytohemagglutinin (PHA) and concanavalin A (Con A), which are capable of inducing the formation of actively proliferating lymphoblasts from T lymphocytes, and pokeweed mitogen (PWM), which in the presence of helper T cells induces polyclonal B lymphocyte proliferation and differentiation into plasmablasts and plasma cells. We show that lysozyme (both human and from hen egg white) inhibits the T lymphocyte response to PHA and Con A and the PWM-driven B cell proliferation and differentiation in the presence of T cells. All of these inhibitory effects are strictly dose dependent and seem likely to be due to interactions of lysozyme with cellular surface receptor sites. Both the enzymatic activity and the marked cationic properties of the lysozyme molecule seem to be required for these effects. It is suggested that lysozyme, which is naturally released by mononuclear phagocytes, might participate physiologically in the mononuclear phagocyte-lymphocyte interactions and be involved in the modulation of the lymphocyte activation process.

Materials and Methods

Lysozymes. Human lysozyme (HL) was purified in our laboratory from the urine of two patients with monomyelocytic leukemia who showed intense lysozymuria. Urine samples were stored in the frozen state (−20°C) and thawed just prior to starting the purification. The purification procedure used was a modification of that described by Canfield *et al.* (16). After adjusting the pH to 6.3 and removing the sediment by centrifugation, 10-liter volumes of urine were supplemented with 400 g of Bio-Rex 70 (Bio-Rad Laboratories, Richmond, CA) previously washed and equilibrated with a 0.05 *M* solution of NaH_2PO_4 adjusted to pH 6.3. The mixture was shaken overnight at 4°C and then allowed to settle for 2 hr. The supernatant was discarded. The resin was washed with the solution described above and then packed in a 2.5 × 80-cm column. The protein was eluted with 0.4 *M* $(NH_4)_2CO_3$. The fractions of eluate showing lysozyme activity, determined according to Parry *et al.* (17), were pooled, adjusted to pH 7.0, and then passed through another ion exchange chromatographic column of CM-32 cellulose (Pharmacia Fine Chemicals, Uppsala, Sweden) equilibrated with 0.05 *M* NH_4HCO_3. The protein was eluted with a linear gradient (0.05–0.5 *M*) of $(NH_4)_2CO_3$. The fractions containing lysozyme were pooled, after which the material was concentrated and the salt removed by Diaflow ultrafiltration using UM2 filters (Amicon N.V., The Hague, Holland). The enzyme was lyophilized and the powder was stored at 4°C.

Another sample of HL used in this study was a generous gift of Dr. E. F. Osserman (Columbia University, New York, NY), in whose laboratory the enzyme had been purified (18) from the urine of patients with monocytic or monomyelocytic leukemia.

Commercial preparations of hen egg white lysozyme (HEWL) were used from three distinct manufacturers: one sample was from SPA (Milan, Italy), another was from Sigma Chemical Co. (St. Louis, MO), and a third sample was from Miles Laboratories (Elkhart, IN).

Heat-inactivated lysozymes were obtained by boiling for 10 min stock solutions of HL or HEWL in 0.14 *M* phosphate buffer (pH 6.5). Trypsin-digested lysozymes were obtained from stock solutions supplemented with low doses of trypsin (Sigma Chemical Co.) and maintained at room temperature until all lytic activity was lost; ovomucoid (Sigma Chemical Co.) was then added to the mixture to inactivate trypsin.

Lymphocyte Preparation. Lymphocytes (mixed populations from the peripheral blood of a number of healthy volunteers of both sexes) were prepared essentially as described by Moretta *et al.* (19). Total lymphocytes were used in the experiments with PHA and Con A, whereas B and T cells were separated for the experiments with PWM. Mononuclear cells were isolated on a Ficoll-Paque (Pharmacia) density gradient and total lymphocytes were obtained by removing adherent cells after incubation at 37°C for 45 min in plastic flasks. After a 45-min incubation of total lymphocytes with neuraminidase-treated sheep erythrocytes, the rosetting T cells were separated from the nonrosetting B cell-rich fraction on a second density gradient. The rosettes in the pellet were dissociated by warming and shaking, and purified T lymphocytes were then obtained by another density gradient at 37°C. The residual T cells were removed from the B cell-rich fraction by rosetting once again with neuraminidase-treated sheep erythrocytes and density gradient centrifugation.

Experiments on Lymphocyte Stimulation Induced by PHA or Con A. Total lymphocytes were cultured in flat-bottomed Microtest II tissue culture plates (Falcon 3040; Becton-Dickinson, Oxnard, CA). Each culture well contained 10^5 cells in 200 μl of RPMI 1640 culture medium (Gibco Bio-Cult, Paisley, Scotland) supplemented with 20% heat-inactivated fetal calf serum (Flow Laboratories, Rockville, MD), 2 m*M* glutamine (BDH Chemicals, Poole, England), and 50 μg/ml gentamicin (Schering Corp., Kenilworth, NJ). Ly-

sozymes or other substances examined for their effects on mitogen responsiveness were present at the desired concentration in the culture medium used to resuspend lymphocytes prior to dispensing them into the wells. As an alternative procedure, lymphocytes were previously incubated for 1 hr at 37°C in the culture medium containing the test substance to be examined at the concentration desired, then repeatedly washed (at least three times) with phosphate-buffered saline, and finally resuspended in the medium and dispensed into the wells. PHA or Con A (both from Difco Laboratories, Detroit, MI) were added from stock solutions prepared so that 100 μl/well yielded final concentrations within the range 1–5 μg/ml for PHA and 2.5–10 μg/ml for Con A, depending on the optimum stimulating effect determined for either mitogen in preliminary titration assays. Culture microplates were then incubated at 37°C in a humidified 5% CO_2 atmosphere. After 54 hr, 1 μCi of [^{3}H]thymidine (sp act, 10 mCi/mmol; The Radiochemical Centre, Amersham, England) was added to each well in a 100-μl volume, and the microplates were incubated for an additional 18 hr. The cultures were terminated by collecting cells on glass fiber filters using a Skatron harvester (Flow Laboratories) and the incorporation of [^{3}H]thymidine was determined in a scintillation counter (LS 7000; Beckman Scientific Instruments Division, Irvine, CA). The counts per minute represented the mean value from at least triplicate cultures and were expressed as percentages of the value corresponding to the lymphocyte response to either mitogen alone.

Experiments on B Cell Differentiation Induced by PWM. Cultures were prepared using the same medium and microplates as described in the paragraph above. The techniques used to set up cell cultures and to detect plasmablasts and plasma cells were mostly the same as those described previously (20). B cells (10^5 in 100 μl) and T cells (5×10^4 in 100 μl) were mixed in each well and cultured in the presence of 5 μl of PWM (Gibco Bio-Cult)/well. Lysozymes or other test substances were added to the cultures in 100-μl volumes/well from suitable solutions in RPMI 1640. In some experiments B or T cells were preincubated for 1 hr at 37°C in medium containing the test substances to be examined, repeatedly washed, and then resuspended and cultured. Microplates were incubated at 37°C in a 5% CO_2 atmosphere. After 7 days the cells were harvested, counted in hemocytometer chambers, and assessed for viability by trypan blue exclusion. Following cytocentrifugation, the cells were fixed in cold ethanol containing 5% acetic acid, rehydrated in phosphate-buffered saline, and stained at room temperature with goat anti-human $F(ab')_2$ conjugated with fluorescin isothiocyanate (molar F to P ratio of 2.0). Slides were examined with a Leitz Orthoplan microscope (Ernst Leitz, Wetzlar, Federal Republic of Germany). The percentage of plasmablasts or plasma cells, i.e., cells with detectable amounts of cytoplasmic immunoglobulin (cIg^+ cells), was determined and their absolute number per well was calculated as a function of the total number of viable cells recovered.

Other Test Substances and Chemicals. Tri-*N*-acetylglucosamine (chitotriose) and antilysozyme antisera were prepared in our laboratory. Chitotriose was obtained by partial acid hydrolysis of chitin (ICN Pharmaceuticals, Plainview, NY) followed by charcoal column fractionation, as described by Rupley (21). Specific anti-HL or anti-HEWL antisera were obtained from rabbits. The initial dose of antigen was given intramuscularly as a water-in-oil emulsion with complete Freund's adjuvant (Difco Laboratories). Two booster doses were given subcutaneously as emulsions with incomplete Freund's adjuvant (Difco Laboratories) 10 and 30 days later. Blood for serum was collected 5 to 7 days after the second booster.

Four basic semisynthetic derivatives of HEWL (guanidyl-lysozyme (S-112), lysozyme methyl ester (S-114), guanidyl-lysozyme methyl ester (S-115), and trimethyl-lysozyme (S-318)) were a kind gift of Dr. P. Ruozi and Dr. T. Bruzzese (SPA, Società Prodotti Antibiotici, Milan, Italy).

The lysozyme from the fungus *Chalaropsis* was purchased from Serva Feinbiochimica (Heidelberg, Federal Republic of Germany). Lysostaphin, β1,4-*N*-acetylneuraminidase from *Clostridium perfringens*, ribonuclease from bovine pancreas, α-lactalbumin from bovine milk, protamine from salmon, and poly-L-lysine (mol wt 4,000–15,000) were purchased from Sigma Chemical Co.

Results

Effects of HL on PHA-Mediated Lymphocyte Stimulation. In the first set of experiments (Fig. 1A, open circles), the lymphocyte proliferation in response to PHA alone or PHA in the presence of different HL concentrations was compared. Low doses of the enzyme were found to strongly inhibit the [^{3}H]thymidine incorporation by human lymphocytes cultured for 72 hr with the mitogen. Moreover, these effects were dose dependent since maximal inhibition (over 80%) was obtained with 5 μg/ml and lower and higher concentrations had progressively less effect. In control cultures without PHA, none of the HL concentrations tested modified the background [^{3}H]thymidine uptake detected in cultures without lysozyme.

In another set of experiments (Fig. 1A, closed circles) the effect of HL was tested using lymphocytes preincubated for 1 hr at 37°C with different concentrations of the enzyme, washed at least three times, and then cultured with PHA. The results were very similar to those obtained when HL was added to the cells in combination with the mitogen at the initiation of the cultures. In fact, not only was the response to PHA of HL-pretreated lymphocytes inhibited, but also the in-

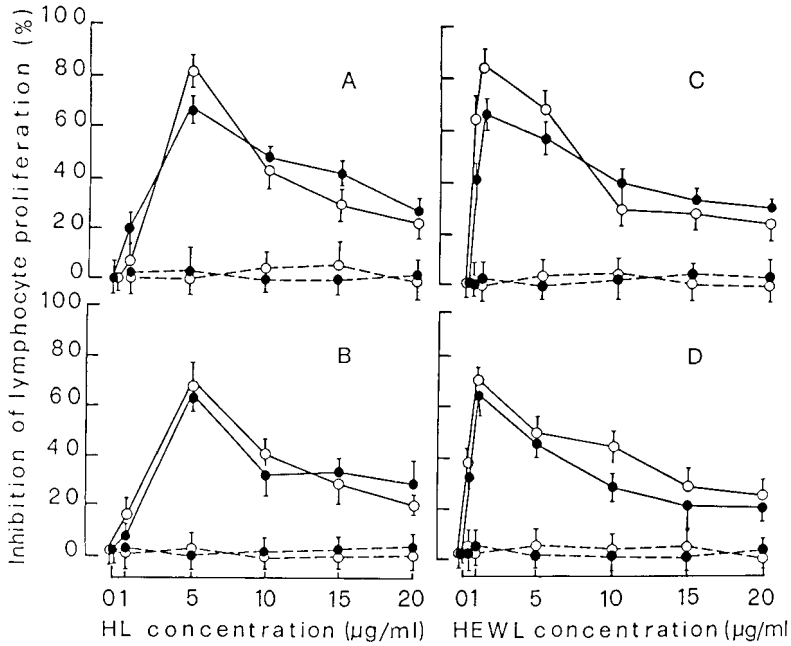

Figure 1. Effects of HL and HEWL on the lymphocyte response to PHA (A, C) and Con A (B, D). Results are expressed as percentage of inhibition of lymphocyte proliferation compared with lysozyme-untreated control. Lysozymes were added to mitogen-stimulated (O——O) or unstimulated (O- - -O) cultures. Alternatively, lymphocytes were pretreated with HL or HEWL, washed, and then cultured in the presence (●——●) or in the absence (●- - -●) of the mitogen.

hibition (only slightly less than in the previous experimental conditions) was dose dependent, and the most effective dose of HL was the same (5 μg/ml) as that obtained when the enzyme was present in the cultures along with PHA. In unstimulated cultures [^{3}H]thymidine incorporation by HL-pretreated lymphocytes was virtually the same as that measured in non-pretreated lymphocytes.

Unlike the native molecule, both the heat-inactivated and the trypsin digested enzyme (tested at the same concentrations as the native molecule) had no effect on PHA-mediated lymphocyte stimulation.

Further experiments were carried out to check the effect of HL on PHA-mediated lymphocyte stimulation when the enzyme was added to the cultures at different times following addition of PHA. HL was used at the concentration (5 μg/ml) which had proved to be the most effective in preventing lymphocyte response to PHA in the previous trials. Figure 2 shows that when HL was added at 1, 4, or 8 hr after PHA, the mitogenic response was inhibited to the same extent as when lysozyme and PHA were present together at the beginning of the culture. A progressive decrease in the inhibitory effect was observed when HL was added after 16 or 24 hr.

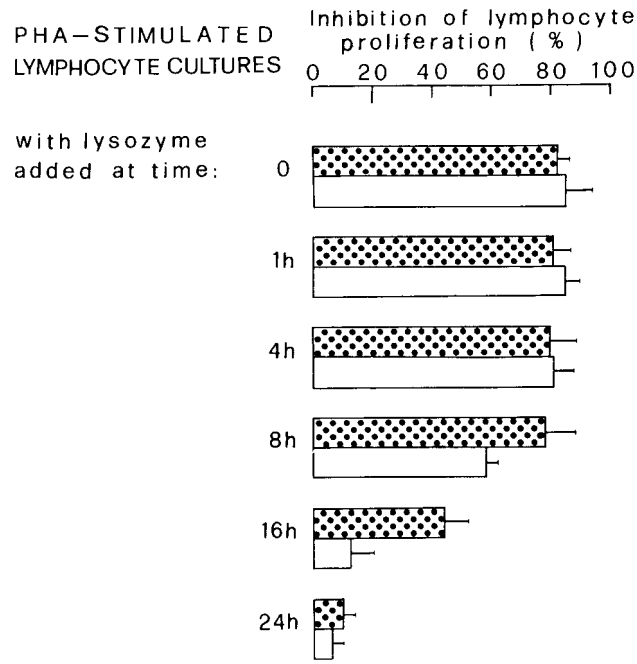

Figure 2. Effects of HL and HEWL on the PHA-mediated lymphocyte stimulation when each enzyme was added to the cultures at different times following addition of the mitogen. Results are expressed as percentage of inhibition of lymphocyte proliferation compared with control without lysozyme. ▒, effects in the presence of 5 μg/ml HL. □, effects in the presence of 1 μg/ml HEWL.

Effects of HL on Con A-Mediated Lymphocyte Stimulation. As shown in Figure 1B, the lymphocyte response to Con A was inhibited by HL in a similar fashion as the response to PHA. The [^{3}H]thymidine incorporation by 72-hr lymphocyte cultures stimulated with Con A was strongly inhibited by HL, both when the enzyme was present in the cultures and when it was used to pretreat the cells as described for PHA. Moreover, the inhibitory effect of HL was clearly dose dependent, and the HL concentration eliciting maximal inhibition was again 5 μg/ml. HL was completely ineffective when it was used after heat inactivation or

trypsin digestion. The only differences from the analogous experiments performed with PHA was that the percentage of inhibition of the response to Con A was slightly less than that observed with PHA, particularly when the enzyme was present in the cultures along with the mitogen.

Addition of HL at different intervals after initiation of Con A-stimulated cell cultures also caused effects similar to those previously described for PHA, with a progressive decrease of the inhibitory activity on the Con A-mediated lymphocyte stimulation starting approximately at 16 hr.

Effects of HEWL on PHA- or Con A-Mediated Lymphocyte Stimulation. HEWL was found to influence the response of human lymphocytes to both PHA and Con A (Fig. 1, C and D) in a manner similar to that described above for HL. A strict dose dependence of the inhibitory effect was observed both when the enzyme was present in the cultures along with the mitogen and when lymphocytes were stimulated with PHA or Con A after pretreatment with HEWL followed by repeated washings. As with HL, the HEWL concentration yielding the maximal inhibitory effect was the same in all experiments; however, this concentration (1 μg/ml) was lower than with HL. Heat inactivation or trypsin digestion of HEWL caused complete loss of its effects on both PHA- and Con A-mediated lymphocyte stimulation.

When HEWL was added to lymphocyte cultures at different times after PHA (Fig. 2) or Con A, a decrease in its inhibitory effect was initially noted with both mitogens when the time interval was 8 hr and became progressively more evident with longer intervals.

Experiments with Lysozyme Inhibitors. Chitotriose, whose capability of antagonizing lysozyme activity is well documented (22), and specific antisera raised in rabbits against HL or HEWL were used as lysozyme inhibitors. The effects of HL or HEWL in the presence of either inhibitor on the PHA- or Con A-mediated lymphocyte stimulation were tested. The two lysozymes were used at the concentrations which had the maximal inhibitory effects in the above-described experiments (5 μg/ml for HL and 1μg/ml for HEWL). The inhibitors were used at concentrations established from preliminary experiments in which both the efficacy in preventing the bacteriolytic activity of HL and HEWL and the possible toxicity on lymphocytes were evaluated. Moreover, when used alone at these concentrations, neither inhibitor proved to have any influence on the lymphocyte response to PHA or Can A.

Specific antibodies (1/64 dilution of whole serum) completely abolished the inhibitory effects of both HL and HEWL on the lymphocyte response to both PHA and Con A, both when the lysozyme-antibody mixture was present in the cultures along with the mitogen and when it was used for cell pretreatment. On the contrary, in double cross tests of specificity (i.e., HL with anti-HEWL and HEWL with anti-HL), the inhibitory properties of both lysozymes proved to be virtually unaffected. Strong, although incomplete, reductions in the inhibitory effects of the two lysozymes on lymphocyte response to the two mitogens were obtained with chitotriose (50 μg/ml). The results obtained in PHA-stimulated cultures are shown in Figure 3.

Effects of HL and HEWL on B Lymphocyte Differentiation Induced by PWM. HL and HEWL were tested for their effects on the *in vitro* differentiation of B lymphocytes into plasmablasts or plasma cells (cIg^+ cells) induced by PWM in the presence of T cells. In 7-day culture combinations set up with 10^5 B cells, 5×10^4 T cells, and PWM, both HL and HEWL inhibited the B cell differentiation (Fig. 4). It is worth noting that the inhibitory effects of HL and HEWL were dose dependent in these experiments too, and the most effective concentrations of the two lysozymes were the same observed in the experiments with PHA and Con A. The number of cIg^+ cells (ranging from 6.5 to 7.5 $\times$

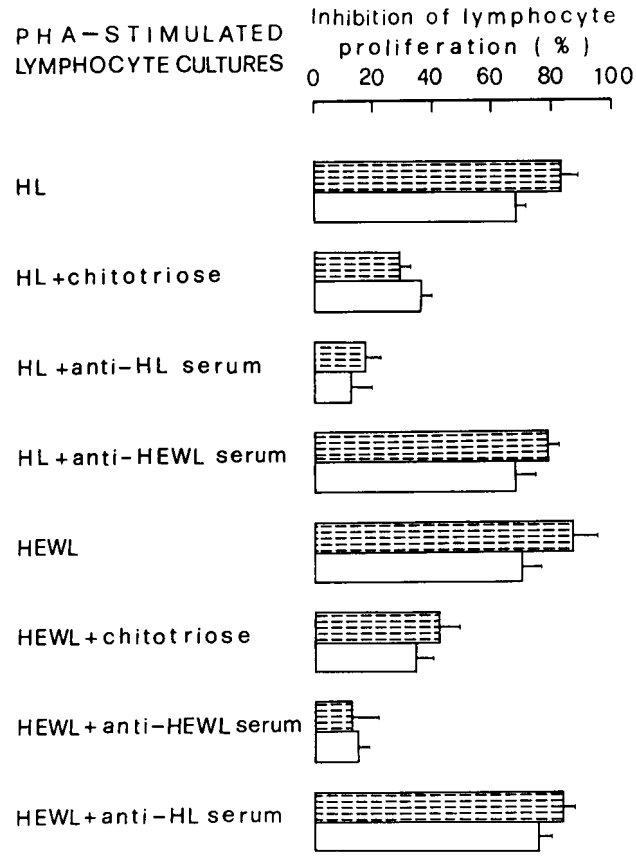

Figure 3. Effects of HL (5 μg/ml) and HEWL (1 μg/ml) on the lymphocyte response to PHA as influenced by the presence of chitotriose (50 μg/ml) or antilysozyme rabbit sera (1/64). Results are expressed as percentage of inhibition of the lymphocyte proliferation compared with lysozyme-untreated control. ▨, Lymphocytes cultured with the mitogen and the lysozyme (plus lysozyme inhibitor, when indicated). □, Lymphocytes preincubated at 37°C for 1 hr with the lysozyme (plus lysozyme inhibitor, when indicated), washed, and then cultured with the mitogen.

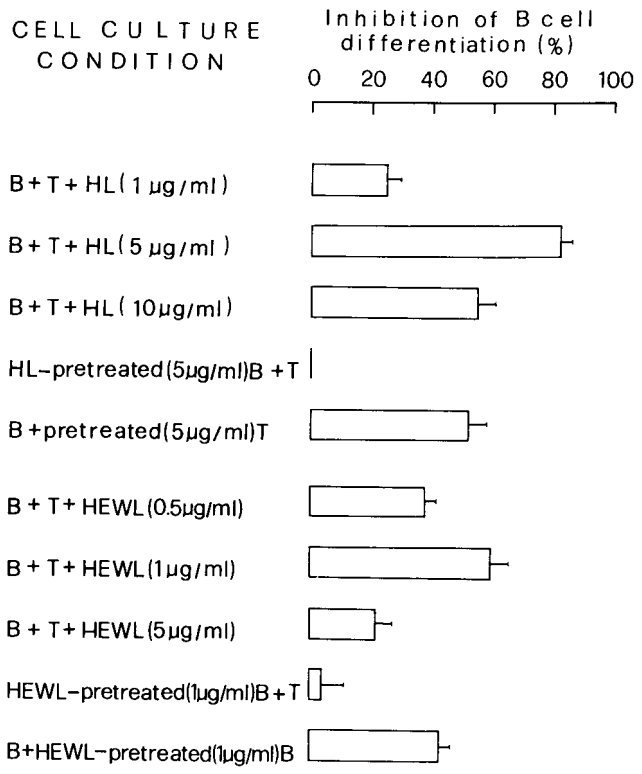

Figure 4. Effects of HL and HEWL on the B lymphocyte differentiation into plasmablasts or plasma cells induced by PWM in the presence of T cells. Results are expressed as percentage of inhibition of B cell differentiation compared with lysozyme-untreated control.

10^4 in the lysozyme-untreated controls) was reduced by more than 80% by HL (5 µg/ml) and by more than 50% by HEWL (1 µg/ml). Again, trypsin inactivation completely prevented the inhibitory effects of both HL and HEWL.

Although less marked, inhibitory effects were also observed when T cells were preincubated for 1 hr with 5 µg/ml HL or 1 µg/ml HEWL, washed, and then mixed with B cells in helper culture combinations. In contrast, no significant effects were observed when B cells were similarly pretreated with either lysozyme.

Effects of Other Bacteriolytic Enzymes, Lysozyme-Like Substances and Cationic-Proteins on the Lymphocyte Response to Mitogenic Lectins. Besides HL and HEWL—which are similar proteins, with the same specificity of enzymatic activity (β1,4-*N*-acetylmuramidase, EC 3.2.1.17), an almost identical molecular weight (about 14,000) and isoelectric point (about 11), and largely overlapping amino acid sequences (3, 22)—two additional natural bacteriolytic enzymes (the so-called "lysozyme" produced by the fungus *Chalaropsis* and lysostaphin), another mucopolysaccharidase (*Clostridium perfringens* neuraminidase), and a natural lysozyme analogue (bovine α-lactalbumin) have been tested. A wide range of concentrations was assayed with all substances. The *Chalaropsis* lysozyme has a molecular weight of approximately 23,000, is a noncationic protein (isoelectric point 7.5), and displays both β1,4-*N*-acetylmuramidase and β1,4-*N*,6-*O*-diacetylmuramidase activities (23). Lysostaphin, which is produced by some coagulase-negative staphylococci, acts mainly as a glycyl-glycine endopeptidase, has a molecular weight of approximately 30,000, and an isoelectric point of about 10 (24, 25). The *C. perfringens* enzyme has β1,4-*N*-acetylneuraminidase activity (EC 3.2.1.18), a molecular weight of about 64,000, and an isoelectric point of about 5 (26). α-Lactalbumins, although lacking both enzymatic activity and cationic properties (isoelectric point about 5), have amino acid sequences and three-dimensional structures very similar to those of typical lysozymes such as HL and HEWL, to which they are believed to be phylogenetically related (27). None of the substances mentioned above, at the different concentrations tested, had any significant effect on lymphocyte responses to mitogenic lectins or on [^{3}H]thymidine incorporation in unstimulated cultures.

Similar experiments were carried out using four semisynthetic derivatives of HEWL at concentrations ranging from 0.5 to 20 µg/ml. All compounds bear a greater positive charge than the native molecule; one (S-112) retains enzymatic activity, whereas three (S-114, S-115, and S-318) are enzymatically inactive. Only S-112, at the concentration of 1 µg/ml, displayed a clear inhibitory effect on the lymphocyte responses to PHA and Con A, whereas S-114, S-115, and S-318 had no effect (Fig. 5). In unstimulated cultures, none of the four substances modified the [^{3}H]thymidine incorporation at the different concentrations tested.

Three additional cationic proteins unrelated to lysozymes, namely, polylysine (mol wt 4,000–15,000), protamine (mol wt about 10,000), and ribonuclease (mol wt about 14,000), were tested at different concentrations, but proved to have no effect on the lymphocyte

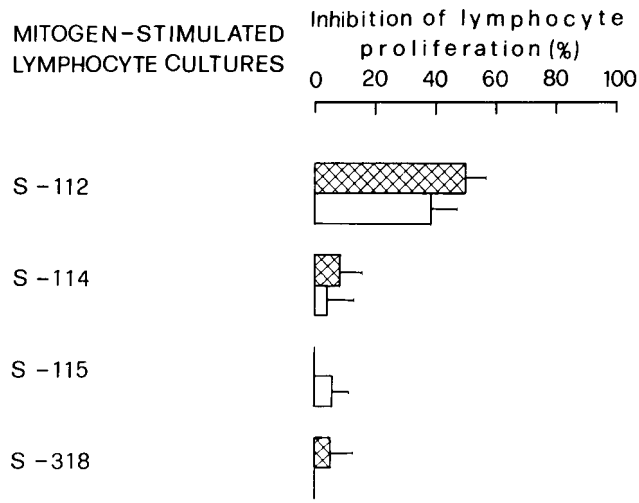

Figure 5. Effects of four semisynthetic derivatives of HEWL (all used at the concentration of 1 µg/ml) on the lymphocyte response to PHA (▨) or Con A (□). Results are expressed as percentage of inhibition of lymphocyte proliferation compared with control with no test substance added.

response to PHA or Con A or on [^{3}H]thymidine uptake in unstimulated cultures.

Discussion

This study shows that lysozyme has a potent, dose-dependent inhibitory effect on lymphocyte response to mitogenic lectins. The reliability of this finding is also underlined by the fact that a similar effect was observed by using different lysozyme preparations. For example, in the experiments with HL, two different sources of the enzyme (one prepared in our laboratory and the other purified in another laboratory by a different procedure) always had comparable effects. Likewise, HEWL samples from three different manufacturers did not display significant differences when parallel trials were carried out.

Since differentiation and dedifferentiation events are largely involved in the lymphocyte response to mitogenic stimulation, the present findings support our previous proposal that lysozymes participate, as a general role, in the regulation of molecular processes of morphogenesis and differentiation (10, 15). Interestingly, the omnipresence of lysozyme in nature and in phylogenesis (3, 22, 28) corresponds notably to the strong evolutionary conservation of cell surface glycoproteins involved in the processes of morphogenesis (29). It could be further speculated that in lower organisms and in fetal tissues, where lysozyme appears early (30), the lysozyme function might chiefly be concerned with mechanisms of shape attainment or arrangement; whereas in fully developed higher organisms lysozyme could play its role in cell and tissue differentiation processes, particularly in those involved in the immune response. On the other hand, in the course of phylogenesis, the major lysozyme-producing cells in vertebrates, i.e., the mononuclear phagocyte system of cells (31), have acquired tight and specialized functional interactions with the phylogenetically younger lymphocyte-plasma cell-immunoglobulin system.

The mechanisms whereby lysozyme inhibits the lymphocyte response to mitogenic lectins are probably complex (and their explanation is beyond the scope of this study), but some interpretations are suggested by our present findings. The possibility of a direct interaction of lysozymes with lectins was ruled out by the fact that HL- or HEWL-pretreated lymphocytes responded to PHA or Con A in the same way as cells cultured along with lysozymes. Similarly, the pretreatment of T cells with either HL or HEWL abrogated the subsequent response to PWM. These experiments also indicated that the inhibitory effect on the PWM-driven B cell differentiation depended on the effect of the enzyme on helper T cells rather than on B cells.

The affinity for carbohydrates of both lysozymes (2) and lectins (32) might be of some importance. In a previous report, treatment with HEWL has been shown to reduce Con A binding capacity of chick fibroblasts (7). In another study, prevention by Con A of the HL- or HEWL-induced agglutination of isolated mitochondria was thought to reflect the fact that both lysozyme and lectin interacted with carbohydrate moieties of the mitochondrial membranes (6).

In our study, experimental evidence indicates that the enzymatic activity of lysozyme is essential for its inhibitory effects on the lymphocyte response to mitogens. In fact, the lysozyme effects were blocked by lysozyme inhibitors, and no effect was observed either with heat- or trypsin-inactivated lysozymes or with a nonenzymatic protein such as α-lactalbumin, closely related to HL and HEWL in its primary and secondary structure. In addition, among the four semisynthetic derivatives of HEWL tested, only the enzymatically active compound was effective. It is worth noting that another bacterolytic enzyme, lysostaphin, (acting as an endopeptidase) displayed no effect, whereas a further lytic enzyme produced by *Staphylococcus aureus* and acting as a β1,4-*N*-acetylglucosaminidase (mol wt approximately 80,000, isoelectric point about 10) (25, 33) was reported to cause a strong, dose-dependent inhibition of the lymphocyte response to PHA, Con A, and PWM (34). This may suggest that the required enzymatic activity takes place on mucopolysaccharidic sites where a glucosaminidase (EC 3.2.1.14) such as the *S. aureus* enzyme acts similarly to muramidases (EC 3.2.1.17) such as HL or HEWL. The failure of another mucopolysaccharidase such as neuraminidase (EC 3.2.1.18) to cause any effect might reflect its different specificity of action, but its lower isoelectric point might be important as well. In fact, besides the enzymatic activity, the positive charge of the molecule also seems to be implicated in the lysozyme-induced inhibition of proliferative response of lymphocytes to lectins. A distinction between functional effects of lysozyme due to the enzymatic activity or to the basic nature of the molecule has been suggested by early studies (35), and the fact that lysozyme is strongly positively charged has been thought to be responsible for some of its biologic effects (11, 35, 36). HL, HEWL, and the above-mentioned *S. aureus* glucosaminidase all have a similar inhibitory effect on the lymphocyte response to mitogenic lectins and are all strongly basic proteins. On the other hand, the lysozyme of *Chalaropsis*, which has muramidase activity but is not a cationic protein, and polylysine, protamine, or ribonuclease, which are basic proteins but have no enzymatic activity on mucopolysaccharidic substrates, are all unable to affect the lymphocyte response to mitogens. Both the enzymatic activity and the basic charge seem to be necessary for the lysozyme molecule to exert its inhibitory effect on the lymphocyte response to mitogenic lectins. Neither property alone seems to be sufficient to cause these effects. It is possible that the positive charge is involved in the attachment of the lysozyme molecule to a receptor site on the cell surface. Once attached, lysozyme

might enzymatically degrade some mucopolysaccharidic fraction in the receptor site, thus rendering cells unresponsive to mitogenic stimulation.

One interesting aspect of the lysozyme-induced inhibition of the lymphocyte response to mitogenic lectins is its strict dependence upon the enzyme concentration (observed with both HL and HEWL) and its reversal with increased enzyme concentrations. Such particular behavior is not easy to explain and is the object of further investigations in our laboratory. One possible explanation is that the inhibitory effect may require the specific receptor to be triggered enzymatically in the presence of an adequate positive charge, but such triggering could be hampered when the positive charge increases above a certain critical level. Another possibility is that lymphocytes have two types of receptors: one with a greater affinity for lysozymes, responsible for inhibition of the lymphocyte response to mitogens, and another with a lower affinity for lysozymes, antagonizing the effects induced by the stimulation of the former receptor. The strict concentration dependence of the inhibitory effect of lysozyme, and the tendency to progressively lose this effect at concentrations over 10–20 μg/ml, might also explain apparent discrepancies between our findings and those of others (12) which indicate a stimulating effect of lysozyme on mixed lymphocyte cultures (in these latter experients, in fact, maximal enhancement was obtained with 250 μg/ml HEWL).

T cells seem to be the major targets of the inhibitory action of lysozyme. Lysozyme binding to and enzymatic activity on surface molecules of T cells might inhibit responsiveness to mitogenic lectins. Such inhibition can occur through different mechanisms currently under investigation: in particular, lysozyme might prevent the interaction of the mitogen with the T cell surface, or reduce the T cell sensitivity to growth factors such as interleukin 2 released during mitogen stimulation, or impair the release of growth and differentiation factors of T cell origin. Moreover, in spite of the removal of adherent cells during lymphocyte preparation, our findings cannot completely exclude the possibility that an additional target of lysozyme may be the accessory cells of the mononuclear phagocyte system. In particular, lysozyme might interfere with the release of soluble mediators of monocyte or macrophage origin, such as interleukin 1, whose crucial importance in the activation of lymphocytes has been extensively documented (37–40). The fact that mononuclear phagocytes are the major natural lysozyme-producing cells (31) does not invalidate this theory, since in the case of neutrophils, which also release lysozyme, the enzyme has been shown to function in a negative feedback system (9). The special ability of mononuclear phagocytes to synthesize and release lysozyme and the effects reported herein of lysozyme on the mitogen-induced lymphocyte activation suggest that this enzyme may contribute to the modulation of the lymphocyte activation process. Assuming that T cell triggering in the antigen-dependent responses does not fundamentally differ from that in the lectin-dependent responses, we can speculate that lysozyme has a physiologic effect in the modulation of T cell responses *in vivo*. It is worth noting that the inhibitory effect of HL on the lymphocyte response to mitogenic lectins was most effective at a concentration close to the physiologic level in human serum. Thus, participation of lysozyme in the complex mononuclear phagocyte-lymphocyte interactions, recently suggested also on the basis of studies with mixed lymphocyte cultures (12), might actually be one important function of lysozyme in vertebrates.

The authors wish to thank Dr. L. Moretta for helpful discussion and critical reading of the manuscript, Dr. E. F. Osserman for his kind gift of a sample of human lysozyme, and Dr. P. Ruozi and Dr. T. Bruzzese for kindly providing samples of semisynthetic derivatives of hen egg white lysozyme.

1. Jollès P. Lysozymes: A chapter of molecular biology. Angew Chem Int Ed **3**:227–239, 1969.
2. Chipman DM, Sharon N. Mechanism of lysozyme action. Science **165**:454–465, 1969.
3. Jollès P, Jollès J. What's new in lysozyme research? Always a model system, today as yesterday. Mol Cell Biochem **63**:165–189, 1984.
4. Takaoka T, Katsuta H, Ishiki S, Konishi T. Effects of lysozyme on the proliferation of fibroblasts in tissue culture. Jpn J Exp Med **42**:221–232, 1972.
5. Osserman EF, Klockars M, Halper J, Fischel RE. Effects of lysozyme on normal and transformed mammalian cells. Nature **243**:331–335, 1973.
6. Adinolfi M, Loeb JN, Osserman EF. Agglutination of rat liver mitochondria by lysozyme. In: Osserman EF, Canfield RE, Beychok S, Eds. Lysozyme. New York, Academic Press, pp463–470, 1974.
7. Asdourian H, Chu L, Lau K, Amos H. Lysozyme: Evidence for effects on chick fibroblasts, HeLa cells, and their products. Biochem Biophys Res Commun **64**:1142–1151, 1975.
8. Klockars M, Roberts P. Stimulation of phagocytosis by human lysozyme. Acta Haematol (Basel) **55**:289–295, 1976.
9. Gordon LI, Douglas SD, Kay NE, Yamada O, Jacob HS. Modulation of neutrophil function by lysozyme. Potential negative feedback system of inflammation. J Clin Invest **64**:226–232, 1979.
10. Satta G, Azzarone B, Varaldo PE, Fontana R, Valisena S. Stimulation of spreading of trypsinized human fibroblasts by lysozymes from *Staphylococcus aureus*, hen egg white, and human urine. In Vitro **16**:738–750, 1980.
11. LeMarbre P, Rinehart JJ, Kay NE, Vesella R, Jacob HS. Lysozyme enhances monocyte-mediated tumoricidal activity: A potential amplifying mechanism of tumor killing. Blood **58**:994–999, 1981.
12. Rinehart JJ, Cerilli JG, Jacob HS, Osserman EF. Lysozyme stimulates lymphocyte proliferation in monocyte-depleted mixed lymphocyte cultures. J Lab Clin Med **99**:370–381, 1982.
13. Osserman EF. Postulated relationship between lysozyme and immunoglobulins as mediators of macrophage and plasma cell functions. Adv Pathobiol **4**:98–102, 1976.

14. Jollès P. A possible physiological function of lysozyme. Biomédecine **25**:275–276, 1976.
15. Satta G, Varaldo PE, Azzarone B, Romanzi CA. Effects of *Staphylococcus aureus* lysozyme on human fibroblasts. Cell Biol Int Rep **3**:525–533, 1979.
16. Canfield RE, Kammerman S., Sobel JH, Morgan FJ. Primary structure of lysozymes from man and goose. Nature (New Biol) **232**:16–17, 1971.
17. Parry RM Jr, Chandan RC, Shahani KM. A rapid and sensitive assay of muramidase. Proc Soc Exp Biol Med **119**:384–386, 1965.
18. Osserman EF, Lawlor DP. Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. J Exp Med **124**:921–951, 1966.
19. Moretta L, Ferrarini M, Mingari MC, Moretta A, Webb SR. Subpopulations of human T cells identified by receptors for immunoglobulins and mitogen responsiveness. J Immunol **117**:2171–2174, 1976.
20. Moretta L, Mingari MC, Moretta A, Cooper MD. Human T lymphocyte subpopulations: Studies of the mechanism by which T cells bearing FG receptors for IgG suppress T-dependent B cell differentiation induced by pokeweed mitogen. J Immunol **122**:984–990, 1979.
21. Rupley JA. The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular-weight substrates for lysozyme. Biochim Biophys Acta **83**:245–255, 1964.
22. Jollès P, Bernier I, Berthou J. Charlemagne D, Faure A, Hermann J, Jollès J, Périn JP, Saint-Blancard J. From lysozymes to chitinases: structural, kinetic, and crystallographic studies. In: Osserman EF, Canfield RE, Beychok S, Eds. Lysozyme. New York, Academic Press, pp31–54, 1974.
23. Hash, JH. Lysozyme *Chalaropsis.* In: Osserman EF, Canfield RE, Beychok S, Eds. Lysozyme. New York, Academic Press, pp95–103, 1974.
24. Browder HP, Zygmunt WA, Young JR, Tavormina PA. Lysostaphin: Enzymatic mode of action. Biochem Biophys Res Commun **19**:383–389, 1965.
25. Wadström T, Vesterberg O. Stdies on endo-β-*N*-acetylglucosaminidase, staphylolytic peptidase, and *N*-acetylmuramyl-L-alanine amidase in lysostaphin and from *Staphylococus aureus.* Acta Pathol Microbiol Scand [B] **79**:248–264, 1971.
26. Nees S, Veh RW, Schauer R. Purification and characterization of neuraminidase from *Clostridium perfringens.* Hoppe-Seyler's Z Physiol Chem **356**:1027–1042, 1975.
27. Brew K, Castellino FJ, Vanaman TC, Hill RL. The complete amino acid sequence of bovine α-lactalbumin. J Biol Chem **245**:4570–4582, 1970.
28. Wilson AC, Prager EM. Antigenic comparison of animal lysozymes. In: Osserman EF, Canfield RE, Beychok S, Eds. Lysozyme. New York, Academic Press, pp127–141, 1974.
29. Edelman GM. Cell adhesion and the molecular processes of morphogenesis. Annu Rev Biochem **54**:135–169, 1985.
30. Glynn AA, Martin W, Adinolfi M. Levels of lysozyme in human foetuses and newborns. Nature **225**:77–78, 1970.
31. Gordon S, Todd J, Cohn ZA. In vitro synthesis and secretion of lysozyme by mononuclear phagocytes. J Exp Med **139**: 1228–1248, 1974.
32. Sharon N, Lis H. Lectins: Cell-agglutinating and sugar-specific proteins. Science **177**:949–959, 1972.
33. Valisena S, Varaldo PE, Satta G. Purification and characterization of three separate bacteriolytic enzymes excreted by *Staphylococcus aureus, Staphylococcus simulans,* and *Staphylococcus saprophyticus.* J Bacteriol **151**:636–647, 1982.
34. Varaldo PE, Biavasco F, Pruzzo C, Satta G. Interference of *Staphylococcus aureus* glucosaminidase with mitogen responsiveness of human lymphocytes. In: Jeljaszewicz J, Ed. Staphylococci and Staphylococcal Infections. Stuttgart, Gustav Fischer Verlag, pp895–899, 1981.
35. Jollès P. Recent developments in the study of lysozymes. Angew Chem Int Ed **3**:28–36, 1964.
36. Kuettner KE, Eisenstein R, Sorgente N. Lysozyme of cartilage and other connective tissues (a speculative review). In: Osserman EF, Canfield RE, Beychok S, Eds. Lysozyme. New York, Academic Press, pp399–410, 1974.
37. Rosenstreich DL, Farrar JJ, Dougherty S. Absolute macrophage dependency of T lymphocyte activation by mitogens. J Immunol **116**:131–139, 1976.
38. Waksman BH, Namba Y. On soluble mediators of immunologic regulation. Cell Immunol **21**:161–176, 1976.
39. Unanue ER, Beller DI, Calderon J, Kiely JM, Stadecker MJ. Regulation of immunity and inflammation by mediators from macrophages. Am J Pathol **85**:465–478, 1976.
40. Mizel SB. Interleukin 1 and T cell activation. Immunol Rev **63**:51–72, 1982.