LYSOZYME is an ubiquitous enzyme found in most biological secretions and leukocytes. This study was aimed at investigating its interaction with other inflammatory mediators on mucosa surfaces, particularly the complement system. Lysozyme has been shown in our present study, to inhibit the haemolytic activity of serum complement in a dose-dependent fashion, when tested within the levels present in normal and inflamed breast-milk samples, and other mucosal secretions. This represents a new anti-inflammatory action of lysozyme in relation to the serum complement, and the exact mode of the interaction need further studies.

Key words: Human lysozyme, Complement system, Inflammation

A novel anti-inflammatory activity of lysozyme: modulation of serum complement activation

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Introduction

Lysozyme is an acid- and heat-stable enzyme that is abundant in the breast-milk and in most other mucosal body-fluids.¹ Lysozyme catalyses the hydrolysis of β (–1,4–) linkage between N-acetylglucosamine and N-acetylmuramic acid in the bacterial cell wall. The enzyme lyses mostly Gram-positive and a few Gram-negative bacteria or induces their aggregation.² In addition, it possesses prominent anti-inflammatory properties.

Lysozyme has been shown to inhibit chemotaxis of activated leukocytes. Other anti-inflammatory functions of lysozyme include inhibition of mitogen-induced lymphoblastogenesis and autologous mixed lymphocyte reaction (at concentrations ranging between 1 and 10 $\mu\text{g/ml}$).³

Its interaction with the complement (Cp) system has so far been found to be indirect, by an inhibition of PMN response towards complement-derived chemotaxins. Our present studies have shown that it is also capable of directly modulating the entire activation of Cp reaction cascade. The exact mechanism for this inhibition is however, yet to be fully examined. Lysozyme, some milk proteins, such as alpha lactalbumin, and low-molecular-weight ligands, such as citrates and phosphates, are known to have high affinity binding sites for calcium,⁴ and may therefore act as inhibitors of complement indirectly, by chelating the divalent ions required for complement activation. The inhibitory effect of lysozyme might also be related to

its ability to degrade certain glycoprotein components of native Cp factors.

The serum Cp system consist of at least 19 proteins, mostly in pre-activated enzymatic forms, activated in a multi-step cascade reaction via either the classical or alternative pathways. The classical pathway is activated mainly by antigen–antibody complexes (IgG or IgM mostly) starting with Clq,Clr,Cls, C4 and C2, and eventually leading to the activation of C3 by cleavage into C3a and C3b. The alternative pathway (APC) utilizes active sites (such as are present on zymosan, yeast, cobra venom, most Gram-negative bacteria, sheep erythrocytes and human cells deficient in the expression of membrane regulatory molecules) in the presence of properdin, serum factors B and D, to activate C3. This step unifies the two pathways and proceeds uniformly thereafter to the formation of (C5b-9) membrane attack complexes (MAC), capable of inserting into biological membranes and producing cell lysis and death. APC activating surfaces are characterized by possession of structures which restrict access to inhibitory factor H to deposited C3b, thereby amplifying the formation of further C3b in the presence of factor B.

Materials and methods

Buffers

Gelatin veronal buffered saline (GVB²⁺) and sucrose gelatin veronal-buffered saline (SGVB²⁺) with Ca²⁺

and Mg^{2+} were prepared as described by Mayer. GVB^{2-} buffer containing 10 mM ethylene diacetyl ethylamine (EDTA) was used as the stopping solution. Sucrose- GVB^{2+} was utilized for the serum complement assay in place of GVB^{2+} buffer, with ionic strength of 0.065 and 0.149 respectively.

Purified human lysozyme

Purified lysozyme crystals was obtained from Serva Feinbiochemica-Heidelberg, Germany. It was dissolved in SGVB^{2+} and added to serum complement assays in final concentrations ranging from 50 to 1500 $\mu\text{g/ml}$.

Sensitized sheep red blood cells

Sheep red blood cells (SRBC) (ORAW 30/31) obtained from Behringwerke, Marburg, Germany were washed twice in GVB^{2+} buffer and resuspended in the same buffer. The SRBC concentration was standardized spectrometrically by adding 100 μl of the SRBC suspension to 1.9 ml water and reading the absorbance at 541 nm (A_{541}) with a spectrophotometer (Stasar III Gilford Instrument Laboratories, Inc., Oberlin, Ohio). GVB^{2+} was added to the SRBC until the A_{541} was 0.468, corresponding to an approximate cell suspension of $1 \times 10^9/\text{ml}$.

A sensitized cell suspension was prepared by a dropwise addition of an equal volume of anti-Forsman haemolysin (diluted 1:300 in GVB^{2+} containing 20 mM EDTA) to the previously prepared SRBC suspension. The cells were incubated at 37°C for 30 min and at $0-2^\circ\text{C}$ for a further 30 min. The cells were then washed once in SGVB^{2+} and resuspended in this buffer. A final suspension of 5×10^8 cells/ml, obtained by an equal addition of an equal volume of buffer, was employed in the test assays.

Normal human serum haemolytic assay

Serum samples were obtained from 10 healthy blood donors. A micro-modification of the standard haemolytic assay was used for the assessment of serum complement activity. A 1:20 dilution of serum was prepared, different volumes (12.5–100 μl) of which was pipetted into the assay tubes, as well as 40 μl of sensitized SRBC, to obtain a total reaction volume of 375 μl , SGVB^{2+} buffer was used for the serum assay, incubating the mixture for 60 min at 37°C , with occasional mixing. After pelleting the remaining intact cells, 200 μl of each supernatant was pipetted into a 96-well flat-bottomed microtitre plates (Greiner Labortechnik, Frickenhausen, Germany) and the amount of the released haemoglobin recorded by a plate spectrophotometer (MR 700 microplate reader, Dynatech Instruments, Inc., Torrance, California) at a test wavelength of 410 against a reference

wavelength of 630. The degree of lysis produced was obtained from the equation

$$Y = \frac{\text{Test OD} - \text{Spontaneous lysis OD} - \text{Serum blank OD}}{\text{H}_2\text{O lysis OD} - \text{Spontaneous lysis OD}} \times 100$$

Assays of serum complement inhibition by lysozyme

Using the modified CH50 assay as described above, a constant volume of serum, containing approximately one CH50 unit, and SRBC, were pipetted into assay tubes. Lysozyme was added before incubation of the reaction mixtures, at final concentrations ranging from 50 to 1500 $\mu\text{g/ml}$.

Result

Lysozyme was added to the serum as a source of Cp in CH50 whole Cp assays. Concentrations ranging from 50 to 1500 $\mu\text{g/ml}$ were added, corresponding to expected physiological and pathological levels of lysozyme in the colostrum, mature or inflamed breast-milk, as well as other normal and inflamed body fluids. Lysozyme was found to inhibit the whole serum complement activity, mainly the classical pathway tested by CH50 assays, in a dose-dependent fashion (Fig. 1). At low concentrations corresponding to physiological levels, the inhibitory effect of lysozyme was relatively minimal. The inhibition thereafter rises sharply with increasing concentrations, within pathological ranges.

FIG. 1. Inhibitory effect of human lysozyme on serum complement activation. ●, % inhibition of serum complement activation (average $\pm$ SEM, $n = 2$).

Discussion

Despite widespread notions about insignificant roles of the inflammatory processes of the Cp system in mucosal secretions,⁵ the Cp system might still represent a major anti-microbial armoury on mucosal surfaces. A recent series of studies have demonstrated *in vitro* Cp-mediated bacteriolytic activities against a serum-sensitive *Escherichia coli*, *Staphylococcus aureus* and *S. epidermidis* in human breast-milk (HBM). Furthermore, non-immune mechanisms of Cp activation have been demonstrated in the mucosal secretions of the lacrimal gland, suggesting that the well-known inhibition of classical pathways of Cp activation at these mucosal sites by secretory components such as lactoferrin, could still be circumvented. In the HBM, apart from the Cp and free fatty acids (FFA), most of the other anti-microbial components of HBM are bacteriostatic. Since bactericidal FFA are also present in the artificial formula feeds, and they do not seem to contribute to the protection of the formula-fed infants, the Cp system should therefore be regarded as a potentially significant source of contribution to the observed resistance of breast-fed infants against infection.

Lysozyme was found to inhibit the classical pathway of serum complement activity in a dose-dependent fashion, particularly within the pathological ranges. The levels of both mucosal lysozyme and Cp components are known to increase during periods of

infection. This would suggest that the mucosal Cp might be actively involved in bacteriolysis, opsonization and modulation of immune responses at mucosal surfaces, in the absence of full-blown inflammation, to protect the body against the constant threat of foreign invaders. The presence of anti-inflammatory secretory components would then serve to protect the tissues against secondary damage during acute overwhelming infections, where excessive inflammatory reactions of the Cp would be undesirable.

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