INHIBITION BY AN EGG LYSOZYME OF THREE STAGES OF AN ENZYMATIC CASCADE OF ACTIVATION OF A CLASSIC PATH OF A HUMAN COMPLEMENT

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It is known, that the lysozyme has alongside with hydrolytic activity an immunomodulation properties. In this connection the ability of an egg lysozyme was explored to act on a complement system. The interaction of a lysozyme with a subcomponent C1q of the first component of a human complement was explored earlier (K_i 0.29±0.09 mM). In operation are studied: (1) "anticomplementary" activity of a lysozyme (K_i 0.30±0.09 mM), (2) activity inhibiting it at the stage of formation C3 convertase of a classic path of a complement (K_i 0.25 ± 0.05 mM), and (3) inhibition at the stage of formation C5 convertase (K_i 0.26±0.01 mM). The detected direct action on a complement of a lysozyme irrelevant with its enzymatic activity, can be one of explanations of an exotic immunomodulation properties of this protein.

The lysozyme (muramidase, EC 3.2.1.17) is a potent antibacterial drug which is capable to destroyt by a hydrolysis of a cellwall of microorganisms. In this connection this enzyme presented in a blood and mainly in secrets of mucosas of an organism often acts together with a complement (see, e. g.,[1]). In the literature there are informations about immunomodulation [2] and anti-inflammatory activity [3] of this enzyme. These properties the authors of the mentioned papers are inclined to explain by ability of a lysozyme to inhibit a complement activation [4]. The interaction of a lysozyme and lactalbumin with a subcomponent C1q of the first component of a human complement was firstly described in [5]. However it was known, that the lysozyme has no ability to activate a classic pathway of a complement [6]. Thus, it was possible to believe, that immunomodulation and anti-inflammatory activity of a lysozyme are explained, first of all, by inhibition of interaction with the first component of a complement on immune complexes. However nothing it was known about ability of a lysozyme to act at other stages of a complement activation, in particular at stages resulting in to releasing of anaphylatoxins—of the basic agents of an inflammation. In a stage of a classic pathway of a complement activation it is possible to underline three key ferment systems: (1) activated component C1, participating in formation of C3 convertase, substrates are C4 and C2, (2) C3 convertase (C2a4b) making C5 convertase, substrate is C3, and (3) C5 convertase (C2a4b3b), originating formation membrane attack complex, substrate is C5. The inhibition of shaping of each of enzymatic systems results in different consequences in a regulation of the relevant processes of a homeostasis, as the hydrolyses products of components C4, C2, C3, and C5, are physiologically active substances, for which one there are receptors on many cells of an organism (see, for example, the review [7]). In this connection the examinations of ability of a lysozyme were carried out to develop anticomplementary activity, and also to inhibit covalent binding of nascent C4b and C3b.

Materials and Methods

In operation have utillized the computer programs for a ELISA method of determination of function activity [8] and determination of parameters of the Michaelis-Menten equation [9], implemented Co. Ltd. Microflora at G. N. Gabrichevsky Moscow Research Institute of Epidemiology and Microbiology, 96-units flat-bottomed micropanel of Russia production (Medpolymer, Moscow), Twin 20, a horse-radish peroxidase Biochemreactive (Latvia), lysozyme Ferein (Russia), the remaining reagents—Russia production of quality are not lower analytical grade. Rabbit IgG antibodies to a human C4 and C3 and the conjugates of these antibodies with peroxidase obtained by conventional methods [10] (more detail is scribed in [8]).

Determination of inhibition of binding nascent C4b. 100 μl a solution of immunochemical pure IgG in 0.05 M sodium carbonate buffer, pH 9.5, in concentration 100 μg/ml was dispenced to each small well of the flat-bottomed polystyrene 96-units micropanel. Covered by a cover and kept for night at 4°C. Two times washed a panel by a veronale buffered solution, pH 7.4, containing 0.15 M NaCl, 0.15 mM Ca^{2+} and 0.5 mM Mg^{2+} (VBS^{2+}), 150 μl in each small well, then a panel unwatered by shake out. To all small units of a panel puted 100 μl VBS^{2+}, 10 μl of guinea pigs serum and kept at room temperature on 30 min, then kept in repair small units removed by shaking out. In other micropanel with U-wells puted series of twofold delutions of freshen human sera in VBS^{2+}, total amount in the small unit was 50 μl. Then to all wells of each series puted 50 μl of a solution of a lysozyme in for each series of concentration, and in the check 50 μl of the

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buffer without an inhibitor, kept in repair small wells of this micropanel transferred to the first micropanel. After an incubation in a thermostat within 1 h at 37°C, twofold washing out by the phosphate buffer, ph 7.4, containing 0.15 M NaCl and 0.05% the Twin-20, and the dehumidifyings of a plane table in each small well puted 100 μl a conjugate of peroxidase with antibodies against a human component in the same buffer. After an incubation in a thermostat within 1 h at 37°C twofold washing out with a detergent and the dehumidifyings of a panel in each small unit imported 100 μl substrate buffer (10 mg ortho-phenylenediamine in 25 ml citrate-phosphate buffer, ph 5.0, and 50 μl 3% H_2O_2). After 30 min incubation in darkness response discontinued by addition into each small unit 50 μl 14% H_2SO_4. The results of response allowed with the help of a spectrophotometer with a vertical beam by gauging of a light absorption at 492 nm. Function activity of a component C4 calculated with the help of the computer program, in a basis which one puts linear regression for dependence of activity on an extinction of a yield of enzymatic response. A constant of inhibition K_i determined with the help of the computer program for calculation of parameters of the Michaelis–Menten equation.

Determination of inhibition of binding nascent C3b. 100 μl solution of a immunochemical pure IgG in 0.05 M sodium-carbonate, pH 9.5, in concentration 100 μg/ml was dispenced on to each small well of flat-bottomed polystirene 96-units micropanel. Covered by a cover and kept for night at 4°C. Two times washed a panel by 150 μl veronal buffered solution, pH 7.4, containing 0.15 M NaCl, 0.15 mm Ca^{2+} (VBS–Ca^{2+}), on in each unit, then a panel unwatered by shaking out of the heels of a fluid. To all small units of a panel puted 75 μl VBS–Ca^{2+}, 15 μl 0.1 M of nickel nitrate (II) and 10 μl guinea pigs serum. After an incubation in a thermostat during 30 min, at 37°C, twofold washing out VBS, containing 5 mM EDTA, (VBS-E) and the kept in repair units removed by shaking out. In other micropanel with U-wells puted seriess of twofold delutions freshen human sera in VBS-E, total amount in the each unit was 50 μl. Then to all units of each series puted 50 μl solution of a lysozyme in for each series of concentration, and in the control 50 μl of the buffer without an inhibitor, kept in repair units of this micropanel transferred to the first micropanel. After an incubation in a thermostat within 1 h at 37°C, twofold washing out by the phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.05% the Twin-20, and the dehumidifyings of a panel in each small cavity puted 100 μl conjugate of peroxidase with antibodies against a human component C3 in the same buffer. Further all as at determination of intercept nascent C4b.

Determination of anticomplementary activity by a hemolytic method circumscribed earlier [11] for determination of complement activation abilitiy, with that modification, that compared results of drop of C4 activity at carrying out of a preliminary incubation of a guinea pig complement with a lysozyme (as it is necessary for doing for determination of ability of an added effector to activate a complement, what judge by consumption of a C4 component) with the data received without such preliminary incubation. The coincidence of results bore for the benefit of intercept by a lysozyme of nascent C4b as in system there was a deficit only of C4, and the remaining components were in abundance and in less sensitive to inhibition.

Results and Discussion

Earlier on the basis of some structural similarities between the C_H2-domain of IgG binding a subcomponent C1q of the first complement component and lysozyme the guess of an opportunity of such interaction for a lysozyme was expressed and it was found [5]. Natural was to expect, that such interaction should not result in activations of system of a complement but only render some inhibition. If the lysozyme was capable to activate a complement system, their collateral abiding in serum and other biological fluids would give in consumption of a complement. Really, was shown [6], that the lysozyme has no ability to activate a complement.

We explored "anticomplementary" activity of a lysozyme in system, where the drop of activity of C4 taking place in a deficit was determined at excess of other components of a complement, at an incubation with an effector—in this case with a lysozyme. This method designed for determination of effector ability to activate complement [11], as it is supposed that at a preliminary incubation of a complement with an effector the drop of activity of 4 happens for case of system activation and C4 consumption. However application of this system without a preliminary incubation has yielded the same results of drop of C4 activity. It could mean that the lysozyme inhibits process of determination of C4 activity which one is elaborated at the presence of an effector. Such appearance can take place as a result of inhibition of an activated species generated at the moment of activation, nascent C4b, by nucleophilic attack its thioester. Detailed such path of inhibition, valid for activated C4b and C3b, and specific recognition of a nucleophilic acceptor bearing to structural specificity of acceptors, surveyed earlier in the relation to C3b [9, 12].

To study intercept of nascent C4b and C3b designed ELISA methods for definition of function activity of com-

Table 1

Constants of inhibition of different stages of activation of a complement by a lysozyme and IgM

Effector	Anticomplementary activity	Binding		
		C1q [5]	nascent C4b	nascent C3b
Lysozyme	0.30 ± 0.09 mM	0.29 ± 0.09 mM	0.25 ± 0.05 mM	0.26 ± 0.01 mM
IgM	—	2.0 ± 0.4 μM	0.9 ± 0.6 μM	1.12 ± 0.02 μM [12]

plement components [8]. The results of determination of inhibition constants of all these stages and a stage of a complement are listed in Table 1. For comparison the similar constants for an human immunoglobulin M are given. The particular participant of these processes IgM has constants of interaction on two order of quantities distinguished from those for a lysozyme. Nevertheless, the detection of ability of a lysozyme soft to inhibite a stage of complement activation a at its three stages can explain anti-inflammatory and immunomodulation activities of a lysozyme.

References

1. Mokracka-Latajka G., Grzybek-Hryncewicz K., and Jankowski S. *Acta Microbiol. Pol.* 1990. **39**. P. 117–120.
2. Dobrotina N.A., Kazatskaia Zh.A., and Emel'ianova G.Yu. *Vopr. Med. Khim. 1987.* **33***, No. 4. P. 66–69.*
3. Ogundele M.O. *Mediators Inflamm.* 1998. **7**. P. 363–365.
4. Ogundele M.O. *Mediators Inflamm.* 1999. **8**. P. 69–75.
5. Kozlov L.V., Sizoi M.N., Zinchenko A.A., and Levkovskii A.V. *Biokhimiya.* 1986. **51**. P. 707–718.
6. Heinz H.P. and Loos M. *Immunobiology.* 1983. **165**. P. 175–185.
7. Kozlov L.V. "Protein of system of a complement." In: *Protein and Peptides.* Moscow, Science. 1995. V. 1. P. 385–395.
8. Kozlov L.V., Lakhtin V.M., Skorokhodova T.G., Batalova T.N., Shojbonov B.B., D'yakov V.L., Gouzova V.A., and Matveevskaya N.S. *Russian J. Bioorg. Chem.* 2000. **26**. P. 555–563.
9. Kozlov L.V., Rostovtseva L.I., Lomaka T.S., and Sutovskaya N.S. *Russian J. Bioorg. Chem.* 1985. **11**. P. 762–768.
10. Getty D., Raykundalia C., and Houba V. *WHO IMM/PIR.* 1983. No. 83.1.
11. Kozlov L.V. and Gouzova V.A. "Determination of ability of complement activation of drugs for intravenous introduction. Methodic guides 98/87. Ministery of Health of Russian Federation.
12. Kozlov L.V. and Lebedeva T.V. *Russian J. Bioorg. Chem.* 1998. **24**. P. 350–355.