

Inhibitory Effect of C-Reactive Protein on Streptolysin O-Mediated Hemolytic Activity. Comparison of Conformational Variants

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It is shown that C-reactive protein binds to streptolysin O, an exotoxin of hemolytic streptococcus, and neutralizes its hemolytic activity. Incubation of C-reactive protein with the working dose of streptolysin O for 15-20 min at 37°C abolished the hemolysis of subsequently added erythrocytes. The concentration of C-reactive protein that reduced hemolysis by 50% was on average equal to 2.28 ± 0.19 µg/ml. C-reactive protein antihemolytic activity was not affected by blocking of its phosphorylcholine-specific sites with free phosphorylcholine, but decreased as a result of blocking with pneumococcal C-polysaccharide and, particularly, with L-α-phosphatidylcholine. This indicates a hydrophobic nature of C-reactive protein-streptolysin O interaction. C-reactive protein subunits retained antihemolytic activity, while the aggregated C-reactive protein lost part of it.

Key Words: *C-reactive protein; streptolysin O; erythrocytes*

C-reactive protein (CRP) belongs to a group of hepatocyte-produced serum proteins which are induced by interleukin-6 during the acute phase of inflammation. CRP possesses opsonizing activity vis-a-vis certain microorganisms and injured autologous cells, and can exert various cytotoxic effects on neutrophils, macrophages, lymphocytes, and platelets [6]. Its main function is to eliminate foreign and autologous necrotized substances from the organism. Streptolysin O (SLO) is an exotoxin of β-hemolytic streptococcus. SLO can exert hemolytic, cytotoxic, and cardiotoxic effects [2]. It is the best studied representative of a group of related bacterial hemolysins that lose their activity after oxidation and restore it after treatment with thiol reagents [10]. SLO toxicity is realized via the formation of plasma membrane pores, through which

cytoplasmic material from the target cell leaks [13]. SLO is neutralized by specific antibodies that are present in the serum of healthy donors and many animals; increased antibody titers are found in patients with acute rheumatism [1]. SLO is also inhibited by such plasma components as cholesterol [9], low density lipoproteins [14], and lysozyme [8]. Circulating immune complexes from patients with rheumatism contain CRP, along with SLO polypeptides [11], pointing to possible SLO-CRP interaction. However, this aspect has not been investigated, and the role of CRP in the composition of the circulating immune complexes remains unclear. In view of this the goal of the present work was an experimental study of CRP-SLO interaction and of the effect of the acute-phase reactant on the toxin-mediated hemolytic activity.

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MATERIALS AND METHODS

CRP was obtained from ascitic fluid of cancer patients. The methods of CRP isolation and puri-

fication, control of homogeneity, and immunochemical purity, as well as of dissociation into subunits have been described earlier [3]. Thermoaggregated CRP was obtained by heating the native product at 63°C for 5 min. Commercial SLO preparation was used according to the manufacturers' directions (Research Institute of Vaccines and Antisera, St. Petersburg); the titration of anti-SLO activities of the substances under study was performed in the same way. The only modification was diminishment of the reagent volumes due to the use of 96-well microplates (Medpolymer, St. Petersburg). Erythrocytes were obtained from the venous blood of healthy donors. Interaction between SLO and CRP was recorded in a solid-phase immunoenzyme assay. SLO was adsorbed in polystyrene flat-bottom 96-well plates that were precoated with cholesterol (Serva) placed in the wells as 0.1 mM ethanol solution and dried, or in untreated polystyrene plates. Wells were washed with phosphate-buffered saline, pH 7.2, followed by blocking of plastic surface with 2% bovine serum albumin solution (Gibco). SLO-coated wells were filled consecutively with native CRP solution (300 ng/ml) in buffered saline with subsequent incubation at 37°C for 2 hours, ass antiserum to human CRP (Research Institute of Vaccines and Antisera, St. Petersburg), and protein A-peroxidase conjugate (Research Institute of Experimental Medicine, St. Petersburg) with intervening washings before the addition of each new reagent. O-phenylenediamine mixed with H₂O₂ served as a substrate for the reaction development. The results were recorded on a Linkey comparator (Ladoga Research and Manufacturing Conglomerate) in a single-wave regime at 405 nm. We also used human anti-influenza γ -globulin (10% solution in ampoules, Research Institute of Experimental Medicine, St. Petersburg), lysozyme (Reanal), phosphorylcholine chloride (Sigma), egg L- α -phosphatidylcholine (EPC; Serva), and pneumococcal C-polysaccharide (Reanal).

RESULTS

The results of an immunoenzyme assay of SLO-CRP interaction (Fig. 1) attest to the binding of acute-phase protein with the toxin. The interaction is dose-dependent and proceeds more efficiently in the uncoated wells. In the cholesterol-precoated wells the specific binding is lower, probably due to the blocking of SLO hydrophobic sites that are also necessary for the interaction with CRP.

The effect of pure CRP preparation on the hemolytic properties of SLO was studied using routine assays of anti-SLO activity in patient sera. As can be seen in Fig. 2. preincubation of the

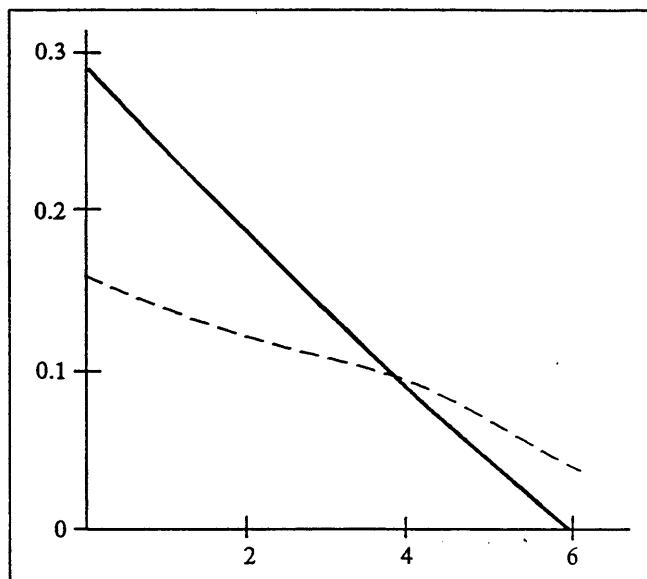


Fig. 1. CRP binding to SLO. Abscissa: log₂ of SLO dilutions used for solid-phase adsorption; initial SLO concentration (log₂=0) corresponds to the working dilution recommended by the manufacturers for testing SLO antibodies. Ordinate: optical density of o-phenylenediamine peroxidation product measured at 405 nm. 1) SLO on plastic precoated with 0.1 mM cholesterol solution in ethanol; 2) SLO on untreated plastic. CRP was introduced into the wells in a concentration of 300 ng/ml. The mean of two experiments is presented.

standard dose of SLO with CRP (37°C, 15 min) led to an abolition of the hemolytic effect, i.e., to toxin neutralization. The CRP concentration reducing the hemolytic effect of the standard dose of

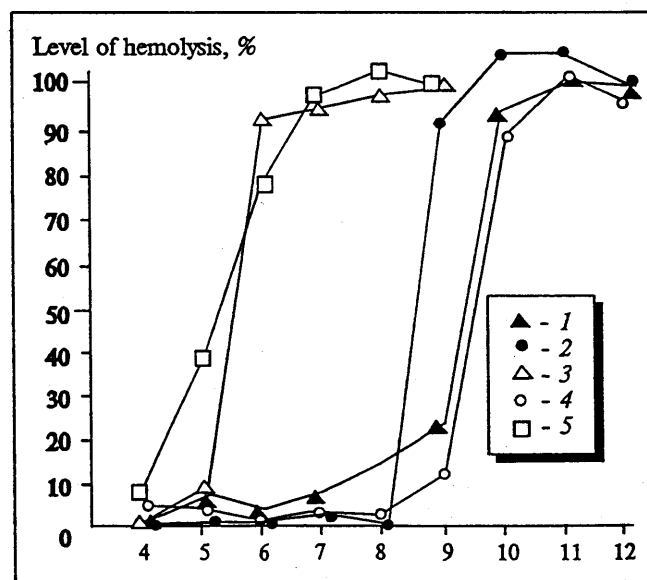


Fig. 2. Inhibition of SLO hemolytic effect by preincubation of SLO with CRP and other inhibitors. Abscissa: log₂ of inhibitor dilution. Initial inhibitor concentration: CRP - 1 mg/ml; human γ -globulin - 10% solution; cholesterol - 7 mg/ml; lysozyme - 2 mg/ml. 1) CRP; 2) human γ -globulin; 3) lysozyme; 4) cholesterol; 5) CRP preincubated for 1.5 hours with 1/6 volume of CRP antiserum. Each curve represents mean data of 4-6 experiments.

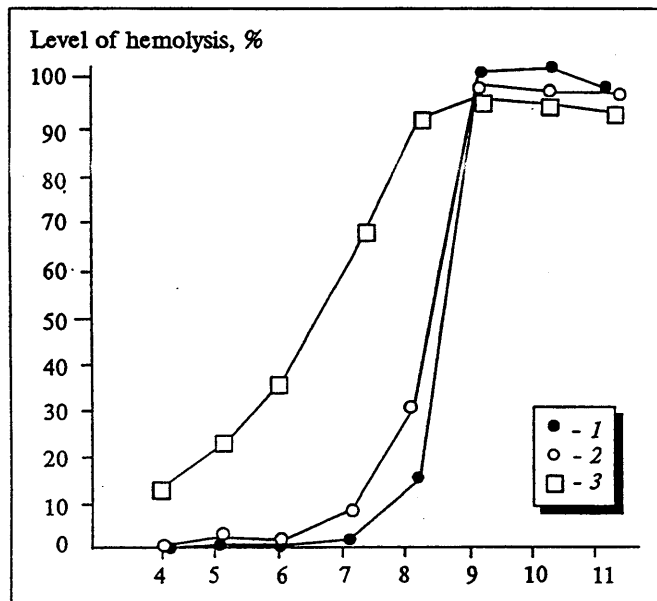


Fig. 3. Comparative efficacy of CRP conformational variants in inhibition of SLO hemolytic effect. Abscissa: log₂ of CRP dilution (initial concentration of each CRP variant is equal to 0.9 mg/ml). 1) native CRP; 2) CRP subunits; 3) aggregated CRP. The mean of 3 experiments is presented.

SLO by 50% (K_i) was equal to $1.58 \pm 0.26 \mu\text{g/ml}$ in 9 experiments with erythrocytes of the same donor, and $2.28 \pm 0.19 \mu\text{g/ml}$ ($1.5\text{--}5.2 \mu\text{g/ml}$) in the experiments with erythrocytes of 26 different donors. In a molar equation, this is on average $1.5 \times 10^{-8} \text{ M}$. For comparison, Fig. 2 shows curves representing the reduction of the hemolytic effect of SLO by known inhibitors. The concentration required to achieve a 50% hemolysis inhibition was higher than that of CRP and reached $230 \mu\text{g/ml}$ for human γ -globulin ($1.5 \times 10^{-6} \text{ M}$), $8.3 \mu\text{g/ml}$ for cholesterol ($2 \times 10^{-5} \text{ M}$), and $42.1 \mu\text{g/ml}$ for lysozyme ($2.9 \times 10^{-6} \text{ M}$). Thus, CRP proved to be a considerably more potent inhibitor of SLO-induced hemolysis than cholesterol and lysozyme. Regarding human γ -globulin, the molar quota of antibodies to SLO in this polyclonal preparation is unknown, and therefore a comparison can be based either on the weight concentrations (see above) or on the assessment of specific antitoxic activity. The latter was estimated as 500 IU/ml for CRP and 1250 IU/ml for 10% human γ -globulin solution, or, after adjustment of the protein concentration to the same level, 500 and 12.5 IU/mg, respectively. The anti-SLO activity of CRP fell after a 1.5-hour preincubation with monospecific CRP antiserum (Fig. 2). CRP is known to be represented in the organism by various conformational forms. These are a native pentamer released by the liver into the circulation and an aggregated form in deposits (regions of inflammation and/or tissue destruction, or

of lipoprotein deposits). Finally, under certain pathological conditions, e.g., during poisoning with heavy metal salts, the protein circulates and is excreted by the kidneys in the form of free subunits [7]. The conformational variants of CRP retain many properties of the native molecule and, in certain cases, can even surpass them [4,5]. In view of this, we undertook a study of the influence of CRP conformational status on its anti-SLO activity. For this purpose, single-unit and aggregated forms were prepared, and their anti-SLO activity was compared to that of native CRP. The results showed that the subunits mostly retain the capability of inhibiting SLO hemolytic activity, while this property was reduced in aggregated CRP (Fig. 3).

Analysis of the mechanism of CRP action using known ligands of this protein showed that free phosphorylcholine in a concentration of 100 mM did not affect anti-SLO activity of CRP. At the same time, pneumococcal C-polysaccharide ($500 \mu\text{g/ml}$) and, especially, EPC (100 mM) inhibited it; this was reflected in the rise of the CRP K_i by 2 and 3.3 times, respectively. This means that, besides a phosphorylcholine-specific site of CRP that apparently participates in the CRP-mediated anti-SLO effect (since phosphorylcholine groups are present in both C-polysaccharide and EPC molecules), an important role in the contact between CRP and SLO should be assigned to the hydrophobic interactions. Evidence in favor of this is presented by inhibition of the CRP anti-SLO effect by the EPC acyl chains. Neither C-polysaccharide nor EPC themselves exerted any effect on SLO hemolytic activity.

The ability of CRP to interact with SLO and to abolish its toxic effect (in the present work we analyzed the hemolytic activity only) shows that the spectrum of protective properties of this reactant of the acute phase of inflammation is much broader than was thought earlier. The data obtained permit us to classify CRP in the system of anti-toxic protective factors and explain numerous clinical data concerning the relationship between CRP and anti-SLO in patients with rheumatism. The presence of CRP in SLO-containing immune complexes [11] also becomes understandable. Antitoxic properties of CRP may be in evidence in the early phase of streptococcal infection, since a rise of CRP in the circulation starts as early as within the first hours of the inflammatory reaction, i.e., long before the appearance of antibodies. Aggregation reduces, but does not totally abolish, the CRP cytotoxic activity, and therefore it is not to be excluded that in tissue deposits of various genesis (e.g., in the vessel walls during arteritis [12]; etc.) CRP retains part of its capacity to bind

SLO, thus, regardless of toxin neutralization, contributing to the development and aggravation of the systemic or local process that itself had been the cause of CRP deposition.

In view of the above, there arises the question concerning the interpretation of the clinical data on anti-SLO, i.e., whether the anti-SLO titer is always associated with the specific antibody production or whether in certain cases it is mainly mediated via CRP release. The most simple, though not the only, methodical approach, the efficacy of which is seen in Fig. 2, may be the use of anti-CRP antibodies in order to block this protein in the sera under study.

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