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Polyreactive Transformation of Class G Immunoglobulins as a Vector for Search of Potential Means for Improving the Activity of Anti-Endotoxin Immunity

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Abstract—Short-term in vitro exposure of a chaotropic reagent to native class G immunoglobulins can significantly increase the activity of anti-endotoxin immunity and cause a decrease in the ability of effector cells for enterobacteria lipopolysaccharides (LPS). This leads to reducing the LPS-dependent activation of these cells and subsequent inflammation. This can be useful not only for the treatment of septic patients, but also for the prevention of the progression of diseases of atherosclerotic nature, because LPS is considered to be the most important factor in the induction of atherogenesis. The polyreactive transformation mechanism of native class G immunoglobulins under the influence of a chaotropic reagent remains unknown. It can consist in the induction of intramolecular conformational rearrangements of native γ-globulins. As a result, "ancestral" sites of low-affinity multispecific binding appear on *Fab* fragments, which determines the appearance of complementarity to evolutionarily stable molecular structures of pathogens. The results indicate the need for further investigation of the polyreactive transformation mechanisms of native class G immunoglobulins and the search for nontoxic chaotropic agents that could potentially be used in clinical practice.

Keywords: endotoxin, lipopolysaccharide, immunoglobulins, systemic endotoxinemia, endotoxin aggression, antiendotoxin immunity

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Recent studies have convincingly shown that dysfunction of physiological defense mechanisms, the development of various diseases, decreased quality of life, and life expectancy are closely associated with failures in the complex system of mutual relations between the intestinal microbiota and numerous cells of the macroorganism, including epithelial, immune and neuroendocrine ones. It is becoming increasingly apparent that intestinal microbiota plays a huge role in maintaining human health in all age categories [1–3]. A significant part of the microbiota of the distal intestines is conditionally pathogenic Gram-negative enterobacteria related to the genera *Escherichia*, *Bacteroides*, *Enterobacter*, *Klebsiella*, *Eubacterium*, *Acidinococcus*, *Fusobacterium*, *Megamonas*, *Megasphaera*, *Proteus,* and *Veillonella* [4, 5]. The main component of the cell wall of this group of microorganisms is lipopolysaccharide (LPS) or endotoxin. LPS is considered one of the most powerful phlogogenic factors of microbial origin. In this case, the biologically active part of the LPS molecule is represented by lipid *A*, which is able to interact with membrane receptors of various mammalian cells, which leads to their activation and the synthesis of a wide range of inflammatory mediators [6].

It is known that in the blood of healthy people there is always a small amount of LPS of intestinal origin [7]. The fundamental role of this phenomenon, called systemic endotoxinemia (SET), in human biology is primarily determined by the ability of LPS of the intestinal microbiota to interact with TLR4, the key cellular receptor of innate immunity, and determine the level of activity of the adaptive unit of the immune system. The pathogenic effect of SET, due to the excessive content of LPS of intestinal and/or other origins in hemocirculation, is called endotoxin aggression (EA), which qualifies as premorbidity or universal pathogenesis factor of many diseases and syndromes [8]. Phenomenological confirmation of this definition has been obtained in clinical studies. The use of means to reduce the concentration of LPS in the blood and normalize the activity indicators of antiendotoxin immunity (AEI) can significantly increase the effectiveness of generally accepted treatment regimens for many diseases, including allergies, chronic inflammatory diseases of the eye and female genital area,

chronic viral diseases, sepsis, etc. [8, 9]. The presence of EA, or only some of its symptoms, has also been detected in patients with autoimmune diseases; type 1 and type 2 diabetes mellitus; with abdominal, cardiovascular and urological surgical pathology in children and adults; with anorexia nervosa (clinical model), myocardial infarction, and progression of atherogenesis [10–15].

It has been found that EA can be eliminated, or its pathogenic effect reduced, by selective hemosorption (LPS filters), immunopreparations (anti-LPS antibody concentrates obtained from human blood, monoclonal antibodies against lipid *A*), enterosorbents, live bifidumbacteria, and choleretic drugs [8, 16–18]. However, there is another possible vector for eliminating EA, which has hardly been developed to date; this is an increase in AEI activity. Indirect signs of this possibility are demonstrated by intravenous laser irradiation of blood, ultraviolet irradiation of blood in vitro and X-ray irradiation of a 5% glucose solution immediately before its subsequent perfusion [8]. Chaotropic agents can also be a potentially promising means of increasing AEI; these are chemical compounds initiating intramolecular conformational rearrangements of native class immunoglobulins G (IGs), as a result of which they acquire complementarity that is typically not characteristic of them. The fundamental possibility of producing polyreactively transformed IGs against protein antigens with short-term exposure to a chaotropic reagent (potassium thiocyanate) was discovered earlier in experiments in vitro [19, 20]. In this regard, the aim of this study was to study the ability of a chaotropic reagent to increase AEI activity.

METHODS

The object of the study was the pooled blood serum of 40 permanent donors of the Crimean Republican blood transfusion station (Simferopol), as well as individual blood serum samples of two groups of volunteers (60 subjects of both sexes), obtained by the standard method and stored in the presence of 0.1% sodium azide at a temperature of +4–8°C. The first group of volunteers consisted of 30 apparently healthy subjects who did not have verified acute or chronic diseases. The second group included 30 subjects who suffered from chronic colonic obstruction (CCO) and needed surgical intervention. Native IG was isolated from serum samples by four reprecipitations with ammonium sulfate (40% of saturation); their qualitative composition was monitored by polyacrylamide gel electrophoresis followed by protein staining with Coomassie R-250 dye [21]. The quantitative protein content in all cases was determined by Bredford's method [22].

After isolation, native IGs were divided into two parts; one of them served as a control, and the other was used to obtain polyreactively transformed immunoglobulins (PRIGs). For this purpose, an equal volume of 7 M potassium thiocyanate was added to 0.5 mL of the native IG solution (20 mg/mL), thoroughly mixed, incubated for 10 min at room temperature +(18–22)°C, and then centrifuged for 10 min at 1500*g*. The chaotropic reagent was removed by gel filtration on a column (13 × 1.4 cm) with Sephadex G-25 in 0.01 M phosphate buffer (pH 7.4) containing 1% NaCl. During the experiments, native IGs and PRIGs were stored at +(4–8)°C in the presence of 0.1% sodium azide.

The estimated ability of a chaotropic reagent to increase the activity of AEI was evaluated in vitro by two different methods: enzyme-linked immunosorbent assay (ELISA) and flow laser cytofluorimetry. ELISA was performed according to the following scheme [23]. Into the wells of RIA/EIA polystyrene microplates, medium binding (Corning, United States) 100 μL of a solution of commercial LPS preparations of *Escherichia coli* K235, *Salmonella Minnesota*, and *Salmonella enteritidis* (Sigma Chemicals, United States) were added at a concentration of 10 μg/mL in 0.05 M carbonate buffer (pH 9.6) containing 70% ammonium sulfate. The control antigen was a solution of ovalbumin (10 μg/mL) in 0.05 M carbonate buffer (pH 9.6), which was introduced into wells of 100 μL. After that, the plates were incubated for 18 h at 37°C. To remove unbound antigens and block free sites of nonspecific binding, the wells were washed (4 times, 1 min) with 0.033 M phosphate buffer (pH 7.4) containing 1% NaCl and 0.05% Tween-20 (PBS-T). Then, 100 μL of a solution of native IG or PRIG (25 μg/mL) in PBS-T and immunoperoxidase conjugate of purified goat affinity antibodies against human IgG (Sigma Chemicals, United States) in PBS-T (dilution, 1 : 8000). Each reagent was subjected to a 60-min incubation at 37°C. Nonspecifically bound components were washed off with PBS-T after each step (five times, 2 min). To record peroxidase activity, 100 μL of a mixture of the substrate and buffer solution (0.1 M phosphate–citrate buffer (pH 6.0) containing 3,3',5,5'-tetramethylbenzidine at a concentration of 0.1 mg/mL and 0.005% H_2O_2) was incubated for 60 min at 37°C. The development of staining was stopped by adding 25 μL of 3 M sulfuric acid to the wells. The optical density of the end product of the enzymatic reaction was determined using a Multiskan FC microplate analyzer (Thermo Fisher Scientific™) at a wavelength of 450 nm. The levels of binding of native IGs or PRIGs are presented in units of optical density of the final product of the enzymatic reaction (arbitrary units). The analysis results were considered correct if, for three controls (*Low*, *Medium*, and *High*), the extinction indices at a wavelength of 450 nm were in the established range of values.

The LPS-binding potential of granulocytes and monocytes of human peripheral blood was determined by flow laser cytofluorimetry in accordance with our previously developed protocol [24]. The leukocyte

Table 1. Indicators of binding of native class immunoglobulins G and nolireactively transformed immunoglobulins with LPS conditionally pathogenic gram-negative enterobacteria and ovalbumin ($M \pm m$)

Antigen	Native class G immunoglobulins	Polyreactively transformed immunoglobulins
	the level of binding, conv. units	
LPS *E. coli* K235	0.184 ± 0.02	1.178 ± 0.11*
LPS *S. minnesota*	0.105 ± 0.01	0.269 ± 0.03*
LPS *S. enteritidis*	0.151 ± 0.04	1.195 ± 0.09*
Ovalbumin	0.100 ± 0.02	0.925 ± 0.07*

The table shows the average values obtained as a result of 15 independent experiments. * Differences are significant ($p < 0.001$).

fraction was isolated from peripheral blood of healthy subjects ($n = 50$) stabilized with sodium citrate using the standard erythrocyte lysis procedure in the presence of ammonium chloride (*Current Protocols in Cytometry*, 1997), followed by washing the cells from detritus, hemoglobin, and the remains of the lysing solution with isotonic phosphate-buffered saline (PBS). As a fluorescent probe for the detection of LPS-binding receptors, we used *E. coli* K235 LPS conjugated to fluoresceinisothiocyanate (LPS-FITC conjugate). 12.5 μL of LPS-FITC conjugate was added to 25 μL of the leukocyte suspension (working dilution 1 : 20) and 12.5 μL of a 0.06% solution of bovine serum albumin (Sigma Chemicals, United States), which is necessary to block the non-specific binding of the fluorescent probe. After 30-min incubation in the dark at +(18–20)°C, 1 mL of FSB was added to the tubes and the leukocyte suspension thus prepared was analyzed using a Pas flow laser cytofluorimeter (Partec, Germany). Data collection and analysis was performed using the Partec FloMax V. 2.4d software (Partec, Germany); 10000 cells were analyzed in each sample with recording of the indicators of front and small-angle light scattering on a linear scale (*Fsc* and *SSC,* respectively), as well as fluorescence via the FL1 channel on a four-digit logarithmic scale. After "gating" clusters of granulocytes and monocytes according to the *Fsc* and *SSC* parameters, we determined the average level of fluorescence of these cells (arbitrary units), the numerical value of which characterizes their LPS-binding potential.

The significance of differences between the studied parameters was evaluated using Student's *t* test. The differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

In the first series of experiments, we analyzed the the properties of PRIGs, which were obtained by 10-min exposure of native IGs isolated from the pooled blood serum of healthy donors in the presence of 3.5 M potassium thiocyanate. It was found that short-term contact of native IGs with a chaotropic reagent led to a significant change in their ability to interact not only with a protein antigen (ovalbumin), but also with LPS of a number of conditionally pathogenic enterobacteria (Table 1). The resulting PRIGs bound the LPS of *E. coli* K235, *S. Minnesota,* and *S. enteritidis* much more actively than native IGs (on average, their LPS-binding ability increased by factors of 6.40, 2.56, and 7.91, respectively). This fact is of fundamental importance, since it indicates the possibility of increasing the activity of AEI using chaotropic reagents and the prospect of their use in clinical settings. A large scatter of the efficacy of binding of PRIGs to LPSs of various enterobacteria is still inexplicable. This could be associated with significant differences in the molecular weight of the LPSs of these enterobacteria, and hence with the degree of their biological activity (or toxicity), which is determined by the general and most conserved hydrophobic fragment of the LPS molecule, regardless of its origin. In addition, no convincing literary data have been found on this issue.

Blocking the interaction of LPS with LPS-recognizing receptor structures of effector cells, mainly TLR4, the main cellular receptor of the innate immunity system, is the most likely mechanism of the presumed protective action of PRIGs in sepsis due to Gram-negative microflora and/or EA of intestinal origin. Therefore, at the next stage of studies using flow laser cytofluorimetry, the effect of PRIG on the LPS-binding potential of granulocytes and peripheral blood monocytes of 50 healthy subjects was studied (Table 2). At the same time, the level of binding of fluorescently labeled LPS with LPS-binding receptors in the control group, when both native IGs and PRIGs were absent in the incubation medium, was taken to be 100% (Fig. 1).

The results of the experiments convincingly demonstrate the ability of PRIGs to significantly ($p < 0.001$) reduce the level of binding of fluorescently labeled LPS with both granulocytes and monocytes of healthy subjects. It seems possible to believe that the interaction of PRIGs with LPS leads to the formation of steric hindrances that interfere with the subsequent binding of LPS to LPS-binding cellular receptors. This means that native IGs subjected in vitro to short-

Table 2. Influence of polireactively transformed immunoglobulins on the interaction of fluorescently labeled LPS with LPS-binding receptors of granulocytes and monocytes of peripheral blood of healthy subjects ($M \pm m$)

Experimental conditions	LPS binding potential, arb. units	
	granulocytes	monocytes
In the presence of native class G immunoglobulins, $n = 50$	0.83 ± 0.06 $p < 0.001$	0.90 ± 0.08 $p < 0.001$
In the presence of polyreactively transformed immunoglobulins, $n = 50$	0.70 ± 0.04 $p < 0.001$ $p_1 < 0.001$	0.68 ± 0.06 $p < 0.001$ $p_1 < 0.001$
Only incubation medium (control), $n = 50$	1.12 ± 0.07	1.36 ± 0.07

p, significance of differences from the control; p_1, significance of differences from native G class immunoglobulins.

term treatment with chaotropic agents are potentially able to prevent excessive LPS-dependent activation of effector cells, thereby reducing the excess production of pro-inflammatory mediators and the risk of uncontrolled systemic inflammatory response.

The main results of the study of various aspects of the phenomenon of polyreactive transformation of native IGs were obtained using the pooled blood serum of healthy subjects. At the same time, the structurally functional repertoire of antibody molecules in each individual subject can vary significantly, which, first of all, is determined by the differences in the antigen-binding sites of immunoglobulins formed during an adaptive immune response to antigenic load [25]. In the next series of experiments, we conducted a comparative study of the LPS-binding properties of PRIGs, which are formed when a chaotropic reagent acts on native IGs isolated from individual blood serum samples from 30 healthy subjects, as well as 30 patients with CCO with a high risk of sepsis.

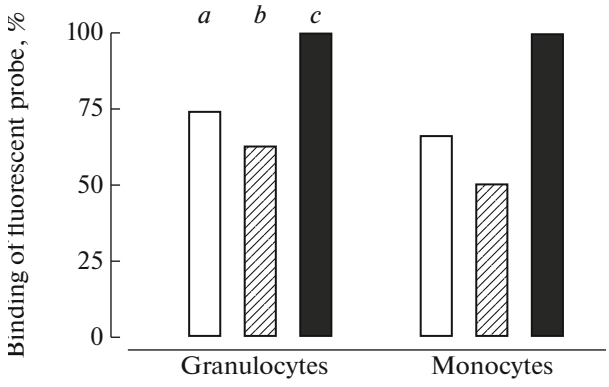

Fig. 1. Comparative characteristics of changes in the LPS-binding potential of granulocytes and monocytes of the peripheral blood of healthy subjects in the presence of (*a*) native class G immunoglobulins and (*b*) polyreactively transformed immunoglobulins. (*c*) Control, incubation medium that does not contain native or polyreactively transformed immunoglobulins.

It was found that the native IG of patients with CCO were less susceptible to polyreactive transformation in the presence of 3.5 M potassium thiocyanate compared to the native IG of healthy subjects (Fig. 2). At the same time, the level of binding of such PRIGs to LPS of *E. coli* K235, *S. Minnesota*, and *S. enteritidis* was significantly lower ($p < 0.001$) in comparison with the PRIGs formed during the polyreactive transformation of native IGs of healthy subjects (on average, lower by 16.5, 37.2, and 25.2%, respectively). Most likely, this is one of the consequences of chronic EA accompanying CCO, which is presumably characterized by a deficiency of not only IG against the hydrophobic structure of the LPS molecule, but also of other IGs [8].

To date, there is no consensus on the mechanisms that underlie the polyreactive transformation of IGs. It is assumed that, in the intradomain structure of antigen-binding sites of γ-globulins, multispecific "ancestral" binding sites that contain leucine-rich repeats (LRRs) may be present [26]. In the framework of this hypothesis, the probability of the existence of "ancestral" binding sites with highly conserved structures of the LPS molecule should be extremely high, since the duration of the presence of blue-green algae, the main source of LPS in the environment, on our planet as is estimated to be billions of years [27]. Chaotropic reagents disrupt the ordered structure of hydrogen bonds in the aqueous environment of γ-globulin molecules, which is accompanied by a weakening of hydrophobic bonds between the VL and VH domains and domain structures of the L and N chains. This, in turn, favors the formation of metastable γ-globulin conformers, in which the "ancestral" multispecific binding sites translocate to the surface *Fab* fragments, determining the appearance of low affinity complementarity with respect to structurally unrelated antigens [26].

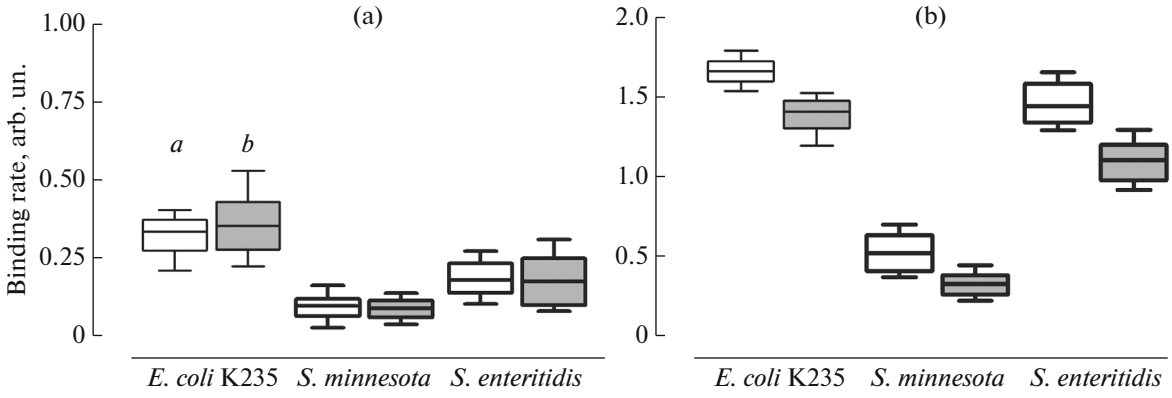

Fig. 2. The main statistical indicators of the interaction of (a) native immunoglobulins of the class G and (b) polyreactively transformed immunoglobulins with LPS of various enterobacteria for (*a*) healthy subjects and (*b*) patients with CTN. The box-and-whiskers diagram presents the average value, the upper and lower quartiles, the minimum and maximum values of the sample.

CONCLUSIONS

The study has demonstrated a fundamentally important fact: short-term exposure in vitro to a chaotropic reagent for native immunoglobulins of class G can significantly increase the activity of AEI and cause a decrease in the ability of effector cells to bind LPS, and, hence, reduce the LPS-dependent activation of these cells and the subsequent synthesis of inflammatory mediators. This can be extremely useful not only for treating septic patients, but also for preventing the progression of diseases of atherosclerotic nature, since the role of LPS as the most important causative factor in the induction of atherogenesis is difficult to overestimate [15, 28]. The mechanism of polyreactive transformation of native class G immunoglobulins under the influence of a chaotropic reagent remains unknown. Presumably, it can consist in the induction of intramolecular conformational rearrangements of native γ-globulins, as a result of which *Fab* fragments "ancestral" sites of low affinity multispecific binding appear, which determines the occurrence of complementarity to evolutionarily stable molecular structures of pathogens. Further study of the mechanisms of polyreactive transformation of native class G immunoglobulins and the search for nontoxic chaotropic agents that could potentially be used in clinical practice are the main goal of the upcoming studies.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare no apparent and potential conflicts of interest related to the publication of this article.

Statement of compliance with standards of research involving humans as subjects. All studies were conducted in accordance with the principles of biomedical ethics formulated in the Helsinki Declaration of 1964 and its subsequent amendments, and approved by the local bioethical committee of the Crimean Federal University named after Far Eastern Branch, Russian Academy of Sciences, Vernadsky (Simferopol). Each study participant submitted voluntary written informed consent, signed by him after explaining to him the potential risks and benefits, as well as the nature of the forthcoming study.

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