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Special Features of Immune Response to the Lethal Toxin of *Bacillus anthracis*

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Abstract—We and other authors have recently shown that the pattern of the immune response to components of anthrax, the *Bacillus anthracis* lethal toxin, is complex. In addition to the neutralizing antibodies, the antitoxin antibody pool contains antibodies enhancing the toxin lethal action. We mapped the epitopes in the protective antigen that are responsible for the induction of both antibody types. In this study, we obtained new data on the cytotoxicity of the *B. anthracis* lethal toxin toward the J774 A.1 cell line in the presence of monoclonal antibodies to various domains of the protective antigen and the lethal factor. The role of the Fc fragment of immunoglobulins in enhancing the lethal toxin action was shown. These results may serve as a basis for the development of a new generation vaccine for anthrax.

Key words: anthrax, epitope mapping, lethal factor, protective antigen, neutralizing and enhancing antibodies

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INTRODUCTION

The anthrax is extremely dangerous acute infectious disease afflicting animals and humans.[2] According to WHO, from 2 up to 20 thousand cases caused by anthrax are annually registered in the world. The anthrax causative factor, *Bacillus anthracis*, can survive in soil for more than 100 years as spores and form the resistant centers of infection, which create a constant danger of epidemics and epizootics. Moreover, the anthrax causative agent is one of probable pathogens that can be used for creation of a highly effective bacteriological weapon [1].

Means of specific preventive treatment of anthrax infections had been developed decades ago. However, they do not meet the requirements of modern medicine and epidemiology. The alive anthrax vaccine CTI used in Russia and CIS countries possesses an increased reactivity and keeps danger reversion [2]. The chemically adsorbed vaccine AVA used in United States and countries of Western Europe needs in frequent revaccinations and is insufficiently characterized in respect of content of antigenic components [3]. In addition, both those and other vaccines are insufficiently effective and have a number of side effects. They should be exchanged by more effective and safe vaccinal preparations with the completely characterized composition.

One of directions in the modification of means of specific preventive treatment of infectious diseases is the creation of chemical vaccines on the basis of protective antigens. As a basis for the development of the anthrax vaccines of a new generation might be one of the primary factors of pathogenicity of this microorganism, the exotoxin of *B. anthracis*. It consists of three polypeptides: a protective antigen (PA), the lethal factor (LF), and the edema factor (EF). After the processing of the precursor of the protective antigen PA83, activated PA63 (further it will simply be called PA) can bind to the receptors on the cell membrane, form heptamers composing a pore, and provide for the LF and EF translocation into endocellular compartments. EF is a Ca^{2+}-dependent adenylate cyclase, and LF, a highly specific zinc-dependent metalloprotease.

It is known that the most expressed protective effect at anthrax therapy is determined by the antibodies to PA [3]. In addition, a substantial protective effect can also be achieved by the use of LF as an antigen [4]. Thus, it is necessary to take into account the fact that, in addi-

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[2] Abbreviations: GST, glutathione S-transferase; MAb, monoclonal antibody; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; anthrax, anthrax; EF, edema factor; LF, lethal factor; LFn, lethal factor and its N-terminal domain; and PA, protective antigen.

Properties of monoclonal antibodies to PA and LF determined in this and previous [6] studies

Antigen used for the animal immunization	MAb	Immunoglobulin isotype	Specificity	Action on lethal toxin in vitro	
				neutralization	enhancement
PA	1D6	IgG1	III domain PA	–	+
	1E10	″	IV domain PA	+	–
	4C2	″	Full-size PA	–	–
	4D2	″	Full-size PA	–	–
	4E2	″	Full-size PA	–	–
	5G2	″	Full-size. PA	–	–
PA-LFn	6G7	IgG2b	IV domain PA	+	–
	6G8	IgG2b	IV domain PA	+	–
	1F2	IgG2a	III domain PA	–	+
	6G9	IgG2b	I omain LF	+	–
	10E9	IgG2b	The same	+	–
	8D5	IgG1	″	+	–
	8D2	IgG1	″	+	–
LF	3B4	IgG1	″	+	–
	2D3	Not determined	″	–	–
	1C5	″	″	–	–
	2B10	″	″	–	–
	4B4	″	″	–	–
	5C9	″	″	+	–

tion to the neutralizing effect, antibodies to separate toxin components, namely to PA, can enhance the toxin action, by analogy to the phenomenon of the antibody-dependent potentiation of viral infections [5]. In this connection, it is necessary to carry out a thorough epitope mapping of PA to reveal the sites responsible for the induction of both neutralizing and unwanted antibodies that enhance the toxin action.

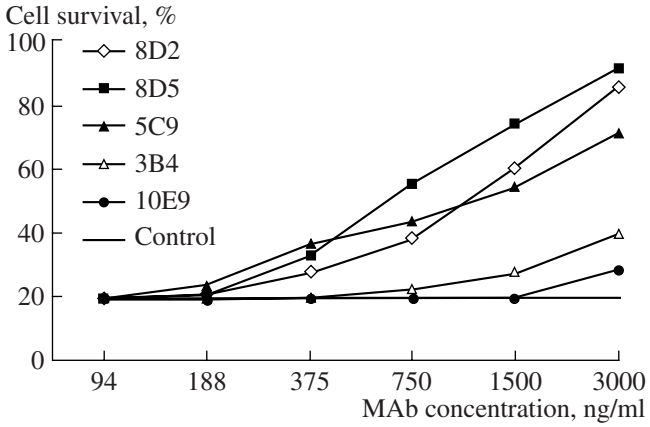

Fig. 1. Effect of lethal toxin of *B. anthracis* on the J774 A.1 line cells in the presence of MAb to LF. The concentration of lethal toxin 0.3 LD_{50}.

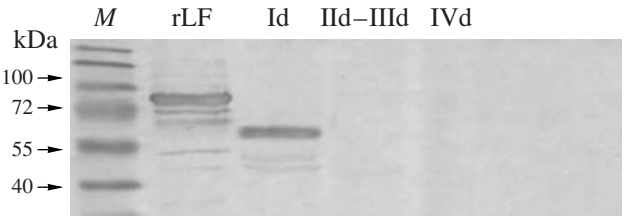

Fig. 2. Western blot of the total protein preparation from the strains of *E. coli* producing recombinant LF and its domains stained by MAb 5C9 to LF. *M*, molecular mass markers; rLF, recombinant lethal factor; Id–IVd, domains I–IV of the lethal factor.

The task of revealing epitopes of the anthrax toxin responsible for the induction of "neutralizing" and "potentiation" antibodies can be successfully solved with the help of MAbs. We have earlier obtain a MAbs potentiating the anthrax toxin action in vitro and carried out for the first time the mapping of the PA epitope that cooperates with the potentiating antibody [6].

In this work, we expanded the panel of hybridomas producing MAb to PA and LF. The activity of the obtained antibodies was investigated toward the lethal toxin in vitro, and their domain specificity was determined. The PA epitopes cooperating with potentiating and neutralizing MAbs were mapped.

RESULTS AND DISCUSSION

Obtaining and Characterization of Monoclonal Antibodies to Components of Anthrax Toxin

Two groups of mice were immunized with the corresponding full-size recombinant antigens, produced in *E. coli* to obtain MAbs to PA and LF. As a result of hybridization of the splenocites of hyperimmune mice and cells of the myeloma line X63-Ag8.653, 16 MAbs to *B. anthracis* PA and LF lethal toxin were obtained.

Taking into account the above-described antibodies to PA [6], the panel of 19 hybridoma clones steadily producing antibodies to PA and LF (table) was in total obtained and characterized.

None of the resulting MAbs possesses a cross specificity to both antigens. The majority of antibodies belonged to the subclass IgG1; four, to subclass IgG2b and one, to subclass IgG2a. It is interesting that antibodies of subclasses IgG2b and IgG2a were obtained only as a result of immunization by the oligomeric complex PA63 with *N*-terminal domain LF (LFn).

The screening of MAbs for the effect of neutralization or potentiation of action of LF on a cell line J774 A.1 in MTT test showed that 11 of 19 obtained MAbs possess the specific activity. The choice of cell line was dictated by that the cells of the macrophage J774 A.1 line are sensitive to the LF action, which enables the experiments on cytotoxicity.

Two earlier characterized antibodies to PA (6G8, 6G7) and one newly obtained antibody to PA (1E10) effectively protected cells from the toxin action. The complete toxin neutralization was achieved in all cases at equimolar concentration of antigen and antibody. Two antibodies (one of which was described earlier (1F2), and another obtained during immunization of

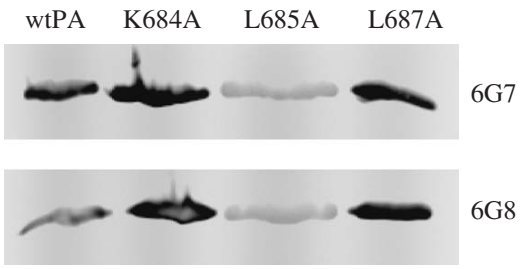

Fig. 3. Interaction of neutralizing antibodies 6G7 and 6G8 with mutant forms of IV PA domain. Above the lanes, the mutation type is shown. wtPA, native domain.

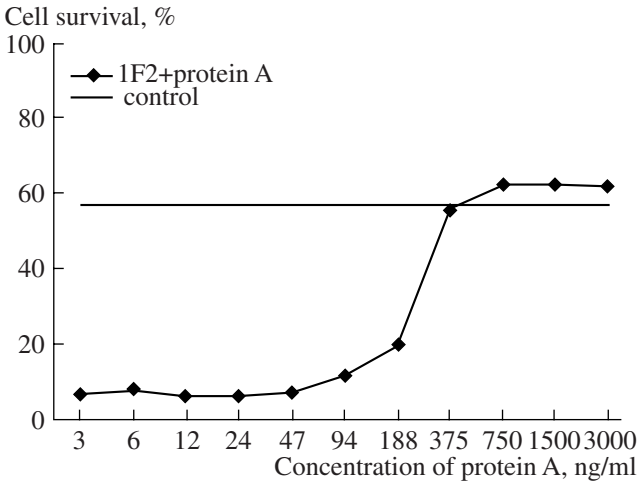

Fig. 4. Change in the potentiating effect of MAb 1F2 in the system lethal toxin–J774 A.1 line cells upon the blockage of Fc fragment by protein A.

mice with recombinant PA in this research, 1D6) potentiated the toxin action on the J774 A.1 cells.

Antibodies to LF as a whole turned out to be less effective in neutralization of toxic action on the cells. Four of ten MAbs did not affect the cytotoxicity, the others more or less effectively neutralized action of the lethal toxin. The most expressed neutralizing effect demonstrated MAbs 8D2 and 8D5; they blocked the action of toxin at concentration of 3 μg/ml by 90% (Fig. 1). No antibodies possessing a potentiating effect were revealed.

Epitope Mapping of Monoclonal Antibodies to PA and LF

The domains PA and LF are separate subunits with the expressed structural and functional organization [7]. Since the functional roles of domains PA and LF are substantially known [7], it is of interest to correlate the neutralizing and potentiating effects of antibodies with their specificity to this or that fragment of PA or LF. We constructed the panel of domains PA and LF in the form of solid proteins with GST. The binding of antibodies with the PA and LF domains was analyzed by Western blotting.

All the antibodies to LF with no dependence of the type of antigen used at immunization (full-size LF or LFn) were specific to the first LF domain (see, e.g., Fig. 2).

The neutralizing antibodies to PA (6G7, 6G8, and 1E10) were specific to IV domain, while the epitopes of potentiating antibodies (1F2 and 1D6) were located in III domain. The antibodies that do not influence the activity of lethal toxin in the MTT test cooperated in immunoblot only with full-size PA.

A more comprehensive epitope mapping within the framework of the domain IV PA was carried out for antibodies 6G7 and 6G8. It was revealed that both antibodies recognize on PA the conformationally dependent epitope between the amino acid residues 631 and 735. Any subsequent variants of reduction of the epitope length led to a loss of the antibody binding with

fragment PA. It is known [8] that the comprehensively characterized neutralized antibody 14B7 also recognizes the conformational epitope in this area of PA (amino acid residues 671–721). The residues K684, L685, and L687 were established to be critical for the 14B7 binding to PA.

Variants of the domain IV PA that contained the earlier described replacements K684A, L685A, and L687A [8] blocking the PA binding to 14B7 were obtained for a comparison of two epitopes. The Western blotting of the mutant variants of the domain IV with antibodies 6G7 and 6G8 showed that the replacements K684A and L687A do not affect the antibody affinity for the domain IV PA, while the L685A replacement causes a reduction of the affinity of both antibodies for PA (Fig. 3). Thus, the epitopes for the 14B7 antibody and the 6G7 and 6G8 antibodies are superimposed but not coincide.

At the same time, the amino acid residues D683, D685, and Y688 located within the borders of the epitope 6G7 and 6G8 are critical for the interaction of PA and its cellular receptor [8]. The designed ternary mutant D683A, D685A, and Y688A of the domain IV PA did not interact with antibodies 6G7 and 6G8 in the Western blotting. The results indicated that the epitopes recognized by the antibodies 6G7 and 6G8 and by the 14B7 antibody differ, but are partially superimposed, while the recognition site for antibodies 6G7 and 6G8 substantially coincides with the site of PA interaction with its cell receptor. It is remarkable that the D683A, D685A, and Y688A mutations only insignificantly influence the ability of the antibody 14B7 to recognize the IV PA domain [8].

The mapping of the epitope recognized by the potentiating antibody 1F2 revealed a fragment of the domain III PA (amino acid residues 520–590) as minimally necessary for the recognition by this antibody. The epitope recognized by the 1D6 antibody was also localized in the third PA domain. A comprehensive mapping of this epitope did not carried out; however, preliminary experiments on the competitive binding of these antibodies (additivity coefficient of 35%) indicated the enough closely located but, nevertheless, different recognition sites.

Study of the Role of Fc MAb Fragment in Potentiating the Lethal Toxin Toxic Effect on Macrophagelike Cell Line J774.A.1

The potentiating mechanism was analyzed on the basis of hypothesis that the Fc fragment participates in increased efficiency of antibody binding of the toxin with cell membrane [5, 9]. According to this hypothesis, such bacterial antigens as staphylococcal protein A, can block the binding of an antibody with the cellular Fc receptor through the binding to the constant part of heavy chain of antibody (Fc fragment). Thus, the addition of protein A to the system potentiating antibody–lethal toxin–J774 A.l cells (the cell line contains receptor Fc γ RI [10]) should result in neutralization of the potentiating effect.

To test this hypothesis, a cytotoxic test with the use of MAb lF2 preliminarily preincubated with various concentrations of protein A was carried. The experiments confirmed that the blocking of Fc fragment of antibodies by protein A reduces the potentiating effect down to its full disappearance (Fig. 4).

Modern data determine three areas within the PA structure, antibodies to which can neutralize the anthrax action. Antibodies to domain Ib prevent the LF binding to PA63, antibodies to the domain IV block the PA binding to cellular receptors [8], and the antibodies to domain II interfere with PA processing [11]. The results confirm the presence of PA immunodominant neutralizing epitopes in the structure of the domain IV, which are overlapped with area of PA binding to a cellular receptor. In this connection, it is of interest to study the efficiency of the protective immune response upon the immunization of animals by the isolated domain IV PA, since the literature data on the efficiency of the domain IV PA as antigen are rather contradictory [12, 13].

Both characterized potentiating antibodies are specific to the same PA fragment, the central part of the domain III. It was shown that certain sites of the third PA domain are responsible for the oligomerization of toxin (from 510 to 518 and from 590 to 600 amino acids) [14]. The functions of other motifs in the domain III remain now unknown. The epitope for the potentiating antibodies we described is just located in the area 520 to 590 aa and is not included in the motifs participation in oligomerization. Therefore, it is supposed that this PA region is simply useful for the anchoring of antibodies in order to its contact with Fc receptor could promote an additional binding of toxin to a specific membrane receptor and not prevent the processing and assembly of toxin. This fragment of domain III is a candidate for its exclusion from the structure of the recombinant vaccine. The modification of the epitope that does not exert a substantial effect on the general of antigenic structure of PA requires a high resolution mapping of the potentiating epitope and the subsequent mutagenesis of separate amino acid residues responsible for the recognition by the potentiating antibody.

We carried out an experiment on blocking the potentiation effect by protein A and showed that it is the spatial orientation of toxin relative the cell membrane that is first of all responsible for the effect of potentiation. Really, the ability to contact Fc receptors is inherent in many antibodies; however, only antibodies to the domain III of PA exerted a potentiating action. Our data suggest that, first, a rather limited quantity of PA sites can be responsible for the development of the undesirable immune response and, second, the revealing of such sites is a practically feasible task.

It follows from the table that the immunization by the PA63–LFn complex leads to a more effective immune response than the immunization by separate toxin components. In this case, the character of immune response (the presence of antibodies with isotypes Ig2) indicates a deeper clonal selection upon the immunization by PA63–LFn in comparison with the immunization by separate toxin components. The mechanism "internal" adjuvant effect of immunization by the PA63–LFn complex demands a further study. It is necessary to note that the effect of molecular adjuvant is also described for the edema factor, the third component of the toxin of anthrax causative factor [15]. It is probable that a combination of various toxin fragments could considerably increase the efficiency of the recombinant anthrax vaccine.

Thus, during our work, we obtained a new information on antigenic structure of lethal toxin *B. anthracis* and about localization concrete antigenic epitopes PA and LF responsible for the formation of neutralizing and potentiating antibodies.

EXPERIMENTAL

Genetic engineering constructs. Full-size genes of PA and LF, and also their fragments were obtained by the PCR method, using as a matrix the total DNA of the CTI-1 strain. The obtained PCR fragments were processed by the corresponding restriction nucleases and ligated into a pET22b-gst-cmyc vector [6] in the case of gene fragments of or into a pET22b-His6 vector for the full-size PA and LF. Thus, all the PA and LF domains were produced as chymerical proteins containing a GST polypeptide at the *N*-end, whereas the full-size proteins bore a hexahistidine sequence at the *N*-end. Point mutations were introduced into the PA gene with the help inverse PCR of a vector pET22b-His_6-PA from the primers containing the corresponding replacements.

Isolation and purifitaion of recombinant PA and LF proteins and their fragments. Recombinant proteins were produced in a *E. coli* BL21DE3 strain and purified according to the protocols suggested by the manufacturer of family of vectors pET, bearing a hexahistidine sequence (Novagen). A metal-chelating sorbent Talon (Clontech) was used. The purification of fragments PA and LF for epitope mapping was carried out on a column with glutathion–Sepharose according to the procedure of manufacturer (Amersham).

Obtaining of monoclonal antibodies to $(PA63)_7$:LFn, pPA and pLF. Mice of line BALB/c were immunized by a $(PA63)_7$:LFn complex, pPA, or pLF by a scheme: 1st day, 50 μg of an antigen in PBS, emulsified in full Freund's adjuvant, hypodermically; 21st day, 50 μg of an antigen in PBS, emulsified in incomplete Freund's adjuvant, hypodermically; 51st day, 50 μg of an antigen in PBS, intravenously; and 58th day, 50 μg of an antigen in PBS, intravenously. Hybridization was carried out by a standard procedure [16].

Immunoblotting and EIA. SDS-PAGE in 7.0–10.5% gel was carried out by the method of Laemmli. Electrophoretic carrying of proteins onto a membrane (Hybond-C, Amersham) was carried out on Mini Trans-Blot equipment of firm BioRad according to the manufacturer's protocol. IEA was carried out by a standard procedure [17]. Intensity of staining was analyzed at a wavelength of 492 nm (photometer Pikon, Russia).

The isolation of monoclonal antibodies from ascite fluid was carried out on column Protein G Sepharose according to the manufacturer's recommendation (Amersham). Antibodies isotypes were determined using a set of manufacturer's Roche Diagnostic Corporation (United States).

Analysis of neutralizing and potentiating activity of antibodies in vitro was carried out in a standard MTT test according to the technique of Tada [18] on a Uniplan spectrophotometer (Pikon, Russia). MAbs in various initial concentrations (from 94 to 3000 ng/ml) were incubated with a mixture of PA and LF (3000 ng/ml of each component) for 1 h at 37°C and then added to cells of a mouse line J774 A.1 and incubated for 18 h. The percent of the survived cells was determined after the end of incubation by the conversion of MTT to formazan. The optical absorption was measured at wavelength of 595 nm using a Pikon photometer.

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