

Determination of Tetanus Toxin and Toxoid by ELISA Using Monoclonal Antibodies

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Abstract—The procedure for obtaining monoclonal antibodies TT-1, TT-2, and TT-3 against tetanus toxin/toxoid is described. It is shown that the commercial DTP vaccine and tetanus toxoid conjugated with a low-molecular-weight hapten can both be used as immunogens. Monoclonal antibodies TT-1 and TT-2 neutralized tetanus toxin in vivo. The monoclonal antibodies obtained were used to design and compare several schemes of quantitative determination of tetanus toxoid and toxin by ELISA. A more sensitive competitive ELISA allowed the detection of as much as 0.01 EC/ml toxoid and 50 LD₅₀/ml toxin.

Tetanus toxin, along with botulinal toxin, is one of the most toxic substances. It is produced under anaerobic conditions by *Clostridium tetani* in the form of a single-chain polypeptide with a molecular weight of 150 kDa. The toxin isolated from culture medium under laboratory conditions consists of two subunits, A (50 kDa) and BC (100 kDa), or the light (L) and heavy (H) chains, linked through a disulfide bond. The spores of the pathogen that get through injured skin into tissues are transformed to bacilli, which synthesize the toxin into the surrounding necrotized tissue. After binding the fragment C of the toxin (an approximately 50-kDa C-terminal moiety of the heavy chain) by the ganglioside-containing receptor of nerve termini, the toxin undergoes endocytosis followed by its retrograde distribution over axons and accumulation in the central nervous system. The fragment A, an enzymatically active moiety of the toxin, represents a zinc-dependent protease that cleaves synaptobrevin, thus blocking the secretion of inhibitory neurotransmitters (glycine and GABA) into the synaptic gap [1]. Such disturbance in the conduction of nerve impulse results in the development of spastic paralysis, characteristic of tetanus.

Immunity to tetanus is determined solely by the antibodies against the toxin. A lethal dose of tetanus toxin, calculated for the human, is lesser than 2.5 ng/kg. Concentrations that low are insufficient for the induction of the synthesis of antibodies. For this reason, immunization as a way of both prophylaxis and treatment of patients with clinical symptoms of the disease is the only way to fight against tetanus. The effectiveness of immunization largely depends on the quality of preparations of tetanus toxoid.

The protective properties of one or another fragment of the toxin have been long studied. There is no consensus of opinion regarding the question as to which moi-

ety or fragment of the toxin molecule primarily induces the synthesis of protective (neutralizing) antibodies and determines the development of protective immunity. Some researchers sought to reveal the protective epitopes by determining the protective activity of monoclonal antibodies generated against different parts of the toxin molecule. Table 1 summarizes the data published in some articles devoted to this problem.

Note that, despite the differences in the techniques used to obtain hybridomas, immunogens (tetanus toxoid, tetanus toxin fragments, or complexes tetanus toxin–antibody), and animal species, all these antibodies neutralized TT in in vivo test, displaying specificity for A-, B-, or C-fragments of the toxin. Part of the protective monoclonal antibodies reacted with the epitopes expressed on A- and B-fragments and A- and C-fragments as well. In addition, some authors reported the protective effect of mixtures of monoclonal antibodies that did not exhibit protective properties when used separately [2, 4, 8, 12, 13].

Thus, it does not seem possible to draw a correlation between the toxin-neutralizing activity of antibodies and the specificity for a certain part of the tetanus toxin molecule.

The key moment in manufacturing immune biological preparations is the control and standardization of their qualitative and quantitative parameters. It should be mentioned that toxins and toxin-derived toxoids are characterized in in vitro and in vivo tests, which reflect their different characteristics that do not correlate unambiguously with one another. In particular, the activity of native toxins is assessed in experiments with animals and is expressed in 50% lethal doses (LD₅₀). The properties of toxoids, which are also assessed in experiments with animals, are expressed in binding

Table 1. Specificity of monoclonal antibodies neutralizing tetanus toxin *in vivo*

Origin of antibodies	Specificity of antibodies for tetanus toxin fragments						
	A	B	A, B	A, C	C	NH ₂ -HC	COOH-HC
Human	1 [7] 5 [12]	1 [4] 1 [6] 3 [7]		1 [12]	2 [3] 1 [4] 1 [8]	1 [2]	
Mouse	1 [8] 1 [10]	1 [10]	4 [5] 2 [8]		3 [5] 1 [10] 1 [11]	6 [8]	4 [8]
Rat					4 [9]		
In total	8	6	6	1	13	7	4

units (BUs), which reflect the interaction with the standard antitoxic serum in the presence of the toxin. The very procedure of testing preparations in animals is fairly long-term (4 days), which does not allow the process of accumulation of the product in the course of culturing the producer to be monitored promptly. One of the *in vitro* tests that are frequently used for characterizing the antigenic properties of toxin and toxoid is the reaction of flocculation, with the activity of the preparations being expressed in Lf units. These tests do not provide information on the purity of preparations and the amount of contaminants and ballast components (antigens of microbial cells, protein components of culture medium, etc.) [14, 15].

An alternative method of tetanus toxin and tetanus toxoid quantification is an enzyme immunoassay (ELISA). Sandwich ELISA based on monoclonal [16] and polyclonal [17] antibodies have been proposed.

The goal of this work was to obtain monoclonal antibodies against tetanus toxin/toxoid, to study their immunochemical and effector properties, and to compare several schemes of quantitative determination of toxin/toxoid by ELISA using the monoclonal antibodies obtained.

MATERIALS AND METHODS

In the study, we used RPML-1640 growth medium (R 8005), calf fetal serum (F 2442), hypoxanthine–aminopterin–thymidine (an additive to growth medium; H 0262), *o*-phenylene diamine (P 1526), glutamine (G 9273), and Streptavidin peroxidase conjugate (S 5512) from Sigma (the United States); rabbit antiisotypic sera from Bio-Rad (the United States); Acel-Imune acellular DTP vaccine from Lederle Laboratories Division, American Cyanamid Company (the United States); aluminum hydroxide gel Alu-Gel-S (12261), polyethylene glycol 1550 (33132), and dimethyl sulfoxide (20385) from Serva (Germany); biotin *N*-hydroxysuccinimide ester (732494), and biotin aminocapronate (1008960) from Roche (Germany); rabbit antiserum against mouse IgG, donkey antiserum against rabbit immuno-

globulins, horseradish peroxidase (EC 1.11.1.7), BSA, and gelatin were manufactured in Russia. The preparations of tetanus toxin and toxoid, manufactured at the State Unitary Enterprise “Research and Production Association Immunopreparat” (Ufa, Russia), were calibrated and provided by Tarasevich State Research Institute for Standardization and Control of Medical Biological Preparations.

Antibodies were obtained in a series of experiments on designing a panel of monoclonal antibodies against the antigens: (1) Acel-Imune acellular DTP vaccine, containing 15 Lf/ml tetanus toxin, 10 Lf/ml tetanus toxoid, 6.5 µg/ml pertussis toxoid, 70 µg/ml filamentous hemagglutinin, 3.2 µg/ml pertactin, and 1.6 µg/ml agglutinin 2 of *Bordetella pertussis*, and (2) tetanus toxoid, conjugated as a carrier to a low-molecular-weight hapten (fumonisin B₁).

Acel-Imune vaccine was injected intraperitoneally to BALB/c mice at an age of 1 and 3 weeks (0.5 ml) and 7 weeks (0.25 ml). Three days after the last immunization, splenocytes were hybridized with P3-X63-Ag8.653 myeloma cells.

The immunization of mice with the conjugated tetanus toxoid was conducted for seven to ten months. The conjugate dose was gradually decreased from 100–150 µg to 5–1 µg by the end of the course, whereas time intervals between injections increased from two weeks to two months. At the first injection, the immunogen (emulsified in 0.5 ml of complete Freund's adjuvant) was injected subcutaneously in several points on the back. At subsequent immunization procedures, aqueous solutions of the conjugate were injected intraperitoneally. On day 4 after the last injection, splenocytes were fused with P3-X63-Ag8.653 myeloma cells, as described earlier [18].

Culture liquids were tested and immunochemical properties of monoclonal antibodies were determined and studied by ELISA in 96-well plates (Dynatech, Germany). Plates were sensitized with solutions of the antigen and antibodies, prepared in 0.05 M carbonate–bicarbonate buffer (pH 9.5), by incubating them at 4°C for 16 h. Wells were washed three to five times with

0.15 M phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20 (PBS-T). The solutions of the antigen, antibodies, and enzyme conjugates were prepared in PBS-T containing 1% BSA. Incubation with immunologic reagents was conducted at 37°C for 1 h. At the last stage of analysis, the mixture of substrates containing 0.4 mg/ml *o*-phenylene diamine in phosphate-citrate buffer (pH 5.0) supplemented with 0.005% hydrogen peroxide was used. After 45 min of incubation, the reaction was stopped by the addition of 50 µl of 4M H₂SO₄ into each well. The optical density was measured at 492 nm on a Dynatech MR 5000 photometer (Germany).

During primary selection of hybrid cultures, their ability to produce antibodies against tetanus toxoid was assessed. For this purpose, wells of plates were filled with toxoid solution (0.2 BU/ml). After incubation, plates were washed and filled with culture medium of growing hybridomas at corresponding dilutions. After washing, rabbit antibodies against mouse IgG, conjugated with horseradish peroxidase, were added. Analysis was finished as described above. The intensity of the synthesis of antibodies was evaluated by titer values. The maximum dilution of antibodies, at which the absorbance values were greater than the baseline values by 0.2 units of optical density, was taken as a titer. Subsequent selection of cultures was performed based on the results of the interaction between monoclonal antibodies and tetanus toxin in ELISA.

The most active producers were cloned by the method of limiting dilutions, accumulated, and stored at -90°C.

Monoclonal antibodies, obtained from culture liquids by precipitation with ammonium sulfate at 50% saturation or in the form of ascites, were purified on protein G agarose. Preparations of antibodies were loaded on a column with sorbent equilibrated in 0.9% NaCl (pH 7.2) and eluted with a similar buffer supplemented with 0.025 M citric acid (pH 2.0). The eluate was neutralized with 3 M NaOH, concentrated using PEG 40000, and dialyzed against PBS (pH 7.5).

Immunoglobulin isotypes were determined by ELISA using rabbit antiisotypic sera (Bio-Rad) and antibodies against rabbit IgG conjugated with horseradish peroxidase.

The effector properties of antibodies were assessed in the test of toxin neutralization in mice. Preparations of monoclonal antibodies in serial dilutions were mixed with tetanus toxin (LD_{50/100}, the dose of the toxin that causes 50% death of animals when injected in mice together with 0.01 UI of standard antitetanus serum), incubated at 37°C for 1 h, and injected in mice subcutaneously, at the inner side of the hind leg. Antitetanus serum with an activity of 0.1 IU/ml served as a reference preparation. The animals were watched for 4 days. The antibodies that prevented animal's death within this time interval were regarded as protective.

Monoclonal antibodies were conjugated with horseradish peroxidase by periodate method, as described

earlier [19]. Briefly, horseradish peroxidase (2 mg; EC 1.11.1.7; Olaine, Latvia) was diluted in H₂O (0.5 ml), mixed with freshly prepared 0.2 M NaIO₄ (0.1 ml), and stirred for 20 min at room temperature. After dialyzing the mixture against 1 mM acetate buffer (pH 4.5) at 4°C overnight, its pH was adjusted to 9.5 with Na₂CO₃. Antibodies (IgG, 4 mg) were dissolved in 0.01 M carbonate-bicarbonate buffer (pH 9.5), containing 0.13 M NaCl, and mixed with the dialysate immediately after the pH adjustment. After incubation of the mixture for 2 h under stirring, NaBH₄ (4 mg/ml, 50 µl) was added, and the resulting solution was incubated at 4°C for another 2 h under periodic stirring. The conjugate thus obtained was dialyzed against PBS (pH 7.2), taken in excess of 2000- to 5000-fold. The conjugate was stabilized by addition of BSA to a final concentration of 5 mg/ml, mixed with an equal volume of glycerol, and stored at -20°C.

The above procedure was used to obtain peroxidase conjugates of antibodies against mouse IgG, human IgG, and rabbit IgG.

To perform biotinylation of monoclonal antibodies, a solution of affinity-isolated antibodies in PBS (pH 7.5-8.0) was mixed either with biotin *N*-hydroxysuccinimide ester or biotin aminocaproate in dimethyl formamide (which were taken in a tenfold molar excess), stirred at room temperature for 2 h, and dialyzed against PBS (pH 7.2). The resulting solutions of conjugates (MAB-B and MAB-C7-B, respectively) were mixed with equal volumes of glycerol and stored at -20°C.

Biotinylated antibodies were used to reveal differences in the specificity of monoclonal antibodies. This test was performed both in competitive and sandwich ELISA.

The ability of monoclonal antibodies to compete with biotinylated monoclonal antibodies for binding with solid-phase tetanus toxoid or to block the binding sites for biotinylated monoclonal antibodies (after forming a complex with tetanus toxoid) was indicative of their equal or similar epitopic specificity.

Similar principles of analysis were used for quantitative determination of tetanus toxin and toxoid.

To determine optimal parameters, we chose the ratios between the antigen and antibodies that gave optical density values ranging from 0.8 to 1.2. The optimal parameters were then used in competitive ELISA. For this purpose, 100 µl of tetanus toxoid (0.03-10 BU/ml) or tetanus toxin (10-3000 LD₅₀/ml) in PBS-T was mixed in wells with 100 µl of antibodies (monoclonal antibodies, biotinylated monoclonal antibodies, MAB-C7-B, or antitetanus human gamma globulin). Bound antibodies were detected using the conjugates of horseradish peroxidase with monoclonal antibodies, streptavidin, or antihuman IgG.

The level of binding of antibodies in wells containing no toxin or toxoid was taken as 100%. The percentage of binding of antibodies was calculated for each

concentration of the toxin or toxoid, and the dependencies were plotted using the Microsoft Excel 97 software. The concentration of tetanus toxin (or toxoid) that causes a 20% inhibition of binding (reliably differing from 100% control) was taken as the analysis sensitivity (or limit of determination).

To select the concentration of antibodies to be used in sandwich ELISA during the sensitization of the plates, monoclonal antibodies at serial dilutions (1/10–1/10000) were added into wells. Tetanus toxoid, bound by these antibodies, reacted with antitetanus human gamma globulin. The reaction was stopped by incubation with antihuman IgG conjugated with horseradish peroxidase. The dilution of monoclonal antibodies that caused maximal staining of the substrate mixture was regarded as optimal and used in further experiments for sensitizing wells of plates. Solutions (100 μ l) of tetanus toxoid (0.003–30 BU/ml) or tetanus toxin (10–3000 LD₅₀/ml) in PBS -T were added to the wells sensitized with monoclonal antibodies. Bound antigens were detected using monoclonal antibodies, biotinylated monoclonal antibodies, MAB-C7-B, or antitetanus human gamma globulin, and the corresponding peroxidase conjugates.

For each variant of the analysis, the dependences of the optical density of the reaction products on tetanus toxin/toxoid concentration were plotted. The minimal amount of antigen, the level of reaction with which was greater than the baseline level by 0.2 units, was determined.

RESULTS AND DISCUSSION

The final goal of this study was to design an immunological test suitable both for a prompt diagnostics of the accumulation of tetanus toxin during producer culturing and for assessment of the quantitative and qualitative parameters of the toxoids obtained. In view of this, out of the entire panel of antitoxoid monoclonal antibodies we selected those that interacted with native tetanus toxin. The test of neutralization in mice served as an unambiguous confirmation of the fact that the selected antibodies interact with the toxin rather than with the contaminants contained in preparations of tetanus toxoid and toxin or with new conformational epitopes formed during detoxification of the toxin with formalin. In terms of the final goal of the study, it was not important whether the protective antibodies showed specificity for the A-, B-, or C-fragment of the toxin. Monoclonal antibodies TT-1 were obtained as a result of immunization of BALB/c mice with the vaccine Acel-Imune, whereas TT2- and TT-3 antibodies were produced in response to injecting the mice with a low-molecular weight compound conjugated with tetanus toxoid (which served as a protein carrier). Monoclonal antibodies TT1 and TT2 were classified with immunoglobulin class G1; TT3, with IgM. All hybridomas displayed the ability to produce ascites, and culturing the hybridomas in mice ensured accumulation of antibodies over several passages. The titer of antibodies in

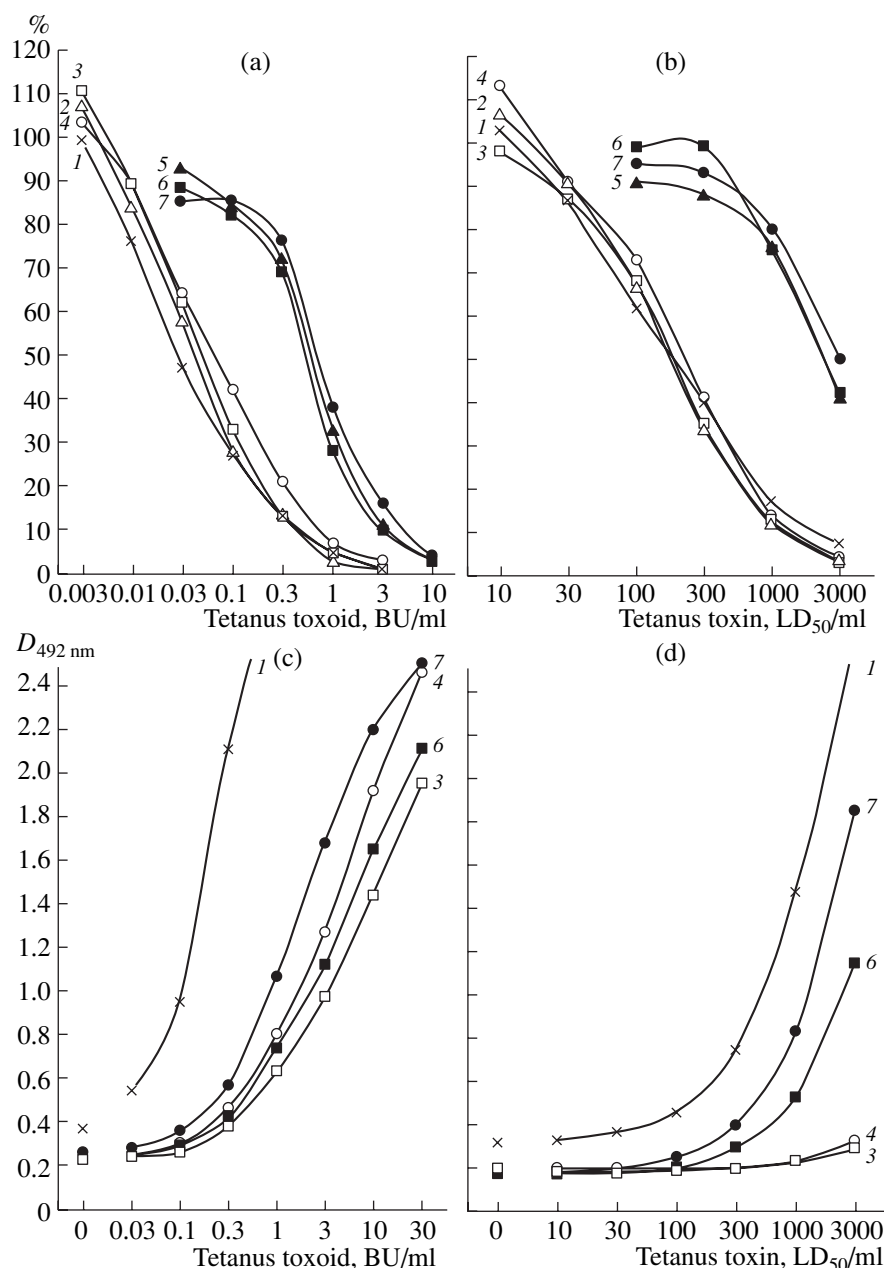
ascites preparations reached 2×10^4 in the case of TT-3 and 10^6 in the case of TT-1 and TT-2. In vivo tests in outbred mice showed that monoclonal antibodies TT-1 and TT-2 neutralized the lethal effect of tetanus toxin.

To synthesize conjugates of monoclonal antibodies with biotin and an aminocaproate derivative of biotin, isotype IgG1 antibodies TT-1 and TT-2 were used. Cross-reactivity was detected in competitive ELISA solely between antibodies and homologous biotinylated conjugates (MAB-B and MAB-C7-B). Thus all three monoclonal antibodies interacted with different determinants of the toxin. These data were also confirmed in sandwich ELISA. Solid-phase monoclonal antibodies blocked binding sites on toxin molecule, thereby preventing the interaction between the conjugates obtained using the same antibodies (unlike heterologous conjugates). This finding also indicates that the epitopes in the structure of the antigen, against which monoclonal antibodies TT-1 and TT-2 were generated, were singular.

To design an analytic system for quantitative determination of tetanus toxin and toxoid, we considered two schemes of analysis—competitive and sandwich ELISA with the use of monoclonal antibodies obtained and commercial preparation of antitetanus human gamma globulin—and performed a comparative analysis of them.

Competitive ELISA. Monoclonal antibodies TT-3 were unsuitable for this type of analysis because of the absence of inhibition of binding of these IgM antibodies by free antigen even at high concentrations (10 BU/ml tetanus toxoid). For this reason, antitetanus human gamma globulin, monoclonal antibodies TT1 and TT-2, and biotinylated conjugates of these antibodies were compared (Figs. 1a, 1b). As seen from the diagrams, neither MAB-B nor MAB-C7-B possessed marked advantages over the nonconjugated antibodies; they had close sensitivity both for tetanus toxin and toxoid (Table 2). In all cases, ELISA based on the use of monoclonal antibodies TT-1 and antitetanus human gamma globulin was more sensitive by more than an order of magnitude than the variant based on the use of TT-2. Thus, this approach allowed approximately 0.01 BU/ml tetanus toxoid and as much as 50 LD₅₀/ml tetanus toxin to be detected.

Sandwich ELISA. The selection of combination of antibodies, used for sensitizing the plates, and biotinylated monoclonal antibodies for detecting bound antigen is a key moment in designing this type of analysis. Our studies showed that, irrespective of the type of biotin-labeled solid-phase antibodies, monoclonal antibodies TT-3 gave the best results. Thus, the main difference between the variants of this group of analyses consisted in the second antibodies (Figs. 1 c, 1d). As expected, antitetanus human gamma globulin allowed the detection of antigens with a higher sensitivity than the reagents based on monoclonal antibodies. The polyclonal nature of antibodies assumes the possibility of multisite interactions with the toxin molecule, which is



Titration of (a, c) tetanus toxoid and (b, d) tetanus toxin in (a, b) competitive and (c, d) sandwich ELISA. Designations: (1) antitetanus human gamma globulin; (2) TT-1; (3) TT-1-B; (4) TT-1-C7-B; (5) TT-2; (6) TT-2-B; (7) TT-2-C7-B.

expressed in a more intensive reaction. In addition, the sensitivity of detection of tetanus toxoid (0.04 BU/ml) and toxin (150 LD₅₀/ml) with polyclonal antibodies was greater by at least a factor of 5 and 2, respectively, than in the case of the conjugates of monoclonal antibodies TT-1 and TT-2 (Table 2). In addition, the advantage in the sensitivity in comparison to monoclonal antibodies is more pronounced when tetanus toxoid is determined. This is due to the fact that antitetanus human gamma globulins contain antibodies against tetanus toxoid, which do not react with tetanus toxin, because they are generated against the epitopes

occurred in the structure of native toxin as a result of its detoxification with formalin. Conversely, monoclonal antibodies were selected by their ability to bind the toxin and neutralize it (i.e., they showed specificity to the structures that were not modified as a result of detoxification of the toxin).

Note that the detection of the toxoid using the conjugates of both monoclonal antibodies was almost equally efficient. The sensitivities of these analyses differed only slightly (within 0.2–0.5 BU/ml). However, in this variant of analysis, the conjugates of monoclonal antibodies TT-1 lacked their ability to interact with the

Table 2. Sensitivity of quantitative determination of tetanus toxoid and toxin using competitive and sandwich ELISA

Scheme of analysis	Competitive ELISA		Sandwich ELISA	
	Tetanus toxoid, BU/ml	Tetanus toxin, LD ₅₀ /ml	Tetanus toxoid, BU/ml	Tetanus toxin, LD ₅₀ /ml
Antitetanus human gamma globulin	0.008	43	0.04	150
TT-1	0.016	67	–	–
TT-1-B	0.015	51	0.48	>3000
TT-1-C7-B	0.013	57	0.3	>3000
TT-2	0.26	1000	–	–
TT-2-B	0.14	820	0.43	640
TT-2-C7-B	0.17	820	0.22	300

native toxin. It can be assumed that this phenomenon was due to a certain orientation of the toxin on the TT-3 antibodies, when the epitopes recognized by the TT-1 antibodies become inaccessible for them.

Thus, the results of this study showed that, similar to conventional tetanus toxoid, the conjugate of tetanus toxoid with a low-molecular-weight hapten, which was used as an immunogen, allowed us to obtain and select monoclonal antibodies that may be used for effectively detection of both the toxoid and the toxin of *Cl. tetani* and exhibit neutralizing activity in vivo.

To design a method of quantitative determination of tetanus toxoid and toxin on the basis of these monoclonal antibodies using ELISA, we considered two schemes of analyses and determined the parameters and conditions of the most sensitive scheme. Competitive ELISA allowed more sensitive detection of the antigens of interest, compared to sandwich ELISA (specifically, 0.01 BU/ml tetanus toxoid and 50 LD₅₀/ml tetanus toxin). After recalculation to weight units, this parameter equals approximately 2 ng/ml (LD₅₀ for mouse is 2 ng tetanus toxin/kg), which corresponds to the sensitivity of sandwich ELISA that is reached when two types of monoclonal antibodies (human and mouse) are used [16]. The monoclonal antibodies obtained in this study and the schemes of enzyme immunoassay, developed on the basis of these antibodies, may be used for assessment of the qualitative and quantitative characteristics of tetanus toxin and toxoid at the stage of production and in commercial immunobiological preparations.

REFERENCES

- Montecucco, C., Papini, E., and Schiavo, G., *FEBS Lett.*, 1994, vol. 346, pp. 92–98.
- Trabaud, M.A., Lery, L., and Desgranges, C., *APMIS*, 1989, vol. 97, no. 8, pp. 671–676.
- Gustafsson, B., Whitmore, E., and Tiru, M., *Hybridoma*, 1993, vol. 12, no. 6, pp. 699–708.
- Ziegler-Heitbrock, H.W., Reiter, C., Trenkmann, J., Futterer, A., and Riethmuller, G., *Hybridoma*, 1986, vol. 5, no. 1, pp. 21–31.
- Ahnert-Hilger, G., Bizzini, B., Goretzki, K., Muller, H., Volckers, C., and Habermann, E., *Med. Microbiol. Immunol.* (Berlin), 1983, vol. 172, no. 2, pp. 123–135.
- Gigliotti, F. and Insel, R.A., *J. Clin. Invest.*, 1982, vol. 70, no. 6, pp. 1306–1309.
- Matsuda, M., Kamei, M., Sugimoto, N., Ma, Y., and Hashizume, S., *Eur. J. Epidemiol.*, 1992, vol. 8, no. 1, pp. 1–8.
- Volk, W.A., Bizzini, B., Snyder, R.M., Bernhard, E., and Wagner, R.R., *Infect. Immun.*, 1984, vol. 45, no. 3, pp. 604–609.
- Sheppard, A.J., Cussell, D., and Hughes, M., *Infect. Immun.*, 1984, vol. 43, no. 2, pp. 710–714.
- Kenimer, J.G., Habig, W.H., and Hardegree, M.C., *Infect. Immun.*, 1983, vol. 42, no. 3, pp. 942–948.
- Simpson, L.L., Lake, P., and Kozaki, S., *J. Pharmacol. Exp. Ther.*, 1990, vol. 254, no. 1, pp. 98–103.
- Lang, A.B., Cryz, S.J., Jr., Schurch, U., Ganss, M.T., and Bruderer, U., *J. Immunol.*, 1993, vol. 151, no. 1, pp. 466–472.
- Mizuguchi, J., Yoshida, T., Sato, Y., Nagaoka, F., Kondo, S., and Matuhasi, T., *Naturwissenschaften* (Berlin), 1982, vol. 69, no. 12, pp. 597–598.
- Vorob'ev, A.A., Vasil'ev, N.N., and Kravchenko, A.T., *Anatoksiny* (Anatoxins), Moscow: Meditsyna, 1965.
- Rukovodstvo po vaksinnomu i syvorotochnomu delu* (Guide to Work with Vaccines and Sera), Burgasova, P.N., Ed., Moscow: Meditsyna, 1978.
- Kiessig, S.T., Hentschel, C., Jahn, S., Mehl, M., Starke, R., and Porstmann, T., *J. Basic Microbiol.*, 1991, vol. 31, no. 2, pp. 135–140.
- Pal, A., Kumar, R., and Jaiikhani, B.L., *Indian. J. Med. Res.*, 1990, vol. 91, pp. 124–125.
- Burkin, M.A., Yakovleva, I.V., Sviridov, V.V., Burkin, A.A., Kononenko, G.P., and Soboleva, N.A., *Prikl. Biokhim. Mikrobiol.*, 2000, vol. 36, no. 5, pp. 575–581.
- Nakane, P.K. and Kawaoi, A., *J. Histochem. Cytochem.*, 1974, vol. 22, no. 2, pp. 1084–1091.