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INTERACTION OF RECOMBINANT DIPHTHERIA TOXOIDS WITH CELLULAR RECEPTORS *in vitro*

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The aim of the research was to compare *in vitro* characteristics of reception of the natural diphtheria toxin — DT and its nontoxic recombinant analogs — toxoids. For assessing ligand-receptor interaction the method of immunoenzyme analysis and ELISA was used, where the bonding layer recombinant analogues of diphtheria toxin cell receptor HB-EGF from sensitive and resistant to the toxin of the organisms were served. According to the results of ELISA the natural diphtheria toxin, in contrast to recombinant toxoids — CRM197, and B subunit, interacted with mouse HB-EGF with a very low affinity. While human HB-EGF with an equally high affinity connected as toxoids as native diphtheria toxin. Therefore, the analyzed recombinant analogs of toxin obtained in *E. coli* cells did not reproduce in full measure the receptor specificity of the natural toxin, which should be considered in the case of using these proteins as biotech products.

Key words: diphtheria toxin, B subunit of diphtheria toxin, CRM197, HB-EGF.

The scientific interest in diphtheria toxin (DT) is growing because it allows development new approaches in medicinal and biological research. Development of recombinant analogs of the toxin is of high scientific and economic importance.

DT is a single chain protein of 535 amino acid residues produced by the toxigenic strains of *Corynebacterium diphtheriae* which contain in their genome prophage — with functional gene tox [1]. The molecule of DT consists of two subunits: A and B, which are formed after moderate trypsinization of the toxin. The A subunit is represented only by one structural domain, catalytic or C-domain. The B subunit is formed by two domains: receptor (R-domain) responsible for binding to receptors on the cell membrane, and translocation (T-domain), responsible for transportation of C-domain through the membrane of endosoma.

Binding of DT to its receptor on the plasma membrane causes the internalization of the "DT–receptor" complex by way of endocytosis. The T-domain integrates into the membrane of endosoma and C-domain transfers into the cellular cytosol after the pH level in the lumen of endosomes decreases. The exact molecular mechanism of the DT C-domain transport through the lipid bilayer is still not clear.

Cytotoxicity of DT is caused by the catalytic transfer of ADP-ribose group from NAD^+ to the diftamide (modified histidine residue) of eukaryotic translation elongation factor 2 (eEF-2). Such ADP ribosylation almost fully stops protein synthesis in the cell and causes cellular death.

The receptor of DT is the transmembranous predecessor of heparin-binding growth factor, similar to epidermal growth factor — proHB-EGF [2]. ProHB-EGF is the single chain transmembrane glycoprotein consisting of 208 amino acid residues [3]. It is composed of heparine-binding, EGF-like, transmembrane, and cytoplasmic domains [4]. R-domain of B subunit of toxin binds with EGF-like domain of proHB-EGF. On the cell surface ProHB-EGF is cleaved by metalloproteinases resulting in the soluble growth factor HB-EGF, which bears only heparine-binding and EGF-like domains, and also is the ligand for EGFR and HER4.

Depending on their sensitivity to DT mammals are classified into sensitive and resistant. ProHB-EGF is present on the cell surfaces of both mammal groups. But EGF-like domain of proHB-EGF in these animal groups differs by 10 amino acid residues [5], and this is the main resistant factor to DT.

There is very controversial information on whether the binding of DT with proHB-EGF occurs on the cellular surface, and whether there is internalization of DT and proHB-EGF complex by endosomes of the resistant cells. According to one point of view, receptors of resistant cells are unable to bind with DT [2, 6–10]. Consequently, such inability to bind causes the toxin resistance of some mammals. According to the other point of view, binding of proHB-EGF of resistant organisms to DT happens on the cellular surface and subsequently the DT–receptor complex enters cytoplasm as part of endosomes [11–14]. Thus, the resistance mechanisms are realized only after DT enters the cell.

Recombinant derivatives of diphtheria toxin are important biotechnological tools to study toxin and other medicine needs. Previously we have created genetic constructions coding varied fragments of DT molecule, including various derivatives of B subunit of the toxin [15, 16], and CRM197 [16]. It is determined that such recombinant derivatives are able to bind and internalize *in vitro* with both types of cells, the sensitive [15] and the cells from a resistant organism [14]. But comparing the interaction of cell receptors with the obtained recombinant derivatives and with native DT has not been conducted. Thus, from the obtained results we have had only indirect evidence in favour of binding process of the HB-EGF with DT on the resistant cell surface and the internalizing of DT complex with receptors into the cytoplasm of the resistant cell.

The genetic constructions coding soluble HB-EGF of human and mouse were also created.

The goal of this work is to compare interaction of native DT and that of its recombinant derivatives with the receptors of resistant and sensitive cells.

According to the immunoassay (ELISA), unlike the native DT, the received recombinant derivatives of the DT molecule are able to effectively interact with the recombinant HB-EGF of the resistant organisms. Herewith the native DT had relatively weak reaction with the receptor of resistant cells. Therefore the recombinant toxins expressed in *E. coli* are not fully reproducing properties of the native toxin. The available data are not enough to explain the reasons of the different interactions of the native protein and its recombinant derivatives with the receptor of resistant cells, but we assume that such differences may occur due to the inadequacies of the expression system.

The obtained results indicate that mechanisms of mammal's resistance to the DT are determined by the value of affinity to the cell receptors.

Materials and Methods

Materials and reagents. Bovine serum albumin (BSA), IPTG (Amersham, USA), yeast extract (Becton, Dickenson and Co., USA), molecular weight markers for protein gel electrophoresis (Fermentas, Canada), bactotryptone (Fluka, Switzerland), β-mercaptoethanol, Tween-20 (Helicon, Russia), kanamycin, chloramphenicol, H_2O_2 ("Kyivmedpreparat", Ukraine), glycerol, dimethyl sulfoxide (DMSO), trisodium citrate, KCl, NaCl, Na_2HPO_4, NaOH, KH_2PO_4, ("Miranda-C", Ukraine), sodium dodecyl sulfate ("Reachem", Russia), acrylamide, bacterial culture medium LB, bromide methyl thiazol tetrazolium (MTT), bromothymol blue, skimmed milk, coomassie G250, ammonium persulfate, imidazole, urea, tetramethylethylenediamine, conjugate of recombinant streptavidin and peroxidase, tris (hydroxymethyl) aminomethane, tricine, Ni-NTA agarose, fetal calf serum (FBS), N,N′-Metilenbisakrilamid, RPMI1640 L-glutamine, Triton X-100, 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma, USA), A 96-well microtiter plates for enzyme-linked immunosorbent assay (Spektar, Serbia) and for eukaryotic cell culture (Greiner Bio One, Great Britain) were used.

DT production. Purification of the toxin was carried out in the Mechnikov Institute of Microbiology and Immunology of the NAMS of Ukraine (Kharkiv, Ukraine), from the culture medium of *C. diphtheria* PW8 strain, by [17].

Production of F(ab)'2 fragments specific to DT. Antibodies to the toxin were extracted from the antidiphtheritic horse serum ("Microgen", Russia) by the triple ammonium sulfate precipitation [18]. The hydrolysis of antibodies was performed using trypsin. Covalent attachment to the antidiphtheritic F(ab)'2 fragments of biotin was performed using biotin N-hydroxysuccinimide ester [19]. Biotinylation and preparation of F(ab)'2 fragments was performed by O. S. Oliynyk, Ph. D. in the Palladin Institute of Biochemistry of the NAS of Ukraine.

Preparation and purification of recombinant derivatives of HB-EGF and DT. Preparation of the genetic constructions coding recombinant HB-EGF of human (*h*HB-EGF), B subunit of DT bound with

EGFP fluorescent proteins (EGFP-SbB), and nontoxic point DT mutant, protein CRM197, are described in the previous articles [15, 16, 20]. Preparation of the construction for the expression of mouse HB-EGF (*m*HB-EGF) on the base of the *pET28a(+)* vector follows [20]; cDNA of the gene *m*HB-EGF was obtained from the 3T3 mouse embryonic fibroblast cell culture.

E. coli strain *BL21 Rosetta (DE3)* was used for expression by induction 1 mmol IPTG following [14, 15]. Metal ion affinity chromatography on Ni-NTA agarose was used for purification. Ni-NTA agarose native buffer was used for all manipulations to obtain and purify proteins: 100 mmol Na_2HPO_4, 10 mmol Tris-Cl, 500 mmol NaCl, pH 8.0. Destruction of the bacterial cell walls, membranes and DNA was conducted with ultrasonic treatment in the presence of 1% Triton X-100.

Purification of *h*HB-EGF, *m*HB-EGF and R-domain of DT was conducted in native conditions with soluble fraction of the cell lysate, of proteins EGFP-SbB and CRM197 in the denatured conditions of 8 M urea solution and renaturation *in vitro* using Ni-NTA agarose column, step by step reducing the concentration of urea (8, 6, 4, 2, 1 and 0 M). Purified recombinant products were eluted in 400 mmol imidazole and transferred protein by dialysis in phosphate buffered saline (PBS): 0.01 M Na_2HPO_4, 0.002 M KH_2PO_4, 0.137 M NaCl, 0.003 M KCl, pH 7.4.

Estimation of purity and concentration of the target recombinant proteins in obtained samples was conducted with SDS-PAGE as in [21].

Eukaryotic cell culture. Mammalian cell lines Vero and L929 were obtained from the Bank of Cell Lines from Human and Animal Tissues of Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, of the NAS of Ukraine. RPMI-1640 medium with L-glutamine and 10% FBS, containing 100 mg/l of streptomycin, 10 000 U/l of penicillin G and 250 µg/l of amphotericin B was used for cell growth at 37 °C temperature and 5% CO_2 in the air.

The DT cytotoxicity testing. Cell cultivation was conducted in the presence of native DT (0.3 µg/ml) during 24 hours after cells reached confluence. The toxin concentration in wells was reduced by double dilution from 2.5 to 0.3 µg/ml. The percentage of living cells was determined by incubation in solution prepared with addition of 100 µl of 5 mg/ml of MTT-reagent solution in PBS per 1 ml of RPMI-1640 with L-glutamine. After the incubation the medium was removed and the solution (10% SDS and 0,6 M HCl in DMSO) was added to dissolve the formazan crystals formed by viable cells. The test results are recorded at 570 nm wavelength and 630 nm reference wavelength.

Comparison of the interaction of proHB-EGF and its ligands by ELISA. All manipulations until the visualization were done in 100 µl of FBS, rinsed with distilled water on each stage. Sorption of *h*HB-EGF and *m*HB-EGF was conducted during the night at 4 °C, the concentration was 10 µg/ml. 10 µg/ml BSA was used as a negative control. All subsequent stages of incubation were performed during 1 hour each, at 37 °C. Free protein binding sites of sorption surface were blocked in 1% solution of skim milk. Recombinant derivatives of DT in the presence of 0.04% Tween-20 were added to the wells covered with HB-EGF after blocking; the concentration reduced by double dilution from 10 to 0.156 µg/ml. To detect the recombinant derivatives of DT we used biotinylated F(ab)'2 horse antibodies against DT and streptavidin–peroxidase polymer conjugate of working dilution determined experimentally in the presence of 0.04% Tween-20 in PBS. For visualization, 100 ml of solution, prepared directly before the experiment, were added in every well: 1 ml 0.001% TMB in DMSO per 9 mL of phosphate-citrate buffer (0.1 M Na_2HPO_4, 0.05 mmol sodium citrate, pH 5.0) and 2 µl 30% H_2O_2. The reaction was conducted for 5 min at 37 °C and was stopped by addition of 50 µl of 1 M sulfuric acid. The results of analysis were established at 450 nm wavelength.

Results and Discussion

More and more researchers become interested in DT because it allows creating medical preparations and agents for biological studies. Creating recombinant analogues of the toxin is of high scientific and economic importance. The aim of the present work is to compare the binding of native DT with the mammal cellular receptors with that of its recombinant analogues.

Obtaining and purification of the recombinant proteins. Preparations of *m*HB-EGF and *h*HB-EGF were purified from soluble fraction of the cell lysate. CRM197 and EGFP-SbB formed only in fully insoluble form (as bacterial inclusion bodies) during the expression in cells of *E. coli*; refolding was conducted *in vitro* to return the correct conformation of these two products. The purity and concentration

of preparations of all obtained proteins were satisfactory for ELISA (Fig. 1).

Cytotoxicity of DT. The results of MTT test (Fig. 2) show that DT causes significantly reduced percentage of viable cells of Vero line (> 50% at concentration of 0.3 μg/ml) but no significant changes in L929 cells viability.

L929 and Vero are cell lines, well-characterized sensitive and resistant respectively to DT. L929 clone is derived from cell line of mice L [22]. Cells from L line are resistant to DT and are derived from house mouse (*Mus musculus* L.). Most studies of the resistance to DT are conducted using the L line [2, 8–10]. L929 is the subclone of L line, it is a well-characterized model of mammalian cells with high resistance to DT [23–25].

Vero cells are derived from African green monkey (*Cercopithecus aethiops* L.), these cells are sensitive to DT and are the model of cells highly sensitive to even the slightest amounts of toxin [9, 8, 10, 23, 24].

DT exhibited high cytotoxicity to Vero cells and no cytotoxicity to L929 (Fig. 2), thus it maintained inherent natural functions.

Comparison of the interaction of proHB-EGF and derivatives of DT using ELISA. Increase of the number of ligands bound with proHB-EGF with the increased concentration of ligands in the incubation medium are given on graphs (Fig. 3). Native DT is characterized by the weak interaction with *m*HB-EGF in comparison with its recombinant derivatives (Fig. 3, *B*). DT and its recombinant derivatives both interact with high efficiency with the *h*HB-EGF.

Thus, the recombinant proteins do not reproduce the properties of DT concerning its interaction with the receptors of resistant animal species. The reason for this fact is not yet fully understood. We offer several assumptions:

Recombinant proteins have undesirable amino acid sequences that are absent in native DT. When the targeted nucleotide sequence embeds in the expression vector it was inevitable that some peptide appendages occurred on either side of the targeted polypeptide chain. Histidine tag (6×His-tag) is also among them, which is necessary to use IMAC. In all used constructions 6×His-tag is located at the C-terminus of the molecule (R-domain) near the site of binding with EGF-like domain of proHB-EGF. The elimination of tags is possible through the use of vectors, which are useful for expression of only the needed sequences or these containing protease cleavage site to cleave the unwanted parts. In this case the process of purification will be much harder.

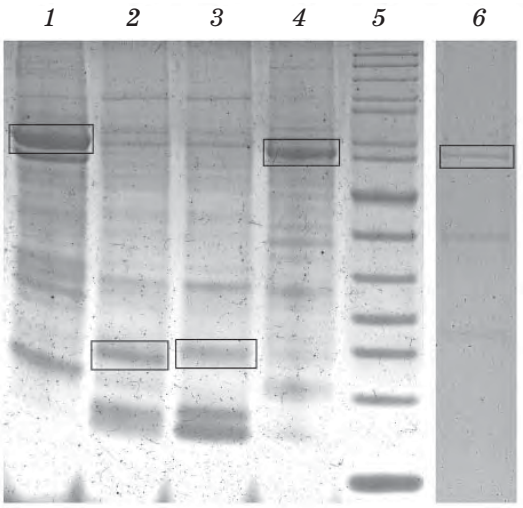

Fig. 1. **Electrophoregram of used proteins:** *1* — EGFP-SbB; *2* — hHB-EGF; *3* — mHB-EGF; *4* — CRM197; *5* — molecular mass markers: 200, 150, 120, 100, 85, 70, 60, 50, 40, 30, 25, 20, 15 and 10 kDa; *6* — DT. Bands corresponding to the target protein are indicated with a rectangular frame

Mutations. Among the toxin's mutations that reduce [25, 26] or enhance [27] the interaction with receptors of sensitive organisms, are known only those that belong to its R-domain. Mutations that could contribute to the appearance of the ability of the toxin to interact with the receptor of the resistant mammals are not described. Nucleotide sequencing of CRM197 (performed at Ukrainian Laboratory of Quality and Safety of Agricultural Products) has shown that R-domain contains such mutations as S374T, I418T, V432A, R494G, and I509M. Each of these mutations could potentially affect binding with the receptor of resistant species.

The incorrect folding of DT polypeptide chain expressed in E. coli. The presence of the native functions is the main issue in obtaining any recombinant protein in foreign cells. DT is a secretory protein (exotoxin) and its predecessor contains a signal peptide [28, 29]. DT folding occurs during the transport of the DT polypeptide chain through the plasma membrane involving Sec protein-translocation pathway [30]. In the case of expression from *pET24a(+)* plasmid in *E. coli* cells, the folding occurs in the cytoplasm or not at all (in which case the bacterial inclusion bodies are forming). The selection of the proper organisms for expression (for example *Corynebacterium glutamicum* [31], of the same genus as *C. diphtheria*) and proper expression vectors (for example *pET-22b* [32]

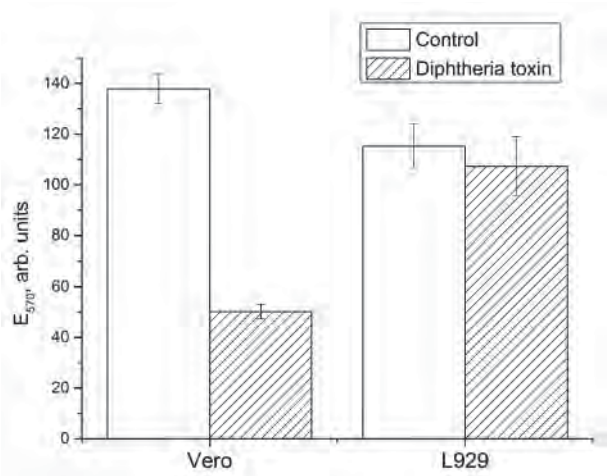

Fig. 2. **Histograms of the results of MTT test: Vero and L929 cells at 0.3 μg/ml DT during experiment, compared to the control (absence of any agents)**
Materials and methods contain detailed description of the cytotoxic test and reliability changes

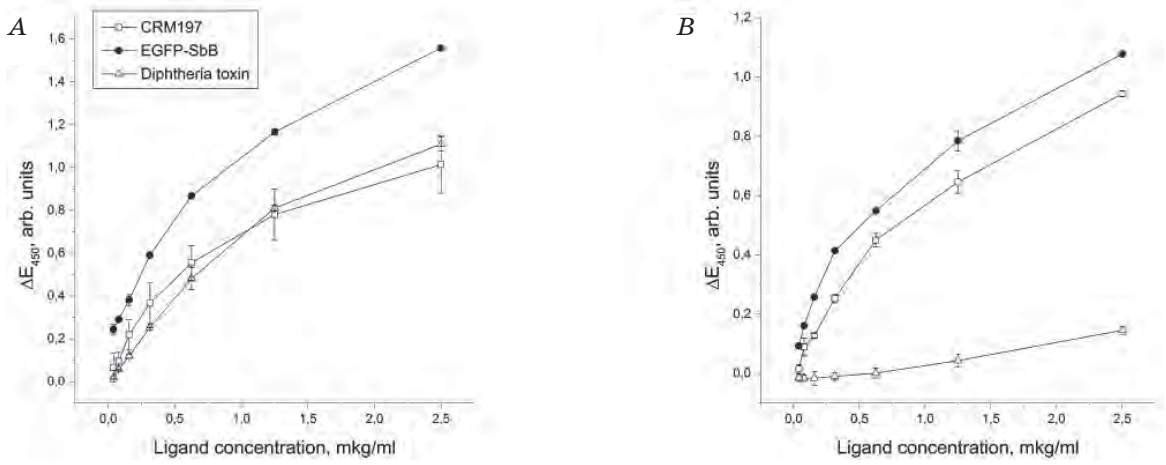

Fig. 3. **Comparison of the interaction of DT with HB-EGF with that of its recombinant derivatives using ELISA:**
ΔE450 — extinction difference at 450 nm between samples: studied (HB-EGF) and control (BSA); *A* — fragment of human receptor hHB-EGF used as antigen; *B* — fragment of mouse receptor mHB-EGF used as antigen

which directs product to the periplasm) are the possible ways to eliminate mis-folding.

The necessity of the functionally active center of DT molecule for the proper interaction with the receptor. There are some facts which support that point mutant CRM197 and native DT interact with cells differently [33–37]. Possibly only the intact molecule of the toxin can properly interact with receptors. Since CRM197 has no functionally active center, we can assume that the sequences of the catalytic center interact with proHB-EGF and determine the resistance of insensitive animals.

Thus, the obtained results show that the recombinant analogues of DT have some functions of the native toxin (interaction with the receptor of sensitive cells and internalization), while other functions are inconstant. The DT receptor of the resistant species of mammals interacts with available recombinant fragments and derivatives of DT more intensively than the native toxin. The results are supporting the existing idea that native DT has much weaker interaction with proHB-EGF of the resistant cells, which is the determining factor of the toxin resistance mechanisms. In transgenic mice that express human proHB-EGF, only in certain specific types of cells could a selective ablation of these cells be performed using DT, as shown in [38]. Ablation is conducted to study the functions of cell populations. According to the results

of this work the recombinant derivatives of B subunit of the toxin will nonspecifically affect the other mice cells, so its usage for such ablation is impractical.

Nowadays, various derivatives of DT are widely used in anticancer therapy to block the interaction of growth factor HB-EGF with EGFR receptors which are involved in carcinogenesis. Soluble HB-EGF derives from proHB-EGF and consequently contains EGF-like domain. That's why soluble HB-EGF also effectively interacts with DT. This fact can be useful during preclinical testing of diphtheria toxoid on mostly resistant to DT laboratory animals (rats and mice) with induced tumors, because the obtained derivatives of DT are also able to interact with HB-EGF of these species. Existing derivatives of DT can effectively block the interaction of HB-EGF (in resistant laboratory animals) with EGFR, which would allow effectively conducting preclinical testing of drugs based on a variety of diphtheria toxoids.

The obtained recombinant derivatives of DT have certain restrictions but they are promising for study of DT and its biological functions not related to such issues as mammal resistance to the toxin, and potential drugs.

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ВЗАЄМОДІЯ РЕКОМБІНАНТНИХ ДИФТЕРІЙНИХ ТОКСОЇДІВ З КЛІТИННИМИ РЕЦЕПТОРАМИ *in vitro*

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Метою роботи було порівняння *in vitro* особливостей рецепції природного дифтерійного токсину — ДТ та його нетоксичних рекомбінантних аналогів — токсоїдів. Для оцінювання ліганд-рецепторної взаємодії застосовували метод імуноензимного аналізу ELISA, де як зв'язувальний шар використовували рекомбінантні аналоги клітинного рецептора дифтерійного токсину HB-EGF від чутливих та резистентних до токсину організмів. За результатами ELISA природний дифтерійний токсин, на відміну від рекомбінантних токсоїдів — CRM197 та субодиниці В, взаємодіяв з HB-EGF миші з дуже низькою афінністю. При цьому HB-EGF людини з однаково високою афінністю зв'язував як токсоїди, так і нативний дифтерійний токсин. Отже, досліджені рекомбінантні аналоги токсину, що їх одержано у клітинах *E. coli*, не відтворювали повною мірою рецепторної специфічності природного токсину, що слід враховувати у разі використання цих протеїнів як біотехнологічних продуктів.

Ключові слова: дифтерійний токсин, субодиниця В дифтерійного токсину, CRM197, HB-EGF.

ВЗАИМОДЕЙСТВИЕ РЕКОМБИНАНТНЫХ ДИФТЕРИЙНЫХ ТОКСОИДОВ С КЛЕТОЧНЫМИ РЕЦЕПТОРАМИ *in vitro*

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Целью работы было сравнение *in vitro* особенностей рецепции природного дифтерийного токсина — ДТ и его нетоксичных рекомбинантных аналогов — токсоидов. Для оценки лиганд-рецепторного взаимодействия применяли метод иммуноэнзимного анализа ELISA, где в качестве связующего слоя использовали рекомбинантные аналоги клеточного рецептора дифтерийного токсина HB-EGF от чувствительных и резистентных к токсину организмов. По результатам ELISA природный дифтерийный токсин, в отличие от рекомбинантных токсоидов — CRM197 и субъединицы В, взаимодействовал с HB-EGF мыши с очень низкой афинностью. При этом HB-EGF человека с одинаково высокой афинностью связывал как токсоиды, так и нативный дифтерийный токсин. Таким образом, исследованные рекомбинантные аналоги токсина, полученные в клетках *E. coli*, не воспроизводили в полной мере рецепторной специфичности природного токсина, что следует учитывать в случае использования этих протеинов как биотехнологических продуктов.

Ключевые слова: дифтерийный токсин, субъединица В дифтерийного токсина, CRM197, HB-EGF.