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EPITOPES IDENTIFICATION OF BROADLY NEUTRALIZING MONOCLONAL ANTIBODIES AGAINST *Corynebacterium diphtheriae* EXOTOXIN

Ya. S. Kulyk[1, 2], *A. A. Siromolot*[1, 2], *D. V. Kolybo*[2]

[1]Taras Shevchenko National University of Kyiv, Ukraine;
[2]Palladin Institute of Biochemistry of the National Academy of Sciences of Ukraine, Kyiv

E-mail: den.kolibo@gmail.com

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Better and high-potency vaccines against diphtheria are urgently needed to provide broader protection against diverse strains and subtypes. Identification of novel broadly neutralizing epitopes targeted by protective antibodies could aid in such efforts.

Aim. In this study we focused on the search of binding sites identification of anti diphtheria toxin monoclonal antibodies and their neutralizing activity to block binding of recombinant exotoxin derivates with host receptors.

Methods. *Vero* cells were cultured in the complete RPMI-1640 medium under standard conditions and used for flow cytometry assay. Recombinant antigens and products of tryptic hydrolysis of CRM197 and SbB were characterized by Ni^{2+}-NTA affinity chromatography and SDS-PAGE under reducing conditions with following ECL Western-Blot using several hybridomas clones of anti-diphtheria toxin monoclonal antibodies.

Results. ECL western blot film results for clone 9.1-E11 showed the specific binding both to whole CRM197 molecule, and to almost all fragments of CRM197 formed as a result of limited proteolysis. In particular, a band corresponding to SbB in molecular weight can be identified. Thus, epitope region of the CRM197 molecule specific to 9.1-E1 mAbs is located within the structure of SbB. At the same time 16.4-E9 clone antibodies had high specificity to R-domain of SbB. In addition, both hybridoma clones antibodies have neutralizing activity against the DT binding subunit, which is a key factor in blocking between cell receptor and it ligand, *C. diphtheriae* exotoxin.

Conclusions. The results obtained indicate that produced antibodies are prospective for improving new diagnostic tools and therapeutic agents, which are used for treatment and understanding of the molecular mechanisms of diphtheria pathogenesis.

Key words: diphtheria toxin, CRM197, SbB, monoclonal antibodies, proHB-EGF, epitopes mapping, neutralizing antibody.

Diphtheria is a potentially fatal infection caused by toxigenic *Corynebacterium diphtheria* strains. The causative agent of diphtheria and diphtheria toxin (DT) are well studied, but thousands of diphtheria cases are still reported annually from several countries in Asia and Africa, along with many outbreaks worldwide [1]. Diphtheria is characterized by the formation of a pseudomembrane in the throat, but cutaneous infections are possible together with high mortality rate, which highlights the need for the development new diagnostic and therapeutic tools. CRM197 (Cross-Reacting-Material 197) is a variant of most studied non-toxic analogue of DT featuring a single site-specific substitution (G52E) which does not affect the deoxyribonuclease activity [2] but suppresses the ADP-ribosylating activity and the toxicity of the parental protein [3]. CRM197 is a commonly used glycoconjugate carrier that improves the immunogenicity of vaccines, particularly in infants [4]. Considering the attention that fusion vaccines are gaining, we thought it of interest to develop and analyze monoclonal

antibodies (mAbs) against DT (or CRM197) that will reveal the key sites of the effect of the toxin on cell receptors and become therapeutic agents for the prevention of the *C. diphtheriae* disease.

Methods

Cell cultures. Vero cell line was obtained from the Bank of Cell Lines of R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of the National Academy of Sciences of Ukraine, Kyiv. Cells were cultured in RPMI-1640 medium (Sigma Aldrich, USA) supplemented with 2 g/l sodium bicarbonate and 10% fetal bovine serum (FBS) with the addition of the antibiotic-antimycotic solution to prevent bacterial and fungal contamination.

Production of recombinant proteins by E. coli cells and their purification. Protocols for CRM197 and recombinant fragments of DT expression in *E. coli* Rosetta BL21 (DE3) cells on LB media by IPTG induction were elaborated earlier and published in previous publications [5]. Purification of proteins by Ni-NTA agarose column chromatography with imidazole elution gradient and determination of protein concentration by tricine SDS-PAGE analysis with TotalLab TL120 software were also performed according to [6]. *Enhanced chemiluminescence western blot* (ECL) protocol was described in [7]. *Limited proteolysis (trypsinolysis)* of CRM197 and SbB was carried out as described in [8] with modification.

Flow cytometry. The protocol of sample preparation for flow cytometry, including cell detachment from culture flasks surface, assessment of cell density in a counting chamber, endocytosis suppression by NaN_3 and low temperature (4 °C), adjusting conditions of incubation medium to reduce a cell surface non-specific protein adsorption by 1% BSA, were described in detail in [5].

Results and Discussions

For evaluation of the selected mAbs samples specificity to fragments of the CRM197, limited proteolytic cleavage by trypsin (EC: 3.4.21.4) of CRM197 and SbB was carried out. Epitope mapping of the CRM197 molecule was conducted by the western blot method (ECL) individually for each of the three selected samples of mAbs (9.2-H4, 9.1-E11, 16.4-E9 clones). The binding specificity of mAbs was determined for whole molecules of CRM197, DT recombinant fragments SbB, SbA, R-domain, as well as cleaved fragments obtained as a result of limited trypsinolysis of CRM197 and SbB. The blotogram for clone 9.1-E11 demonstrated that the chosen antibodies show specific binding not only to the whole CRM197 molecule, but also to almost all fragments of CRM197 formed as a result of limited proteolysis (Fig. 1, *A, B*). In particular, a band corresponding to SbB in molecular weight can be identified. Result indicates that the epitope region of the CRM197 molecule specific to mAbs obtained from clone 9.1-E11 is located within the structure of SbB. Analyzing the blotogram obtained during testing of clone 16.4-E9, high specificity for SbB and R-domain was observed (Fig. 1, *C, D*). Since the R-domain is part of SbB, it can be concluded that the R-domain contains an epitope region specific to mAbs expressed by clone 16.4-E9. We hypothesize that these mAbs can block the binding of DT to cellular receptors.

The natural receptor for DT on the cell surface is the membrane-anchored form of HB-EGF, proHB-EGF, is biologically active, providing mitogenic stimulation to neighboring cells in a juxtacrine mode [9]. Significant amount of proHB-EGF is present on the surface of the *Vero* cell line. To determine the neutralizing activity of the studied mAbs samples, a fluorescent derivative of DT - EGFP-SbB (SbB fused with green fluorescent protein in one open reading frame), which specifically interacts with the above-mentioned receptors was used. Among the tested mAbs, were identified two samples (clones 16.4-E9 and 9.1-E11) the neutralization efficiency of which was almost 100% compared to the control. Thus, mAbs are able to specifically bind to SbB of DT, while preventing its interaction with partner molecules on cell surface (Fig. 2). Our experiments confirm assumption regarding the ability of mAbs samples 16.4-E9 and 9.1-E11 to block the binding of DT with host cell receptors.

Conclusions

Among mAb-producing clones obtained in our laboratory three clones were characterized (9.1-E11, 16.4-E9 and 9.2-H4), which have the highest specificity to CRM197 and to individual target antigens (SbB, and R-domain). Moreover, it was found that clones 9.1-E11, 16.4-E9 have a neutralizing activity to preventing binding of DT to the proHB-EGF

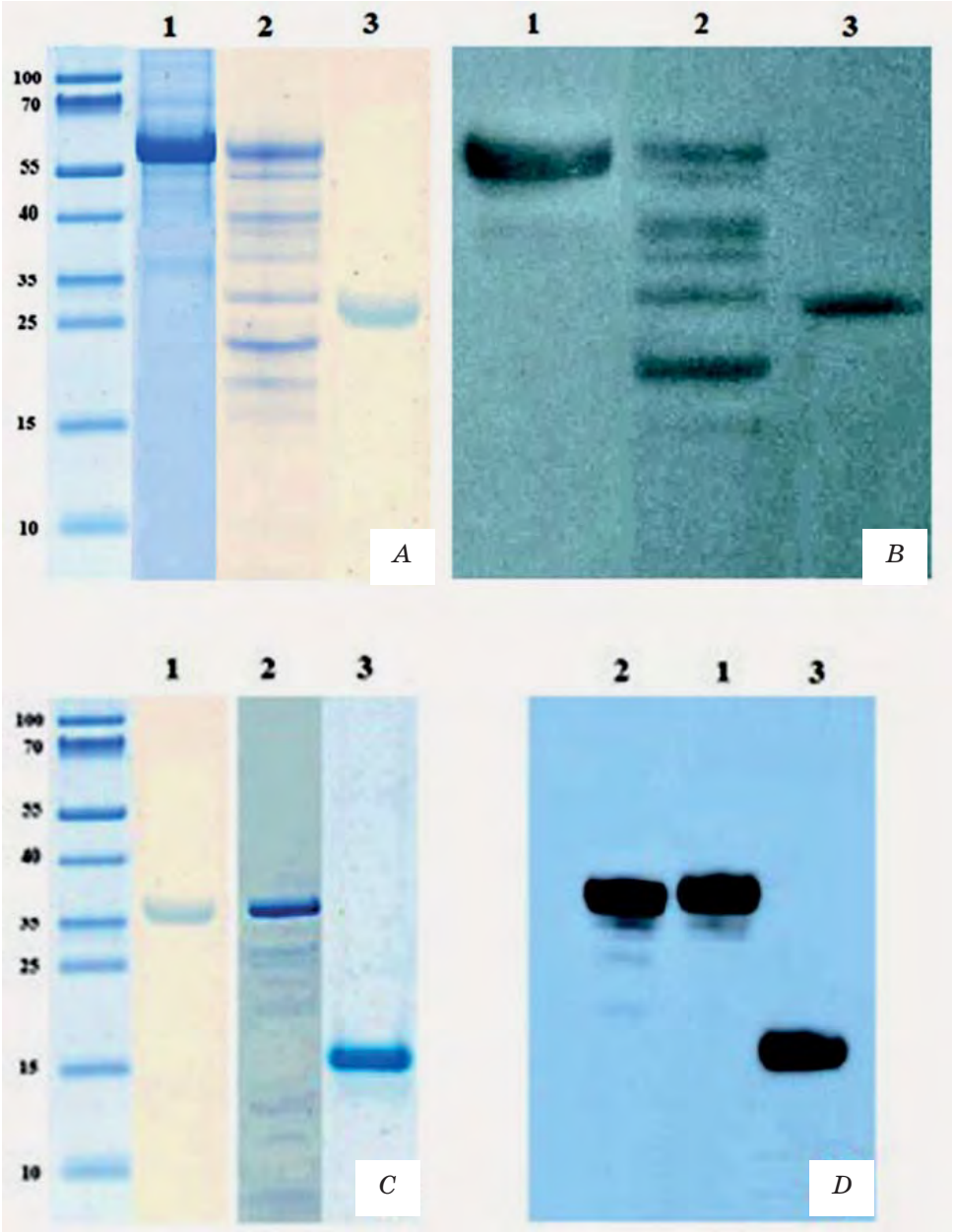

Fig. 1. **Epitope mapping of CRM197 and SbB by mAbs of clones 9.1-E11 (*A, B*) and 16.4-E9 (*C, D*):** *A* — SDS-PAGE of entire CRM197 molecule (1), after trypsinolysis (2), SbB (3); *B* — ECL blotting of corresponding electropherogram by mAbs; *C* — SDS-PAGE of SbB molecule (1), SbB after trypsinolysis (2), R-domain (3); *D* — ECL blotting of corresponding electropherogram by mAbs.

receptor, which is a perspective for improving existing and developing new diagnostic and therapeutic agents based on mAbs, including for prevention, treatment and understanding of the molecular mechanisms of diphtheria pathogenesis.

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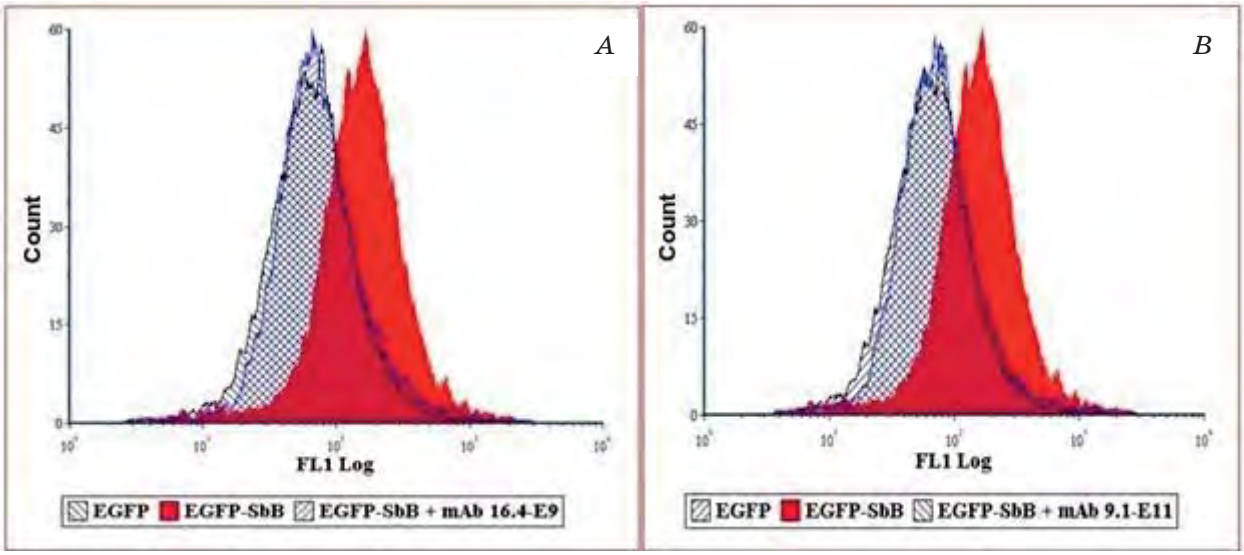

Fig. 2. **Fluorescence intensity of *Vero* cells incubated with SbB-EGFP. Detection of the ability of mAbs from clones 16.4-E9 (*A*) and 9.1-E11 (*B*) to block the interaction of SbB-EGFP with proHB-EGF**

REFERENCES

1. *Sharma N. C., Efstratiou A., Mokrousov I., Mutreja A., Das B., Ramamurthy T.* Diphtheria. *Nature Reviews Disease Primers.* 2019, 5(1), 1–18. https://doi.org/10.1038/s41572-019-0131-y
2. *Bellone M. L, Puglisi A., Dal Pia F., Hochkoeppler A.* Production in *Escherichia coli* of recombinant COVID-19 spike protein fragments fused to CRM197. *Biochemical and Biophysical Research Communications.* 2021, 558, 79–85. https://doi.org/10.1016/j.bbrc.2021.04.056
3. *Uchida T., Pappenheimer A. M. Jr., Greany R.* Diphteria toxin and related proteins. I. Isolation and properties of mutant proteins serologically related to diphteria toxin. *J. Biol. Chem.* 1973, 248, 3838–3844. https://doi.org/10.1016/S0021-9258(19)43810-6
4. *Aw R., Ashik M.R., Islam A. A.Z.M., Khan I., Mainuddin M., Islam Md. A., Mohammad M. Ahasan, Karen M. Polizzi.* Production and purification of an active CRM197 in *Pichia pastoris* and its immunological characterization using a *Vi*-typhoid antigen vaccine. *Vaccine.* 2021, 39(51), 7379–7386. https://doi.org/10.1016/j.vaccine.2021.10.083
5. *Siromolot A. A., Krynina O. I., Kolybo D. V., Komisarenko S. V.* Antiproliferative and apoptotic effects of anti-human HB-EGF neutralizing polyclonal antibodies *in vitro. Experimental oncology,* 2020, 42(1), 25–30. https://doi.org/10.32471/exp-oncology.2312-8852.vol-42-no-1.14145
6. *Labyntsev A. J., Kolybo D. V., Yurchenko E. S., Kaberniuk A. A., Korotkevych N. V., Komisarenko S. V.* Effect of the T-domain on intracellular transport of diphtheria toxin. *Ukr. Biochem. J.,* 2014, 86(3), 77–87. http://dx.doi.org/10.15407/ubj86.03.077
7. *Krynina O. I., Korotkevych N. V., Labyntsev A. J., Romaniuk S. I., Kolybo D. V., Komisarenko S. V.* Influence of human HB-EGF secreted form on cells with different EGFR and ErbB4 quantity. *Ukr. Biochem. J.*, 2019, 91(5), 25–33. https://doi.org/10.15407/ubj91.05.025
8. *Skar D. C., Rohrbach M. S., Bodley J. W.* Limited trypsinolysis of native *Escherichia coli* elongation factor G. *Biochemistry,* 1975, 14(17), 3922–6. https://doi.org/10.1021/bi00688a030
9. *Hamaoka M., Chinen I., Murata T., Takashima S., Iwamoto R., Mekada E.* Anti-human HB-EGF monoclonal antibodies inhibiting ectodomain shedding of HB-EGF and diphtheria toxin binding. *The Journal of Biochemistry*, 2010, 148(1), 55–69. https://doi.org/10.1093/jb/mvq033