THE HYPOTHESIS OF TISSUE-SPECIFIC ACTION OF DIPHTHERIA TOXIN

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Diphtheria is an infection caused by toxigenic strains of *Corynebacterium diphtheriae*. The pathogen releases the toxin that affects heart, kidneys, adrenal gland, as well as spinal and cerebral nerves. Tissue- and organ-specific action of diphtheria toxin is considered to be associated with the blood supply to these organs. We propose the hypothesis that takes into account the physical and chemical properties of the toxin molecule (positively charged R-domain in the B subunit) and cell expression of different types of the HB-EGF receptor and CD9 co-receptor, which are responsible for the toxin penetration into the cell. The proposed hypothesis explains the possible mechanisms of diphtheria complications.

Keywords: diphtheria, diphtheria toxin, *Corynebacterium diphtheriae*, HB-EGF receptor, CD9 co-receptor, tissue specificity, organ specificity.

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ГИПОТЕЗА ТКАНЕСПЕЦИФИЧНОСТИ ДЕЙСТВИЯ ДИФТЕРИЙНОГО ТОКСИНА

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Дифтерия — инфекция, вызываемая токсигенными штаммами бактерии *Corynebacterium diphtheriae*. Возбудитель выделяет токсин, который воздействует на сердце, почки, надпочечники, спинномозговые и черепно-мозговые нервы. Ткане- и органоспецифичность действия дифтерийного токсина принято связывать с особенностями кровоснабжения указанных органов, однако в статье предложена гипотеза, учитывающая физико-химические свойства молекулы токсина (наличие положительно заряженного R-домена в субъединице B молекулы) и представленность в клетках различного типа рецептора HB-EGF и корецептора CD9, с которыми токсин связывается для проникновения в клетку. Дано объяснение возможных механизмов осложнений при дифтерии с учетом гипотезы.

Ключевые слова: дифтерия, дифтерийный токсин, *Corynebacterium diphtheriae*, рецептор HB-EGF, корецептор CD9, тканеспецифичность, органоспецифичность

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Diphtheria is a classic toxemic infection caused by *Corynebacterium diphtheriae*, a gram-positive bacterium, and most importantly by its toxin. The pathogen penetrates the mucous membranes of the oropharynx, nasopharynx, larynx and trachea and sometimes those of eyes and genitals. A local inflammatory focus is formed accompanied by necrotic cell death, blood plasma coagulation, edema, and formation of a whitish-gray coating over the mucosal surface. In the multilayer epithelium of the nasopharynx, epiglottis and vocal cords, the membrane is inseparable from the underlying tissue, while in the monolayer epithelium of the larynx, trachea and bronchi the membrane is easily separated and may cause airway obstruction and asphyxia.

The toxin secreted by toxigenic strains of *C. diphtheriae* enters the bloodstream and selectively affects the heart, kidneys, adrenal glands, and spinal and cranial nerves [1]. In this paper, we present the hypothesis that the tissue and organ specificity of the toxin can be explained by both physical and chemical properties of the molecule as well as by the structure of the cell membranes of the target organs.

Structure of the toxin and its penetration into the cell

Diphtheria toxin (DT) is a protein with a molecular weight of 58 kDa (535 amino acid residues, isoelectric point, pI, of 5.9). It consists of two subunits (A and B); the a subunit exhibits nuclease activity [2] and ADP-ribosyltransferase activity towards elongation factor EF-2. It blocks the cellular mechanism of protein synthesis, which results in cell apoptosis. The B subunit consists of the transmembrane (T) domain and the receptor binding (R) domain responsible for binding to HB-EGF [3].

The a subunit of the toxin is delivered into the cell in several steps (Fig. 1). In the first step (pH of 7.4, DT electrical charge of –9.44), the R domain of the B subunit mediates the binding of the toxin to its receptor HB-EGF and co-receptor CD9 [3]. In the second step, clathrin-dependent endocytosis of the toxin occurs; the clathrin-coated vesicles containing DT are shortly transformed into early endosomal vesicles. While PIP phosphatase and the heat shock protein Hsp70 separate the primary clathrin coat from the vesicles [4], and new components (COPI proteins, Arf, Rab, etc.) begin to

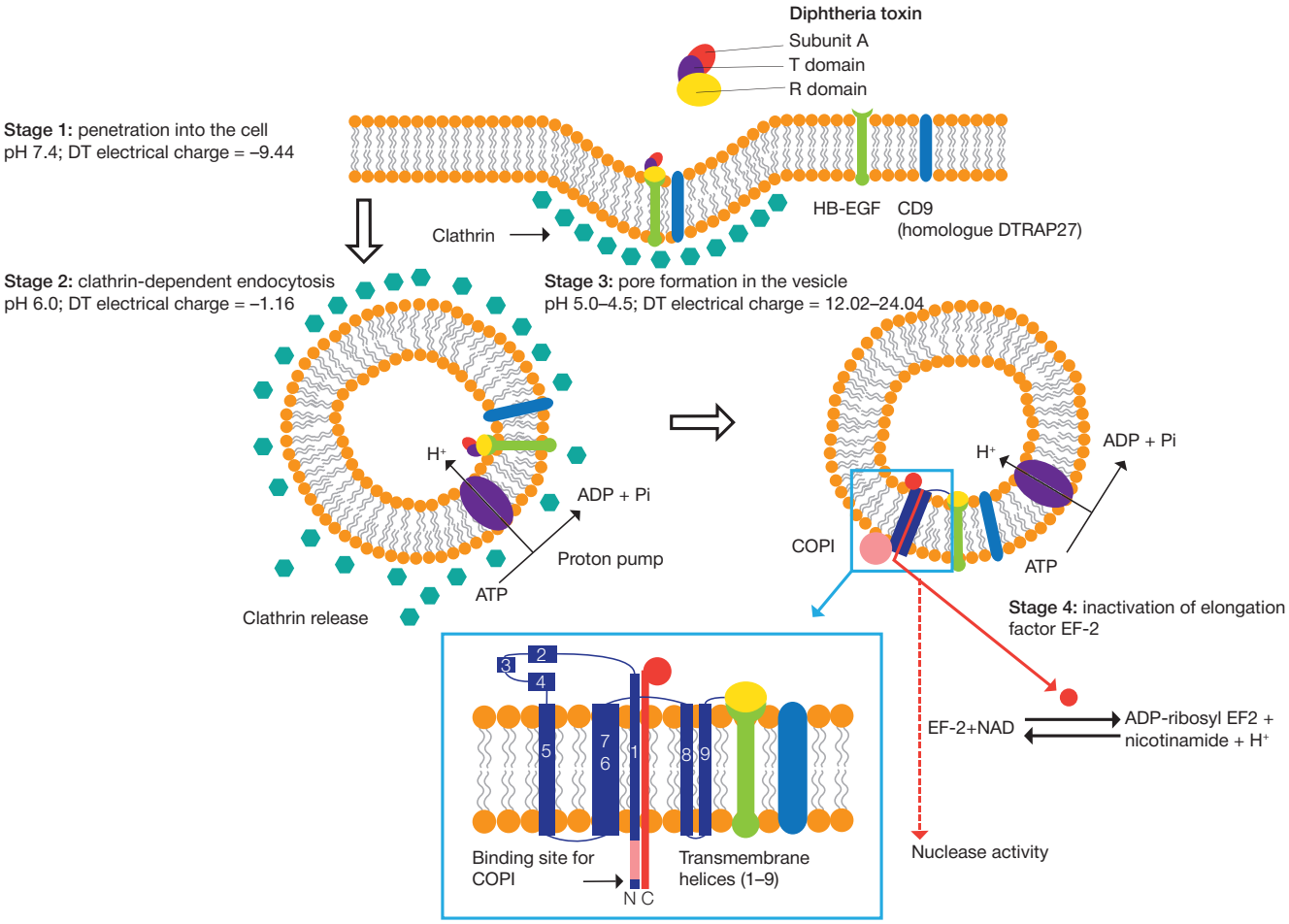

Fig. 1. The mechanism of toxin penetration into the cell

adhere to it [3, 4], the proton pump acidifies the medium inside the vesicle, thus lowering the pH to 6.0 [4] and changing the electrical charge of DP to −1.16. In the third step, the pH within the vesicle drops to 5.0–4.5 [4], and the protein charge becomes positive (from 12.02 to 24.04). These conditions are necessary to modify the conformation of the T domain of the toxin B subunit and to form a pore in the vesicle. The T domain consists of 9 transmembrane helices (TH), which in turn are packed into three layers: the first comprises amphiphilic TH1–TH3, the second comprises hydrophobic TH5–TH7, and the third comprises TH8 and TH9 forming the central core [3]. TH1 closely interacts with the catalytic subunit and also has sites for binding to COPI complex, which plays a crucial role in the A subunit translocation (along with TH2–TH4). In the fourth stage, a free subunit inactivates elongation factor EF-2.

Tissue and organ specificity of the toxin

The tissue specificity of the toxin can be explained by its physical and chemical properties, namely, the presence of the R domain (amino acid residues 432–535 [3]) that ensures the interaction between the toxin and HB-EGF. The positive charge of this domain (8.2 at pH of 7.4, pI of 10.43) determines the ability of the toxin to bind to a negatively charged membrane receptor. If a cell membrane can be imagined as an equivalent to an electrical circuit, then it has both capacitive (C) and resistive properties (R) defined by the components of the membrane itself (phospholipids and proteins). One side of the membrane is charged positively and the other is charged negatively [5]. A deficit of cations on the inner membrane creates a negative charge inside the cell, while their excess on the outer membrane surface creates a positive charge on the outside. Na^+/K^+ ATPase serves as a kind of battery (E) establishing a voltage difference by pumping $3Na^+$ out and $2K^+$ into the cell. Such are the passive electrical properties of the membranes [5]. In turn, the excitable cells are able to redistribute ions during depolarization. This phenomenon is the basis of our hypothesis explaining the tissue specificity of the toxin. The excess of cations outside the membrane that generates the resting potential repels DT, and temporary ion redistribution during the action potential attracts it. There are also specific lipids: gangliosides that impart special properties to highly specialized cells, such as neurons and cardiomyocytes. In these cells, gangliosides may account for 5–10 % of the total lipids [4]. Sialic acids present in the molecular structure of gangliosides generate a strong negative charge, which determines their tropism towards positively charged molecules.

In the modern literature, organ specificity of DT has been linked to the peculiarities of the blood supply to the targeted organs [1]. However, neither liver nor the gastrointestinal tract are affected by the toxin, even though they receive up to 25 and 10 % of the cardiac output, respectively. Here, the assumption is that the organ specificity of the toxin can be attributed to the presence of HB-EGF and CD9 and their ratio in different cell types. According to RefExA database [6], HB-EGF is present in large amounts in the spinal nerves (Spinal_c), thyroid cells (Thyroid), pulmonary epithelium (Lung), smooth muscle tissue (Sm_mscl), cardiomyocytes (Heart), renal epithelium

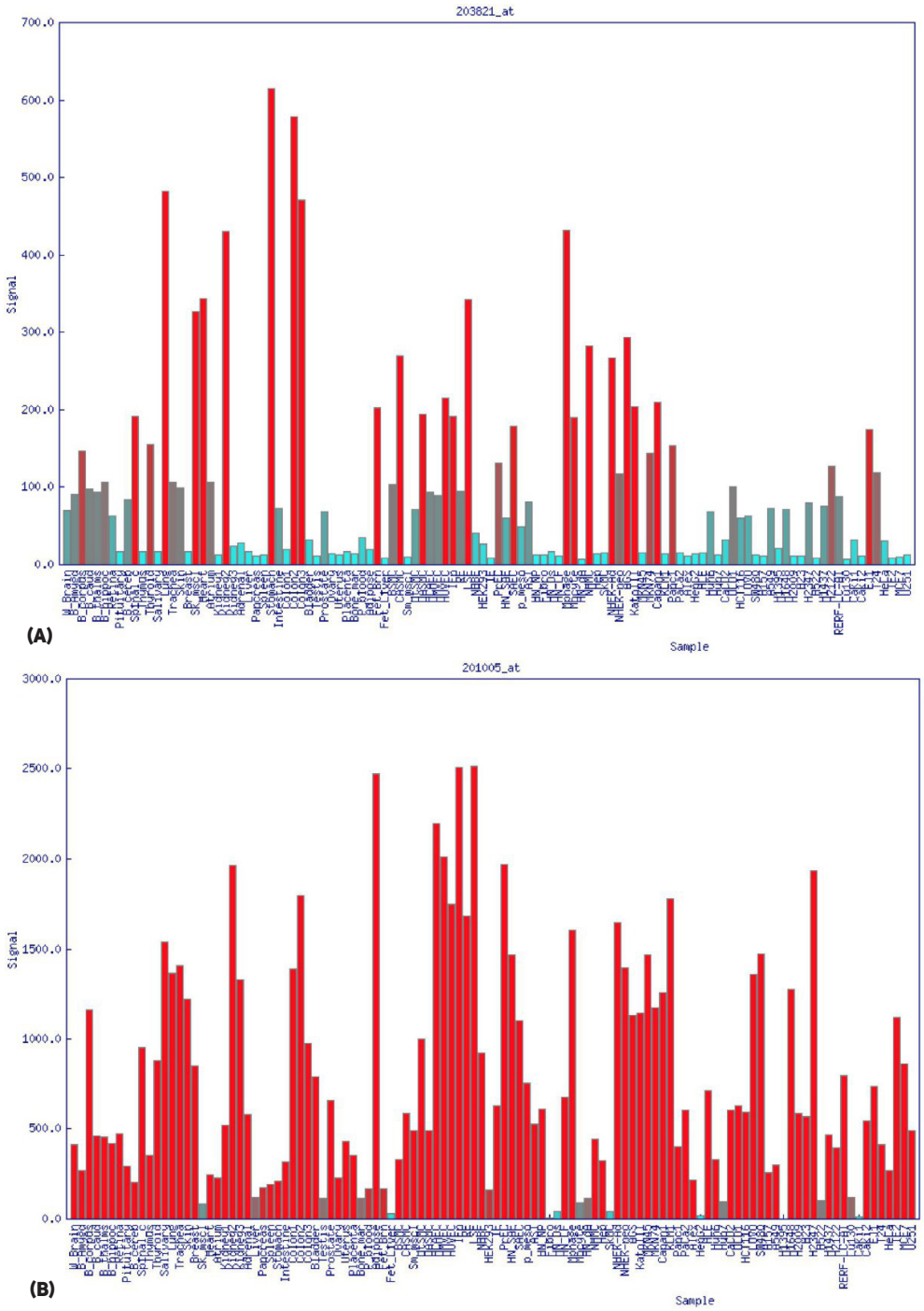

Fig. 2. Expression of the HB-EGF **(A)** and CD9 **(B)** in human tissues (according to RefExA database [6])

(Kidney_2), stomach epithelium (Stomach), and intestinal epithelium (Colon2, Colon3) (Fig. 2A). The concentration of the co-receptor CD9 is high in most cell types, except for the skeletal myosymplasts (Sk_mscl), liver cells (Liver), spermatogenic epithelium (Testis), and hematopoietic bone marrow cells (Bone_mar) (Fig. 2B). To have any effect, the diphtheria toxin requires both HB-EGF and CD9, a criterion which is met by the cells of the heart, lungs, gastrointestinal tract, kidneys, thyroid gland, spinal and cerebral nerves.

Complications of diphtheria in the light of the hypothesis

The first complication of the hypertoxic form of diphtheria is myocarditis [1]. It can be explained by the presence of a plateau phase in the excitation of the cardiomyocytes, which prolongs the duration of depolarization, as compared to that in the cells of other excitable tissues. This is followed by tubular nephrosis [7], adrenal hemorrhage [8], and by neuritis and polyneuritis several weeks after, sometimes even after recovery [9]. This sequence is associated with the substrate specificity of the toxin to EF-2, which is normally present near the ribosomes and the rough endoplasmic reticulum. In neurons, these organelles are located only in the soma; the toxin enters the cell most often through axons and dendrites; in order to reach its substrate, the subunit a then uses intracellular mechanisms of protein transport from the projections to the cell body, i. e., retrograde transport. Use of such mechanisms has been reported for many toxins [10]. However, transport here is very

slow, approximately 1 mm/day, which is why the damage to the nervous system usually occurs at a later stage [9].

Nephrosis may be associated with the renal filter structure: the pores are located in its narrowest segment, and the glomerular basal membrane allows passage of non-negatively charged molecules weighting up to 69 kDa, which is associated with the presence of the negatively charged glycocalyx on the surface of the cell membrane [5]. Evidently, DT is filtered into the Bowman–Shumlyansky's capsule where it enters the proximal tubules by endocytosis facilitated by its interaction with the megalin-cubilin complex [5]. DT is an acidic protein, so the penetration described is possible only if a molecule has a strong positive pole that will be pulled by the glycocalyx. Subsequently, instead of being cleaved by lysosomal enzymes, the a subunit of the toxin penetrates the cytosol of the tubular epithelium, which leads to nephrosis [7].

The above confirms that characteristics of blood supply explain only the adrenal hemorrhage caused by DT [8]. The blood flow through the adrenal glands is extremely vigorous (three major arteries and approximately 25–30 arterioles). This makes the organ vulnerable to pressure changes and susceptible to thrombogenesis under conditions of extensive blood loss, toxemia and shock, when the blood supply to vital organs is amplified to the detriment of other less important organs [11].

CONCLUSIONS

The proposed integrated hypothesis explains both the mechanism of action of diphtheria toxin and its impact on the infectious process in general, which lends a better understanding of the pathogenesis of diphtheria. The proposed hypothesis provides a basis for improved methods of treatment of the hypertoxic form of the disease, and a framework to study the mechanism of action of other toxins.

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