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Review Article

Action of Cholera Toxin B Subunit and Peptide LKEKK on Different Cell Types

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1. Abstract

The review analyzed and systematized data on the action of cholera toxin B subunit (CT-B) and the synthetic peptide LKEKK that corresponds to residues 16-20 in thymosin-α_1 and 131-135 in interferon-α_2 on the functional, NO-synthase and guanylate cyclase activity of T and B lymphocytes, of macrophage-like cell line RAW 264.7, of human Caco-2 and rat IEC-6 intestinal epithelial cell lines. According to the data presented, CT-B and the peptide bind to the cholera toxin receptor of the target cell with high affinity and trigger the following cascade of intracellular reactions: activation of inducible NO synthase → increase in NO production → increase in soluble guanylate cyclase activity → increase in the cyclic guanosine-3',5'-monophosphate level.

2. Keywords: Protein; Peptide; Receptor; Drug; Cholera toxin B subunit; Signal transduction

3. Introduction

Cholera toxin (CT) is a soluble protein produced by gram-negative bacteria *Vibrio cholerae*; it consists of two main subunits, CT-A and CT-B, its molecular mass is 84 kDa [1]. The CT-A subunit determines the symptoms of the disease, while CT-B is a means of delivering CT-A to target cells. CT-A is a 28 kDa protein consisting of two primary domains, CT-A1 and CT-A2; CT-A1 determines the activity of the toxin, CT-A2 acts as an anchor [2]. CT-B forms a ring-like structure composed of five CT-B monomers. Each monomer is a nontoxic protein consisting of 103 amino acid residues [3] and binding to the monosialotetrahexosylganglioside (GM1a, Galβ3GalNAcβ4 (Neu5Acα3) Galβ4GlcCer) which is present in almost all cells [4-6].

Cholera toxin B-subunit (CT-B) is a promising immunomodulating and anti-inflammatory agent. It has been shown that the protein suppresses immunopathological reactions in allergies and autoimmune diseases [7, 8], stimulates humoral immunity and induces anti-inflammatory reactions in vivo, in particular, reduces intestinal inflammation in Crohn's disease [9, 10]. Since CT-B is able to weaken infectious diseases and, at the same time, inhibit the development of autoimmune reactions, the question remains unclear how these two opposing immune processes can be mediated by the same protein.

Earlier in the structural and functional studies of interferon-α (IFNs-α) we obtained the peptide LKEKKYSP corresponding to the fragment 131-138 of human IFN-α_2, capable of high affinity binding to mouse thymocytes [11] and human fibroblasts [12]. The labeled peptide binding was competitively inhibited by unlabeled IFN-α_2, TM-α_1 and CT-B. Comparison of amino acid sequences of the octapeptide and TM-α_1 showed that they contain the same LKEKK fragment corresponding to the sequence 16-20 TM-α_1 and 131-135 IFN-α_2 (Figure 1). We suggested that this fragment may be involved in the binding of TM-$\alpha_1$and IFN-α_2 with a common receptor and synthetic peptide LKEKK may also have the same ability.

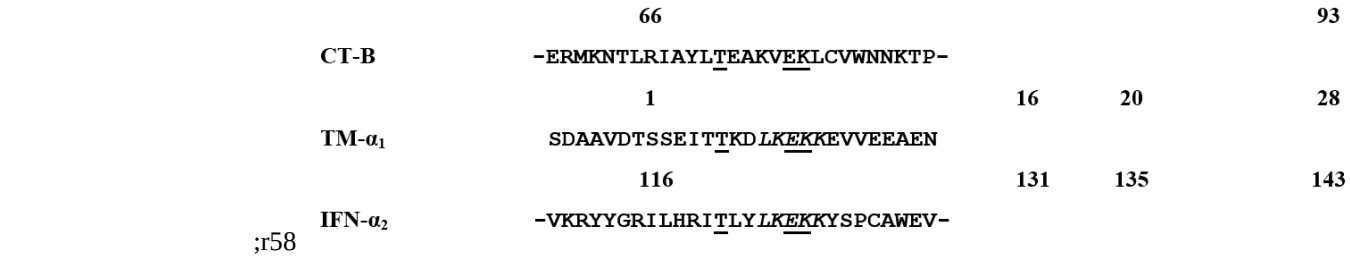

Figure 1. The amino acid sequences of CT-B, human TM-α_1, and human IFN- α_2. Numbers of amino acid residues are shown in figures. The identical residues are underlined. The sequence of peptide LKEKK is shown in italics.

Recently we synthesized peptide LKEKK and found that [^{3}H] LKEKK binds with high affinity to donor blood T lymphocytes [13, 14], rat intestinal epithelial cell membranes [15, 16], rat IEC-6 [17] and human Caco-2 [17, 18] intestinal epithelial cells, murine Raw 264.7 macrophage-like cells [19]. Treatment of cells and membranes with proteases did not affect the [^{3}H]LKEKK binding, suggesting the non-protein nature of the peptide receptor. The results obtained showed that lymphocytes and intestinal epithelial cells have on their surface a non-protein receptor common for TM-α_1, IFN-α_2, and CT-B. It has been suggested that this receptor could be the CT receptor, which is known to be a GM1-glanglioside

The review analyzed and systematized data on the action of cholera toxin B subunit (CT-B) and the synthetic peptide LKEKK on different types of cells *in vitro* and *in vivo*.

4. Action of CT-B and Peptide LKEKK on Human Blood T and B Lymphocytes

The analysis of the binding of ^{125}I-labeled CT-B to T and B lymphocytes isolated from the blood of healthy donors showed the presence of one type of high affinity binding sites (K_d 2.8 and 3.0 nM, respectively) [13, 14]. Unlabeled TM-α_1, IFN-α_2, and the peptide LKEKK competitively inhibited the labeled protein binding both to T (K_i 3.3, 2.9, and 3.6 nM) and B (K_i 3.7, 3.3, and 3.8 nM) cells. The peptide KKEKL with an inverted amino acid sequence tested in parallel did not inhibit the binding of the labeled protein (K_i > 10 μM). These results indicate that TM-α_1, IFN-α_2, and the peptide LKEKK bind with high affinity and specificity to cholera toxin receptor on human blood T and B lymphocytes.

So far, no data have been obtained on the binding of TM-α_1 to gangliosides. At the same time, CT-B is known to suppress the antiviral activity of IFN-α [19-20] by inhibiting its interaction with GM1-ganglioside [21-22]. In addition, the ability of IFN-α to reversibly bind to GM1 ganglioside with high affinity and specificity has been shown. The regions of the molecule directly involved in the binding are the oligosaccharide fragment of GM1 including lactose (β-D-Gal-(1→4)-Glc) and *N*-acetylneuraminic acid [22] and highly conserved IFN-α 131-138 fragment [11], or even shorter 131-135 fragment [13].

The binding of CT-B and the peptide LKEKK to T and B lymphocytes has been found to lead to a dose-dependent increase in the activity of soluble guanylate cyclase (sGC), but does not affect the activity of adenylate cyclase and membrane-bound guanylate cyclase (pGC) [14]. The peptide with an inverted KKEKL sequence tested in parallel did not affect sGC activity, indicating a high specificity of the CT-B and peptide LKEKK action.

The soluble guanylate cyclase (sGC) is shown to be a heterodimer that consists of α- and β-subunits, catalyzes catalyzes a conversion of guanosin-5'-triphosphate (GTP) into the cyclic guanosin-3',5'-monophosphate (cGMP), and is activated by a direct interaction of NO with a hem of the β-subunit [23]. Besides sGC, there are at least seven plasma membrane enzymes that synthesize the second-messenger cGMP [24]. All membrane (particulate) GCs (pGC-A through pGC-G) share a basic topology, which consists of an extracellular ligand binding domain, a short transmembrane region, and an intracellular domain that contains the catalytic (GC) region [23, 24]. There are data indicating that T-cellular inducible NO-synthase (iNOS) and NO play a crucial suppressing role in the control of T- helper differentiation [25-27]. NO was shown to inhibit T cells in the G1-phase and to induce apoptosis through activation of the sGC-dependent protein kinase G [28]. However, NO was found to display a suppressing effect only at high concentrations (>100 μM), whereas its low concentrations (5-25 μM) selectively increased the differentiation of Th1 cells and did not influence the differentiation of Th2 cells [29, 30]. Th1 cells are involved in the development of inflammatory reactions and the elimination of intracellular pathogens, while Th2 cells are closely associated with allergies and the displacement of extracellular parasites [31-33]. Both cell types have the same precursor and differentiate into two different lines, mainly under the influence of cytokines in the microenvironment. During specific antigenic activation of T-cell precursors the differentiation of Th1 cells is stimulated by IL-12, and the differentiation of Th2 cells by IL-4 [34, 35]. Niedbala et al. [29, 30] demonstrated that the activating effect of low concentrations of NO was mediated through cGMP and was manifested selectively on Th1 cells. NO activated sGC, which led to an increase in the level of cGMP that activated expression of the IL-12

receptor β2-subunit but did not influence the IL-4 receptor. Since IL-12 and IL- 4 are key cytokines in the induction of Th1 and Th2 cells, respectively, they are responsible for the selective action of NO on the differentiation of T cells. Thus, low doses of NO promote the differentiation of Th1 cells by the selective induction of IL-12Rβ2 via the sGC-cGMP-dependent pathway.

Earlier studies have shown that after infections in iNOS-deficient mice an increased Th1-response is developing, which is accompanied by an increase in the level of IFN-γ and a decrease in the level of IL4 [36-38]. These data show that NO selectively suppresses the expansion of Th1 cells through negative feedback that can be realized due to inhibition of IL-12 synthesis by activated macrophages [39]. This mechanism might be very useful in inflammatory diseases mainly mediated through Th1 cells. By contrast, a strong Th1 cell response is very desirable for the effective protection of the organism against intracellular pathogens.

The stimulating effect of CT-B and the peptide LKEKK on sGC activity in T-lymphocytes suggests that they are capable of selectively inducing differentiation of Th1 cells through the sGC-cGMP-dependent pathway.

As mentioned above, CTB is currently considered a promising immunomodulatory agent. Therefore, the establishment of the molecular mechanism of action of this protein is important for its implementation in medical practice. The ability of CTB and peptide LKEKK to enhance the activity of sGC in T and B lymphocytes makes appropriate further detailed study of protein and peptide on the sGC-cGMP signal transmission pathway and its mediated activities.

5. Effect of CT-B and Peptide LKEKK on Murine Macrophage-Like RAW 264.7 Cell Line

It is established that that^{125}I-labeled CT-B binds with high affinity to the RAW 264.7 cells (K_d2.3 nM) [40]. The labeled CT-B binding was inhibited by unlabeled IFN-α_2, TM-α_1 and the peptide LKEKK (K_i 0.9, 1.1 and 1.4 nM, respectively), but not inhibited by unlabeled peptide KKEKL (K_i > 1 μM) In the concentration range of 10–1000 nM, CT-B and the peptide LKEKK dose-dependently increased NO production by cells and intracellular sGC activity. The peptide KKEKL tested in parallel was inactive, indicating a high specificity of the CT-B and peptide LKEKK action.

NO is a diffuse messenger, which mediates a wide spectrum of physiological and pathological processes in the nervous, cardiovascular, and immune systems [41]. It has several protective functions: improves tissue perfusion, inhibits thrombocyte aggregation [42], decreases leucocyte adhesion to endothelial cells [43, 44] and proliferation of cells of the smooth muscles, and facilitates preservation of tissue and organ architecture [45]. In addition to regulating normal physiological functions, NO participates in the development of a number of pathological states, such as septic shock, stroke, and neurodegenerative diseases [41, 46-47]. NO is synthesized from *L*-arginine by several isoforms of the NO-synthase (NOS): inducible (iNOS), endothelial (eNOS), and neuronal (nNOS) [41, 48] activating sGC by direct interaction with hem of its β-subunit [23]. cGMP that is accumulated in a cell transmits signals to subordinate elements of the signal cascade: cGMP-dependent protein kinases, cGMP-regulated cationic channels, and cGMP-activated phosphodiesterases [23, 49]. It is irrefutably proved that the effects of low NO concentrations (~5 to 50 μM) are mediated by cGMP [29, 30]. Our results are in good agreement with this data: an enhancement of the NO production from 26 μM in a control to 48 and 45 μM in the presence of 100 nM of CT-B or the LKEKK peptide, respectively, results in an almost twofold increase in the sGC activity [40].

NO was found to increase the content of F-actin in macrophages and, thus, change their ability for adhesion, for the formation of pseudopodia, and phagocytosis [50, 51]. Key regulators of the actin reorganization which resulted in morphological changes in the NO-stimulated cells were considered to be the cGMP-regulated Ca^{2+}-calmodulin [51].

CT-B and the peptide LKEKK in a concentration of 100 nM were shown to significantly increase the ability of the RAW 264.7 cells for the adhesion and the spreading *in vitro*. [40]. Inflammation is known to cause an enhanced directed migration of leukocytes. The activated cells gain an ability to adhere to the vascular endothelium and to migrate in an area of an infection and inflammation, and the cells change their shape from round to stellate during this process. Therefore, such properties of phagocytes as adhesion and spreading adequately reflect their functional status. In addition, the adhesion and spreading of the cells can be somewhat considered as initial stages of phagocytosis: attachment and circumvallation of a particle by pseudopodia.

The ability of CT-B and the peptide LKEKK to influence the phagocytic activity of the RAW 264.7 cells was studied on a model system of the bacterial phagocytosis of the *Salmonella typhimurium* 415 virulent strain *in vitro* [40]. The characteristics of the phagocytosis of the *S. typhimurium* 415 bacteria (PhA, the phagocytic activity; CBA, cytophatic action of the bacteria; PhN, the phagocytic number) by the RAW 264.7 cells in the control and in the presence of CT-B or the LKEKK peptide were given in Table 1. The control experiments demonstrated that the cells actively imbibed the bacteria of this strain. More than a half of the total cell number (PhA 56.3 ± 5.2%) participated in the phagocytosis in one hour, and every phagocyte contained 7 microorgan- isms on average (PhN 7.0 ± 2.2). However, the imbibed microbes were not digested. Moreover, they continued their active reproduction inside the phagocytes, and PhN increased from 7.0 ± 2.2 to 12.1 ± 3.2 between the second and seventh hours of the phagocytosis. The cell infection continued for 2 hours. Then, the infection medium was replaced by the culture medium, and, starting from this moment, the PhN could be increased only due to the reproduction of the previously imbibed microbes. A mass cell death

was observed even to the seventh hour of the phagocytosis (CBA 61.7 ± 5.3%), and all the monolayer was degraded to the 12th hour (CBA ~100%). Thus, the interaction of the microbes with the RAW 264.7 cells in the control resulted in the death of the latter. Quite the opposite picture was observed in the presence of 100 nM of CT-B or the LKEKK peptide. In both cases, the digestive ability of the cells significantly increased, and the salmonella did not proliferate inside the cells (Table 1). These results pointed to the ability of CT-B and the LKEKK peptide to stimulate bactericidal activity of the RAW 264.7 cells *in vitro*.

Table 1. The influence of CT-B and the LKEKK peptide on the phagocytosis of the bacteria of the *S. typhimurium* 415 virulent strain by the LPS-activated RAW 264.7 cells *in vitro** [40].

Protein/peptide, nM	PhA, %	CBA, %	PhN
- (Control)	46.2 ± 4.3	10.3 ± 2.0	3.2 ± 1.3
	56.3 ± 5.2	23.2 ± 3.2	7.0 ± 2.2
	49.2 ± 4.4	47.5 ± 4.1	10.2 ± 3.5
	36.2 ± 3.2	61.7 ± 5.3	12.1 ± 3.2
	0	~ 100	0
CT-B (100)	75.3 ± 6.2♥	3.2 ± 1.1♥	5.2 ± 2.3
	87.5 ± 7.3♥	7.6 ± 2.2♥	6.3 ± 2.2
	64.0 ± 8.2♥	13.2 ± 4.0♥	5.0 ± 2.2♥
	24.3 ± 5.4♥	17.3 ± 4.2♥	2.1 ± 1.3♥
	7.2 ± 3.1	14.3 ± 2.3♥	1.2 ± 0.3
LKEKK (100)	69.4 ± 5.2♥	3.1 ± 2.2♥	4.1 ± 2.2
	80.1 ± 6.3♥	8.2± 3.0♥	5.3 ± 2..
	56.6 ± 3.4	16.3 ± 2.2♥	4.3 ± 2.0♥
	27.2 ± 5.5	18.2 ± 4.1♥	3.2 ± 2.4♥
	6.2 ± 3.2	19.4 ± 3.2♥	1.3 ± 0.4

* The lines for every compound sequentially (top-down) corresponded to the data that were obtained in 1, 2, 4, 7, and 12 h.
♥The difference from the control is significant ($P < 0.05$).

6. Action of CT-B and Peptide LKEKK on Intestinal Epithelial Cells

The effect of CT-B and the LKEKK on rat IEC-6 and human Caco-2 intestinal epithelial cells was studied. ^{125}I-labeled CT-B was found to bind with high affinity to IEC-6 and Caco-2 cells (K_d 3.6 and 3.7 nM, respectively), and in both cases unlabeled TM- α $_{ƒ1}$IFN-α $_{,2}$the peptide LKEKK competitively inhibited its binding [17, 18]. At the same time, the peptide KKEKL did not affect the binding (K_i > 10 μM). Similar results were obtained when studying the binding of ^{125}I-labeled CT-B to membranes isolated from epithelial intestinal ratcells: the binding was characterized by a high affinity for (K_d 3.7 nM) and was inhibited by unlabeled IFN-α_2, TM-α_1 and the peptide LKEKK (K_i, 2.0 1.5 and 1.0 nM, respectively), unlabeled peptide KKEKL was inactive (K_i> 1 μM). In addition, CT-B and the LKEKK peptide did not affect the activity of adenylate cyclase and pGC [16]. At the same time, in the concentration range of 10-1000 nM CT-B and the peptide LKEKK in dose dependent manner increased the activity of sGC in rIEC-6 and Caco-2 cells and production of NO which is an activator of this enzyme; the peptide KKEKL tested in parallel was inactive [18].

It is known that NO is an inhibitory mediator in the intestine, endothelial NO is involved in local regulation of the blood flow of intestinal cells, and NO formed during inflammation contributes to the loss of the integrity of the intestinal mucosa [52, 53]. Increased NO production and subsequent local cytotoxicity are apparently one of the mechanisms for the development of necrotic enterocolitis (NEC) [54]. NO is synthesized from *L*-arginine by NO synthase (NOS), of which there are three isoforms inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) [55]. iNOS is not constitutively expressed; this enzyme is induced in response to inflammation and provides a high level of NO production [54]. The role of NO produced by iNOS in the pathogenesis of NEC was first described by Ford and colleagues who demonstrated increased expression of iNOS in resected human NEC tissue [56]. In animal models of NEC, inhibition of iNOS has been shown to attenuate inflammatory intestinal damage [57-59].

As noted above, the effects of low NO concentrations (~ 5 50 μM) are mediated by cGMP [29, 30]. The pattern of cGMP elevation closely correlated with the enhanced cell activation by NO. Our data [18] also suggest that low levels of NO activate sGC and, therefore, sGC-mediated signal pathway.

TNF-α is known to increase the expression of inflammatory mediators in Caco-2 cells, in particular IL-8 [60]. The anti-inflammatory potential of CT-B and the LKEKK peptide was studied in a model of TNF-α-induced inflammation in human Caco-2 cells *in vitro* [61]. For this, cells were treated with CT-B or peptide LKEKK at the concentration range of 10-5000 μM, and TNF-α (2 ng/mL) was added to induce inflammation. Experiments have shown that pre-treatment of Caco-2 cells with CT-B or the peptide LKEKK at the concentration of 100-5000 μM significantly reduced TNF-α-induced IL-8 secretion in a dose-dependent manner. The peptide KKEKL with inverted amino acid sequence tested in parallel was inactive; which indicates a high specificity of action of the peptide LKEKK. In addition, treatment with CT-B or the peptide LKEKK significantly reduced mRNA levels of IL-8, TNF-α, IL-6, and IL-1β, when compared to cells treated with TNF-α alone. In addition, a significant increase in the expression of the anti-inflammatory cytokine IL-10 was noted. Based on these results, it was concluded that. that both CT-B and the LKEKK peptide have an anti-inflammatory effect *in vitro*.

The effects of CT-B and the peptide LKEKK on dextran sodium sulfate (DSS)-induced production of inflammatory cytokines in the colon were studied. Both the protein and the peptide (20 mg/kg body weight orally for 14 days) significantly reduced TNF-α production when compared to positive control mice. At the same time, protein or peptide treated mice showed less pronounced clinical signs of inflammation. The results are consistent with those obtained *in vitro* experiments and show that both CT-B and the peptide LKEKK have an anti-inflammatory effect *in vivo*.

The ability of CT-B and the peptide LKEKK to increase the activity of sGC in target cells indicates the involvement of this enzyme in the realization of their effects. To prove this hypothesis, the effect of CT-B and the peptide LKEKK on TNF-α-induced secretion of IL-8 in Caco-2 cells with a partial or complete absence of sGC activity was investigated. Inhibition the enzyme activity is achieved using ODQ (1-H-[1,2,4]oxadiazolo[4,3-α]quinoxalin-l-one), an inhibitor of sGC [62]. According to the data obtained, a decrease in enzyme activity was accompanied by a loss in the ability of the protein and the peptide to inhibit the secretion of IL-8. Thus, the inhibitory effect of CT-B and the peptide LKEKK on secretion of pro-inflammatory cytokines in TNF-α-stimulated Caco-2 cells is mediated by the iNOS- sGC pathway.

7. Conclusion

The data presented in this review show that CT-B and the peptide LKEKK corresponding to residues 16-20 in thymosin-α_1 and 131-135 in interferon-α_2 bind to the cholera toxin receptor of the target cell with high affinity and trigger the following cascade of intracellular reactions: activation of inducible NO synthase → increase in NO production → increase in soluble guanylate cyclase activity → increase in the cyclic guanosine-3',5'-monophosphate level (Table 2).

Table 2. Effect of CT-B and the peptide LKEKK on various cell types.

Cell type	Binding affinity	iNOS	sGC	Reference
Human blood T lymphocytes	high	activate	activate	[13, 14]
Human blood B lymphocytes	high	activate	activate	[14]
Murine macrophage-like RAW 264.7 cell line	high	activate	activate	[40]
Human Caco-2 intestinal epithelial cell line	high	activate	activate	[17, 18]
Rat IEC-6 intestinal epithelial cell line	high	activate	activate	[17]

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