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Interaction of Cholera Toxin B Subunit with Intestinal Epithelial Cells

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1 Abstract

We have prepared ^{125}I-labeled cholera toxin B subunit (^{125}I-labeled CT-B, a specific activity of 98 Ci/mmol), and found that its binding to rat IEC-6 intestinal epithelial cells was high-affinity (K_d 3.7 nm). The binding of labeled protein was completely inhibited by unlabeled thymosin-α_1 (TM-α_1), interferon-α_2 (IFN-α_2), and the synthetic peptide LKEKK that corresponds to residues 16-20 in TM-α_1 and 131-135 in IFN-α_2 (K_i 1.5, 1.0 and 2.0 HM, respectively), but was not inhibited by the synthetic peptide KKEKL with inverted amino acid sequence (K_i > 10 μm). Thus, TM-α_1, IFN-α_2, and the peptide: LKEKK bind with high affinity and specificity to CT-B receptor on riec-6 cells. It was found that CT-B and the peptide: LKEKK at concentrations of 10 - 1000 nm increased in a dose-dependent manner the nitric oxide production and the soluble guanylate cyclase activity in the cells.

2 Keywords

Protein: Peptide; Receptor; Cholera toxin B subunit; IEC-6 cell line

3 Abbreviations

AC: adenylate cyclase, CT-B: cholera toxin B subunit, GC: guanylate cyclase, IFN-α: interferon-α, Kd: equilibrium dissociation constant, K_i: equilibrium inhibition constant, NOC-18: 2:2'-(hydroxynitrosohydrazino)bis-ethamine, rIEC-6: rat intestinal epithelial cell line, SEM: standard error of the mean, TM- α_1: thymosin-α_1.

4 Introduction

More than two decades ago octapeptide LKEKKYSP corresponding to the sequence 131-138 of human interferon-α_1 (IFN-α_2) capable of high affinity binding to murine thymocytes and human fibroblasts [32] was obtained. [31] Binding of labeled octapeptide was competitively inhibited by unlabeled IFN-α_2, thymosin-α_1 (TM-α_1) and cholera toxin B-subunit (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.

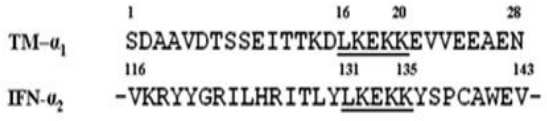

Figure 1: Comparison of amino acid sequences of TM-α_1 and IFN-α_2. The numbers of the amino acid residues are marked with numerals. Sequence of peptide LKEKK is underlined.

Recently we have synthesized the peptide LKEKK and found that [3H] LKEKK binds with high affinity to donor blood T lymphocytes and rat intestine epithelial cell membranes [16,17]. Treatment of cells and membranes with proteases did not affect [^{3}H] LKEKK binding, suggesting the non-protein nature of the peptide receptor. The binding was completely inhibited by TM-

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Table 1 Main characteristics of the synthesized peptides

Peptide	Purity, %	Amino acid analysis data	Molecular mass, D
LKEKK	>98	Glu 1.09 (1), Leu 1.00 (1), Lys 3.27 (3)	645.2 (calculated value ? 644.87)
KKEKL	>97	Glu 1.12 (1), Leu 1.03 (1), Lys 3.32 (3)	648.6 (644.87)

α_1, IFN-α_2, and cholera toxin B subunit (CT-B). Thus, using [^{3}H] LKEKK, we demonstrated the existence of a non-protein receptor common for TM-α_1, IFN-α_2, and CT-B on human T lymphocytes, and rat intestine epithelial cell membranes. We suggested that this receptor could be the cholera toxin receptor, as is known, is a GM1-ganglioside [9,12].

To prove this hypothesis, in this study we have prepared 125I-labeled CT-B and investigated its interaction with rat IEC-6 intestinal epithelial cells in the absence and in the presence of unlabeled TM- α_1, IFN-α_2, peptides LKEKK and KKEKL.

5 Material and methods

5.1 Chemicals

Na ^{125}I (2×10^6 Ci/M specific activity) was from Russian Scientific Center "Applied Chemistry" (St. Petersburg, Russia). All media, sera for culturing cells,1,3,4,6-tetra-chloro-3α,6α diphenilglicoluril (Iodogen), and other chemicals were obtained from Sigma (St. Louis, MO).

5.2 Peptides and proteins

Human thymosin-α_1 were obtained from Immunodiagnostic AG (Germany), cholera toxin B subunit was from Sigma (USA). Peptides LKEKK and KKEKL were synthesized on an Applied Bio systems Model 430A automatic synthesizer (USA) using the Boc/Bzl tactics of peptide chain elongation as described previously [24]. The peptides were purified to homogeneous state by preparative reverse-phase HPLC (Gilson chromatograph, France) on a Delta Pack C18 column, 100A (39×150 mm, mesh size 5 μm; flow rate 10 ml/min, elution with 0.1% TFA, gradient of acetonitrile 10–40% in 30 min). The molecular masses of the peptide were determined by fast atom bombardment mass spectrometric analysis (Finnigan mass spectrometer, San Jose, CA). The data of amino acid analysis (hydrolysis by 6 M hcl, 22h, 110°C; LKB 4151 Alpha Plus amino acid analyzer, Sweden) and mass spectrum analysis are presented in (Table 1).

5.3 Preparation of ^{125}II-labeled CT-B

CT-B (20μg) was labeled by solid phase oxidation method using Na125I (1 mci) and Iodogen [21]. The labeled protein was purified by gel filtration on Sephadex G-25 (0.9 × 10 cm column, 50 mm phosphate buffer, ph 7.4).

5.4 Cell culture

Rat IEC-6 and human Caco-2 intestinal epithelial cell lines were kindly provided by Moscow Research Epidemiology and Microbiology Institute. Cells were maintained at 37°C in 5% CO2 in DMEM medium supplemented with 10% fetal bovine serum. All experiments were performed with cells which were in between 15 and 25 passages. Cells in exponential growth phase used for all experiments.

5.5 Binding assay

The binding of 125I-labeled CT-B to riec-6 cells was assayed in 1 ml of RPMI-1640 medium, containing 10 mm Hepes, 20 mm nan3 and 0.6 mg/ml PMSF (ph 7.4). As following: 100 μl labeled protein (concentration range 10^{-10}-10^{-7}M, each concentration point in triplicate) plus 100 μl medium (for total binding) or 10^{-4}M unlabeled peptide (for nonspecific binding) were added to 800$^{\mu}$l cell suspension (10^6 cells) and incubated at 4°C for 40 min. Then the samples were filtered through Whatman GF/A glass fiber filters to separate cell-bound labeled protein from non-bound (free) one. Filters were washed three times with 5 ml ice-cold saline. Radioactivity was counted using Mini-Gamma counter (LKB, Sweden). The specific binding of ^{125}I-labeled CT-B to cells was determined as the difference between total and nonspecific binding that was measured in the presence of 10^{-4} M unlabeled protein. The specific binding of ^{125}I-labeled CT-B to cells was further characterized by the equilibrium dissociation constant K_d, To determine K_d, the ratio between molar concentrations of the bound (B) and free (F) labeled protein was plotted against molar concentration of the bound labeled protein (B) [20].

5.6 Competition assay

To estimate the inhibitory effects of TM-α_1, IFN-α_2, peptides LKEKK and KKEKL, the cells (10^6/ml) were incubated with 5 nm labeled CT-B and one of the tested ligands (concentration range, 10^{-12} 10^{-5} M; three measurements for each concentration) as described above. The inhibition constant (K_i) was calculated using the formula: $K_i = [IC_{50}]/(1 + [L]/K_d)$, where [L] is the^{125}I-labeled CT-B molar concentration; K_d is the equilibrium dissociation constant of the 125I-labeled CT-B–receptor complex; IC_{50} is the concentration of unlabeled ligand causing 50% inhibition of the labeled protein specific binding [3]. IC_{50} was determined graphically from the inhibition plots. The value of K_d was determined as described above. The data are presented as the means ± SEM of at least three independent experiments.

5.7 Fractionation of riec-6 cells

Cells were resuspended in 10 mm Tris-hcl buffer, ph 7.5, containing 1 mm mgcl2, 1 mm DTT, 5μm pepstatin A, 50 μm PMSF, 10 μm soybean trypsin inhibitor, and 1mm benzamidine. The resulting suspension (5×10^8cells/ml) was homogenized on an ice-cold bath, and the homogenate was centrifuged at 100000 g (10 min, 4°C). Supernatants were sampled, and sediments were resuspended in equal volumes of the buffer solution. The supernatants were used to determine the activity of sgc, and resus-

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pended sediments – to determine the activity of AC and pgc.

5.8 Nitric oxide assay

In oxygenated medium, NO is rapidly oxidized to NO2 . Therefore, riec-6 cell media was collected in 1.5 ml tubes and the samples were assayed in duplicate for nitrite (NO2) using a chemiluminescence nitric oxide analyzer as previously described (Chicoine et al., 2004). Briefly, 50μl of sample were placed in a reaction chamber containing a mixture of sodium iodide (nai) in glacial acetic acid to reduce NO2 to NO. The NO gas was carried into the NO-analyzer by a constant flow of Helium gas. The analyzer was calibrated with a nano2 standard curve.

5.9 Nitric oxide assay

The adenylate cyclase (AC) activity was determined with [α^{-32}P]ATP according to the method described earlier [22]. Isoproterenol activating AC via β-adrenergic receptors was taken as a positive control [30]. The enzyme activity was expressed in nanomoles of catp formed in 10 min per 1 mg of the membrane protein.

The guanylate cyclase (sgc and pgc) activity was measured by monitoring the conversion of [α^{-32}P]GTP to [^{32}P]cgmp ;the product was isolated by precipitation with zinc carbonate and chromatography on a column of aluminum oxide [23,26]. The enzyme activity was expressed as the amount of cgmp produced in 10 min (in nano moles per 1 mg protein). The protein concentration was determined by the Lowry method [14] using bovine serum albumin as a standard. Statistical study was performed by the Student's t-test.

6 Results

The main characteristics of the synthesized peptides are given in (Table 1).

6.1 Binding of ^{125}I-labeled CT-B to riec-6 cells

We found that ^{125}I-labeled CT-B bound specifically to riec-6 cells. The receptor-labeled CT-B complex reached dynamic equilibrium after $\sim$ 40 min of incubation at 4°C and remained in this state for at least two hours. Therefore, to assess the equilibrium dissociation constant (K_d) for the labeled protein binding to riec-6 cells, the reaction was carried out for 40 min. The nonspecific binding of ^{125}I-labeled CT-B under these conditions was 11.3 $\pm$ 0.9 % of its total binding. The Scatchard plot characterizing the specific binding of ^{125}I-labeled CT-B to riec-6 cells is shown in (Figure 2) The linear character of the plot indicates that there is one type of binding sites for labeled protein on the cells, K_d = 3.7 $\pm$ 0.3 nm.

To characterize the specificity of the ^{125}I-labeled CT-B binding to riec-6 cells, unlabeled TM-α_1, IFN-α_2, the peptide LKEKK, and the peptide KKEKL with the reverse sequence were tested as potential competitors. The K_i values (Table 2) demonstrated strong inhibitory capacity of TM-α_1, IFN-α_2, and the peptide LKEKK (K_i = 1.5 $\pm$ 0.3, 1.0 $\pm$ 0.3, 2.0$\pm$ 0.5), whereas the peptide KKEKL did not inhibit the ^{125}I-labeled CT-B binding (K_i > 10 μm), indicating a high specificity of TM-α_1, IFN-α_2, and the peptide LKEKK binding. Thus, TM-α_1, IFN-α_2, and the peptide LKEKK bind with high affinity and specificity to the CT-B receptor on riec-6 cells.

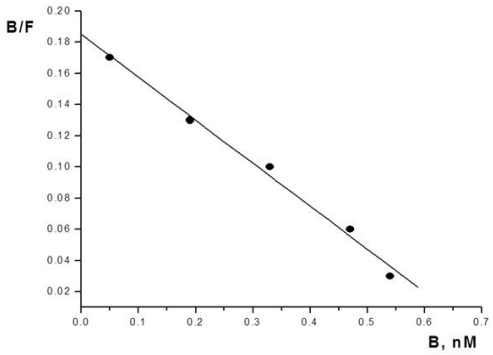

Figure 2: Scat chard analysis of the specific binding of ^{125}I-labeled CT-B to of rIEC-6 cells. B and F molar concentrations of bound and free labeled protein, respectively.

Table 2 Inhibition of ^{125}I-labeled CT-B specific binding to rIEC-6 cells by unlabeled ligands

Ligand	IC_{50} (nM)	K_i (nM)
IFN-α_2	1.5 $\pm$ 0.2	1.0 $\pm$ 0.2
TM- α_1	2.3 $\pm$ 0.3	1.5 $\pm$ 0.3
LKEKK	3.0 $\pm$ 0.2	2.0 $\pm$ 0.3
KKEKL	>100	>100

Note: Values are averages (means $\pm$ SEM) of at least three independent experiments performed in triplicate. Assays conditions are described under Materials and Methods.

6.2 Effects of CT-B, peptides LKEKK and KKEKL on the nitric oxide production in riec-6 cells

The results presented in (Table 3) show that CT-B and the peptide LKEKK at concentrations of 10 - 1000 nM enhanced NO production (measured as NO_2^-) in a dose-dependent manner. The enhancement leveled off at the peptide or CT-B concentration of 100 nm. The peptide KKEKL was inactive.

Table 3 Effects of CT-B, peptides LKEKK and KKEKL on N0 production by rIEC-6 cells

Ligand (nM)	Level of NO_2^- (nmol of NO2/mL medium $\pm$ SEM)		
	CT-B	LKEKK	KKEKL
Control	16 $\pm$ 3		
0.1	14 $\pm$ 3	16 $\pm$2	17 $\pm$ 3
1	15 $\pm$ 2	18 $\pm$ 4	14 $\pm$ 3
10	20 $\pm$ 3	19 $\pm$3	16 $\pm$ 4
100	28 $\pm$ 3*	26 $\pm$ 2*	18 $\pm$ 3
1000	29$\pm$ 3*	27$\pm$3*	15 $\pm$ 3

* Significant differences between experience and control (P <0.05).

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Table 4

Effects of CT-B, peptides LKEKK and KKEKL on the sGC and pGC activity of rIEC-6 cells

Ligand (nM)	Guanylate cyclase activity (nmoles of cGMP per 1 mg protein in 10 min± SEM)					
	sGC			pGC		
	CT-B	LKEKK	KKEKL	CT-B	LKEKK	KKEKL
Control	0.9 ± 0.1			1.5 ± 0.2		
1	0.8 ± 0.2	0.9 ± 0.2	1.0 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	1.4 ± 0.2
10	1.2 ± 0.2*	1.3 ± 0.2*	0.8 ± 0.3	1.5 ± 0.3	1.6 ± 0.2	1.5 ± 0.3
50	1.5 ± 0.3*	1.4 ± 0.3*	0.9 ± 0.2	1.6 ± 0.2	1.5 ± 0.2	1.5 ± 0.2
100	1.7 ± 0.3*	1.5 ± 0.3*	0.9 ± 0.2	1.4 ± 0.3	1.6 ± 0.3	1.4 ± 0.3
1000	1.6 ± 0.4*	1.5 ± 0.3*	0.9 ± 0.3	1.6 ± 0.2	1.7 ± 0.2	1.8 ± 0.3

* Significant differences between experience and control (P <0.05).

Table 5 Effects of CT-B, isoproterinol, peptides LKEKK and KKEKL on the AC activity of rIEC-6 cell membranes

Concentr--ation of compound (nM)	Adenylate cyclase activity (nmoles of cAMP per 1 mg protein in 10 min)			
	CT-B	LKEKK	KKEKL	Isopro terinol
0	1.8 ± 0.2			
0.1	1.9 ± 0.3	2.0 ± 0.3	1.9 ± 0.2	1.8 ± 0.2
1	1.8 ± 0.2	1.9 ± 0.4	1.9 ± 0.3	2.2 ± 0.3
10	1.9 ± 0.2	1.9 ± 0.2	1.7 ± 0.2	2.4 ± 0.2
100	1.7 ± 0.3	1.8 ± 0.3	1.8 ± 0.2	2.8 ± 0.3
1000	1.9 ± 0.2	1.9 ± 0.2	1.9 ± 0.3	3.2 ± 0.4

6.3 Effects of CT-B, peptides LKEKK and KKEKL on the AC, sgc, and pgc activity of riec-6 cells

The results presented in (Table 4) show that CT-B and the peptide LKEKK at concentrations of 10 - 1000 nM increased in a dose-dependent manner the sgc activity in riec-6 cells, but did not affect the pgc activity as vell as the AC activity (Table 5). The peptide KKEKL was inactive. Thus, the activating action of CT-B and the peptide LKEKK on sgc was specific and dose-dependent.

7 Discussion

Cholera toxin (CT) is the soluble toxin secreted by the Gram negative bacteria Vibrio cholerae. CT is an 84 kd protein made up of two major subunits, CT-A and CT-B [29]. The CT-A subunit is responsible for the disease phenotype while CT-B provides a vehicle to deliver CT-A to target cells. CT-A is a 28 kd subunit consisting of two primary domains, CT-A1 and CT-A2, with the toxin activity residing in the former and the latter acting as an anchor into the CT-B subunit [29]. 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 (Lai, 1977) and binding to the monosialotetrahexosylganglioside (GM1a, Galβ3GalNAcβ4 (Neu5Acα3) Galβ4GlcCer) that is broadly distributed in a variety of cell types including epithelial cells of the gut [4,9,12].

CT-B is now viewed as a promising immune modulating and anti inflammatory agent. Recombinant CT-B has been recently found to suppress immunopathological reactions in allergy and autoimmune diseases to stimulate humoral immunity and to induce anti inflammatory responses in vivo, in particular, to mitigate the intestinal inflammation of Crohn's disease in mice and humans [25,29,2,28]. Since CT-B can prevent infection but also autoimmune reactions, the question is how these two apparently opposite immune reactions can be achieved by the same protein. Currently, there is no answer to this question.

The results of this study show that 125I-labeled CT-B binds with high affinity to riec-6 cells (K_d = 3.7 nm, Figure 2), and unlabeled TM- α_1, IFN-α_2, the peptide LKEKK inhibit its binding by 100% (Figure 3, Table 2). Previously, we showed that [3H]LKEKK binds with high affinity to non-protein receptor on donor blood T lymphocytes , and on rat intestine epithelial cell membranes [16,17]. In both cases, the labeled peptide binding was competitively inhibited by unlabeled TM- α_1, IFN-α_2, and CT-B. These data, as well as the fact that cholera toxin has no receptors other than GM1-ganglioside, allow us to conclude that GM1-ganglioside is the receptor common for TM- α_1, IFN-α_2, and the peptide LKEKK. The data in (Tables 3-5) show that CT-B and the peptide: LKEKK at concentrations of 10 - 1000 nm increased in a dose-dependent manner the nitric oxide (NO) production and the soluble guanylate cyclase (sgc) activity in riec-6 cells.

Nitric oxide (NO) is the principal inhibitory neurotransmitter in the gut, endothelial-derived NO is involved in the local regulation of mucosal blood flow and inflammatory-derived NO is involved in the loss of mucosal integrity [27,1]. Increased production of NO and subsequent local cytotoxicity to mucosal epithelial cells has been proposed as one of the putative mechanisms in the development of necrotizing enterocolitis (NEC) [6]. 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) [15]. Of the three isoforms of NOS described, inos is not constitutively expressed, but induced at high

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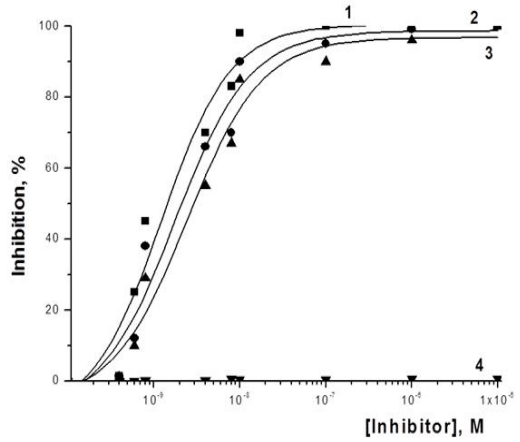

Figure 3: SInhibition of ^{125}I-labeled CT-B specific binding to rIEC-6 cells by unlabeled IFN-α_2 (1), TM-α_1 (2), peptides LKEKK (3) and KKEKL (4).

levels during inflammation resulting in relatively high levels of NO production [6]. The role of inos-derived NO in the pathogenesis of NEC was first described by Ford and colleagues who demonstrated elevated inos expression in resected human NEC tissue compared to non-NEC control tissue [10]. In experimental models of NEC, the inhibition of inos has been found to attenuate inflammatory intestinal injury [7,11,8].

According to the results of this work, CT-B and the peptide: LKEKK increased in a dose-dependent manner the sgc activity in riec-6 cells. Sgc, a heterodimer consisting of α and β subunits, which is activated by the direct interaction of NO with the heme of the β subunit [13]. There is conclusive evidence that effect of low concentrations of NO is cgmp dependent [18, 19]. These authors measured the levels of intracellular cgmp in CD4+ T cells cultured in the presence of graded concentrations of NOC-18. Cgmp concentration was significantly elevated by 5 μm and 10μm of NOC-18; this amount declined to the control level at 100 μm of NOC-18. These results strongly indicate that the enhancing effect of low concentrations of NO is mediated by cgmp. The pattern of cgmp elevation closely correlated with the enhanced cell activation by NO. Our data also suggest that low levels of NO activate sgc.

As we mentioned above, CT-B has a high potential as an immune modulatory and anti-inflammatory agent. In this regard, the study of the activity of the peptide LKEKK capable of binding to GM1-ganglioside and activating sgc is of great interest.

8 Conclusion

The results of the present study show that TM-α_1, INF-α_2, and the synthetic peptide LKEKK that corresponds to residues 16-20 in TM-α_1 and 131-135 in IFN-α_2 bind with high affinity and specificity to cholera toxin receptor on rat IEC-6 intestinal epithelial cells. The CT-B and peptide LKEKK binding to the receptor leads to an increase in activity of soluble guanylate cyclase. Residues 16-20 in TM-α_1 and 131-135 in IFN-α_2 are involved in binding to the receptor.

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9.1 Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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