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Interaction of cholera toxin B subunit with T and B lymphocytes

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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 T and B lymphocytes from the blood of healthy donors was high-affinity (K_d 2.8 and 3.0 nM, respectively). 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, 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 donor blood T and B lymphocytes. It was found that CT-B and the peptide: LKEKK at concentrations of 10–1000 nM increased in a dose-dependent manner the soluble guanylate cyclase activity in T and B lymphocytes.

1. Introduction

Recently we have synthesized the peptide LKEKK corresponding to the sequence 16–20 of human thymosin-α_1 (TM-α_1) and the sequence 131–135 of human interferon-α_2 (IFN-α_2) and found that [^3H]LKEKK binds with high affinity to donor blood T lymphocytes [1]. Treatment of cells with trypsin or proteinase K did not affect [^3H]LKEKK binding, suggesting the non-protein nature of the peptide receptor. The binding was completely inhibited by TM- α_1, IFN-α_2, and cholera toxin B subunit (CT-B). Thus, using [^3H]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. We suggested that this receptor could be the cholera toxin receptor.

To prove this hypothesis, in this study we have prepared ^{125}I-labeled CT-B and investigated its interaction with human blood T and B lymphocytes in the absence and in the presence of unlabeled TM- α_1, IFN-α_2, peptides LKEKK and KKEKL.

2. Material and methods

2.1. 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).

2.2. Preparation of ^{125}I-labeled CT-B

CT-B (20 μg) was labeled by solid phase oxidation method using Na^{125}I (1 mCi) and Iodogen [2]. The labeled protein was purified by gel filtration on Sephadex G-25 (0.9 × 10 cm column, 50 mM phosphate buffer, pH 7.4).

2.3. Isolation of T and B lymphocytes

Mononuclear cells were isolated from the blood of healthy donors as described in [3]. T and B cells were isolated by the method of Patel et al. [4] using dense polystyrene beads coated with mouse antibodies against human CD3 and CD19, respectively. The method yielded a > 95% pure population of target (CD3 +) T and (CD19 +) B lymphocytes.

2.4. Binding assay

The binding of ^{125}I-labeled CT-B to T and B lymphocytes was

Abbreviations: CT-B, cholera toxin B subunit; IFN-α, interferon-α; K_d, equilibrium dissociation constant; K_i, equilibrium inhibition constant; SEM, standard error of the mean; TM- α_1, thymosin-α_1

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assayed in 1 ml of RPMI-1640 medium, containing 10 mM Hepes, 20 mM NaN_3 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 µl 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 lymphocytes 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) [5].

2.5. 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: $K_i = [IC_{50}] / (1 + [L] / K_d)$ [6], where [L] is the ^{125}I-labeled CT-B molar concentration; K_d is the equilibrium dissociation constant of the ^{125}I-labeled CT-B–receptor complex; IC_{50} is the concentration of unlabeled ligand causing 50% inhibition of the labeled protein specific binding. IC_{50} was determined graphically from the inhibition plots. The value of K_d was determined as described above.

2.6. Measurement of soluble (sGC) and particulate (pGC) guanylate cyclase activity

Subcellular fractions from T and B lymphocytes were obtained at 4 °C as described earlier [7]. Cells were resuspended in 10 mM Tris–HCl buffer, pH 7.5, containing 1 mM $MgCl_2$, 1 mM DTT, 5 µM pepstatin A, 50 µM PMSF, 10 µM soybean trypsin inhibitor, and 1 mM benzamidine. The resulting suspension (5×10^8 cells/ml) was homogenized on an ice-cold bath, and the homogenate was centrifuged at 100,000*g* (10 min, 4 °C). Supernatants were sampled, and sediments were resuspended in equal volumes of the buffer solution. The supernatants and resuspended sediments were used for determining the activity of sGC and pGC, respectively.

The guanylate cyclase activity was measured by monitoring the conversion of [α-^{32}P]GTP to [^{32}P]cGMP [8]; the product was isolated by precipitation with zinc carbonate and chromatography on a column of aluminum oxide [9]. The guanylate cyclase activity was expressed as the amount of cGMP produced in 10 min (in pmol per 1 mg protein). The protein concentration was determined by the Lowry method [10] using bovine serum albumin as a standard. Statistical study was performed by the Student's *t*-test.

3. Results

The main characteristics of the synthesized peptides (purity, amino acid content, and molecular mass) are shown in Table 1. The specific activity of ^{125}I-labeled CT-B was 98 Ci/mmol.

3.1. Binding of ^{125}I-labeled CT-B to T and B lymphocytes

We found that ^{125}I-labeled CT-B bound specifically to T and B lymphocytes. The receptor-^{125}I-labeled CT-B complex reached dynamic equilibrium after ~40 min of incubation at 4 °C and remained in this state for at least 2 h. Therefore, to assess the equilibrium dissociation constant (K_d) for the labeled protein binding to lymphocytes, the reaction was carried out for 40 min. The nonspecific binding of ^{125}I-labeled CT-B under these conditions was 9.3 ± 0.9 and 10.2 ± 0.8% of its total binding with T and B lymphocytes, respectively. The Scatchard plots characterizing the specific binding of ^{125}I-labeled CT-B to T and B lymphocytes are shown in Fig. 2. The linear character of the plots indicates that there is one type of binding sites for labeled protein on T and B cells, K_d = 2.8 ± 0.3 and 3.0 ± 0.3 nM respectively.

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)

To characterize the specificity of the ^{125}I-labeled CT-B binding to T and B lymphocytes, unlabeled TM-α_1, IFN-α_2, the peptide LKEKK corresponding to residues 16–20 in TM-α_1 and 131–135 in IFN-α_2, 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-α1, IFN-α2, and the peptide LKEKK (K_i = 3.3 ± 0.3, 2.9 ± 0.3, 3.6 ± 0.5 and 3.7 ± 0.3, 3.3 ± 0.4, 3.8 ± 0.4 nM for T and B cells, respectively), whereas the peptide KKEKL with inverted sequence did not inhibit ^{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 donor blood T and B lymphocytes.

3.2. Effects of CT-B, peptides LKEKK and KKEKL on the sGC and pGC activity of T and B lymphocytes

The results presented in Table 3 show that CT-B and the peptide LKEKK at concentrations of 10–1000 nM increased in a dose-dependent manner the sGC activity in T and B lymphocytes, but did not affect the pGC activity (Table 4). The peptide KKEKL was inactive. Thus, the activating action of CT-B and the peptide LKEKK on sGC was specific and dose-dependent.

4. Discussion

Cholera toxin (CT) is the soluble toxin secreted by the Gram negative bacteria *Vibrio cholerae*. CT is an 84 kDa protein made up of two major subunits, CT-A and CT-B [11]. 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 kDa 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 [12]. CT-B forms a ring-like structure composed of five CT-B monomers. Each

Table 2
Inhibition of ^{125}I-labeled CT-B specific binding to T and B lymphocytes by unlabeled ligands.

Ligand	IC_{50} (nM)		K_i (nM)	
	T cells	B cells	T cells	B cells
TM- α_1	9.2 ± 0.7	10.0 ± 0.9	3.3 ± 0.3	3.7 ± 0.3
IFN-α_2	8.8 ± 0.1	8.9 ± 0.7	2.9 ± 0.3	3.3 ± 0.4
LKEKK	10.1 ± 0.9	10.3 ± 0.8	3.6 ± 0.5	3.8 ± 0.4
KKEKL	> 10,000	> 10,000	> 10,000	> 10,000

Note: Values are averages (means ± SEM) of at least three independent experiments performed in triplicate. Assays conditions are described under "Material and methods" section.

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