Binding of cardiotoxin analogue III from Formosan cobra venom to FL cells

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The binding equilibrium at 37 or 0°C of ^{125}I-cardiotoxin analogue III (CT III) to fetal lung (FL) cells (cultured human amnion cells) was achieved within 1 h, and the binding at 37°C was irreversible. The Scatchard analysis at 37 C on the binding of ^{125}I-CT III indicated that FL cells had two types of binding sites with different association constants. The association constant and the number of high-affinity sites was 1.1×10^{10} mol^{-1} or 2.8×10^6 per FL cell, respectively. At 37 or 0 C, the cytotoxicity of CT III paralleled the amount of bound CT III to FL cells, and at 37 C was inhibited by the presence of acidic phospholipids.

Cardiotoxin Cytotoxicity Acidic phospholipid (FL cell, Cobra venom) Scatchard analysis Binding equilibrium

1. INTRODUCTION

A number of publications have appeared on the amino acid sequences of cytotoxins from various cobra venoms [1]. From the sequence data, the positive charge, hydrophobicity and conformation of the cytotoxin molecule are supposedly essential for the cytotoxicity [2,3]. Therefore, we presumed that cytotoxins attach the acidic hydrophobic components on cell membranes. Braganca et al. [4] reported that phosphatidic acid or phosphatidylserine inhibited the lytic action of P_6 protein on Hoshida sarcoma cells. To ascertain the generality of this phenomenon, we examined the effects of cell membrane components on the cytotoxic activity of CT III against FL cells.

On the other hand, the cytotoxicity of cytotoxins was dependent on cell species [5,6] or temperature [6], and Condrea et al. [7] reported that the amounts of bound ^{131}I-DLF were related to the hemolytic activities against various erythrocytes. To elucidate the relationship between the binding amount and the temperature-dependent cytotoxicity, we investigated the quantitative binding of ^{125}I-CT III to FL cells at 37 or 0°C. Furthermore, we report here the results of Scatchard analysis at 37°C on the binding of ^{125}I-CT III to FL cells.

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Abbreviations: FL, fetal lung; CT, cardiotoxin

2. MATERIALS AND METHODS

CT III was isolated from Formosan cobra venom according to [8]. The strong basic polypeptides isolated from a specific venom or from venoms of different species or subspecies have been designated as cardiotoxin, skeletal muscle depolarizing factor, cobramines A and B, cytotoxin, toxin γ, toxin direct lytic factor, protein 12B and others. In this paper, we use the name 'cardiotoxin (CT)' because of their effect on the heart and because of the precedent established by Lee et al. [9]. Na^{125}I (2 mCi) was purchased from the Radiochemical Centre, Amersham, England. FL cells (cultured human amnion cells) were purchased from Flow Labs (VA, USA) and were

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maintained in monolayers at 37°C in Eagle's minimal essential medium supplemented with 2% calf serum. Phospholipids, ganglioside, phosphatidic acid, cardiolipin, and sialic acid were products of Sigma (USA). All other chemicals were commercial preparations of the highest quality available.

2.1. *Effects of cell membrane components on cytotoxicity of CT III*

FL cells were dispersed with 0.2% trypsin and suspended in phosphate-buffered saline to a concentration of 2.5×10^7 cells/ml. To 0.5 ml aliquots of the suspension, various concentrations of each cell membrane component were added separately, followed by CT III at a final concentration of 20 μg/ml. Then the mixtures were incubated at 37°C for 30 min. The cytotoxic activities were measured by the trypan blue exclusion test as described [8]. The inhibitory effect on the cytotoxicity was expressed as CRD_{50}, i.e. the concentration of the compound required to reduce the cytotoxicity given by 20 μg/ml of CT III to 50% of its value.

2.2. *^{125}I-labeling of CT III*

CT III was labeled with ^{125}I in the presence of chloramine T as described by Hunter and Greenwood [10]. TGhe reaction mixture contained 2 mCi $Na^{125}I$, 200 μg chloramine T and 1 mg CT III in 0.3 ml phosphate-buffered saline. The iodination reaction was allowed to proceed for 1 min at room temperature and stopped by the addition of 0.2 ml sodium metabisulfite (0.5 mg), followed by 0.4 ml of 1% KI solution in phosphate-buffered saline containing 0.25% BSA, referred to hereafter as assay buffer. The labeled CT III was separated from iodine by passage through a Sephadex G-25 column (0.9 × 44 cm) equilibrated with assay buffer. 1 ml fractions were collected, from each of which a 1 μl sample was taken for gamma counting in an Alloka autogamma counter. The specific activity of ^{125}I-CT III was 8×10^5 cpm/μg protein.

2.3. *Binding assay of ^{125}I-CT III to FL cells*

All binding assays were performed in assay buffer, and tubes were siliconized by a Siliconiser (Fuji Systems Corp.). To examine the time course of association of ^{125}I-CT III with FL cells, 1.5 μg ^{125}I-CT III was added to each of a series of tubes each containing 0.5 ml FL cell suspension (2.5×10^7 cells/ml), and incubated at 37 or 0°C for 0.5, 1, 2 or 3 h. After incubation, cells were washed three times with assay buffer by centrifugation at $3000 \times g$ for 5 min. The radioactivity of ^{125}I-CT III bound to FL cells was measured in an Alloka autogamma counter. To study the course of dissociation of ^{125}I-CT III from FL cells, ^{125}I-CT III bound FL cells were incubated at 37°C for 0.5, 1 or 2 h in assay buffer containing 30 μg/ml of CT III. Each incubation mixture was centrifuged at $3000 \times g$ for 5 min and the radioactivities of pellets measured in an Alloka autogamma counter. For Scatchard analysis, 0.5 ml FL cell suspensions (2.5×10^7 cells/ml) were incubated with various amounts (0.06–3.0 μg) of ^{125}I-CT III for 1 h at 37°C. After incubation, cells were washed three times with assay buffer by the centrifugation method. The radioactivity of ^{125}I-CT III bound to FL cells was assessed in an Alloka autogamma counter.

2.4. *Time course of cytotoxicity of I-CT III*

CT III was iodinated with NaI in the presence of chloramine T. Iodinated CT III (I-CT III) was added at a final concentration of 3 μg/ml to tubes each containing 0.5 ml FL cell suspension (2.5×10^7 cells/ml) in assay buffer, and then incubation was carried out at 37 or 0°C for 0.5, 1, 2 or 3 h. After incubation, the cytotoxicity (%) was measured by the trypan blue exclusion test as described [8].

Table 1

Concentrations of cell membrane components required for 50% inhibition of the cytotoxicity of cardiotoxin (CT-III)

Cell membrane component	CRD_{50} (μg/ml)
Phosphatidylserine (PS)	8
Phosphatidylinositol (PI)	13
Cardiolipin (CL)	14
Ganglioside	38
Phosphatidylcholine	>200
Phosphatidylethanolamine	>200
Spingomyelin	>200
Cholesterol	>200
Phosphatidic acid	>200
Sialic acid	>200

CRD_{50} means the concentration required for 50% inhibition of the cytotoxicity given by CT-III at 20 μg/ml

3. RESULTS

CRD_{50} values of the various cell membrane components tested are shown in table 1. Phosphatidylserine (PS), phosphatidylinositol (PI), and cardiolipin (CL) were more inhibitory against the cytotoxicity of CT III than was ganglioside, whereas the CRD_{50} of the other compounds was greater than 200 μg/ml. The time course of the association of ^{125}I-CT III with FL

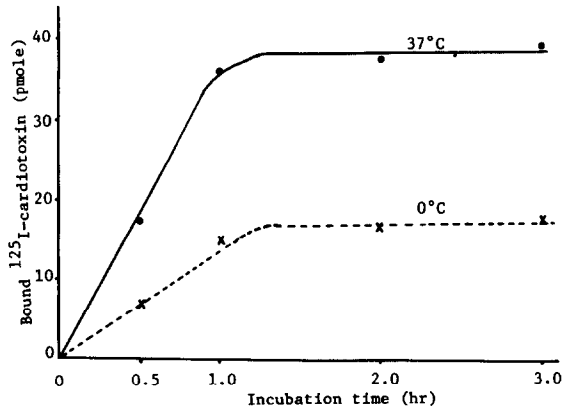

Fig.1. Time course of association of ^{125}I-cardiotoxin analogue III (CT III) with FL cells at 37 or 0°C. ^{125}I-CT III (1.5 μg or 1.2×10^6 cpm) was added to each of a series of tubes each containing 0.5 ml FL cell suspension (2.5×10^7 cells/ml) in assay buffer and incubated at 37 or 0°C for 0.5, 1, 2 or 3 h. ^{125}I-CT III-bound FL cells were washed three times by centrifugation, and their radioactivities measured in an Alloka autogamma counter.

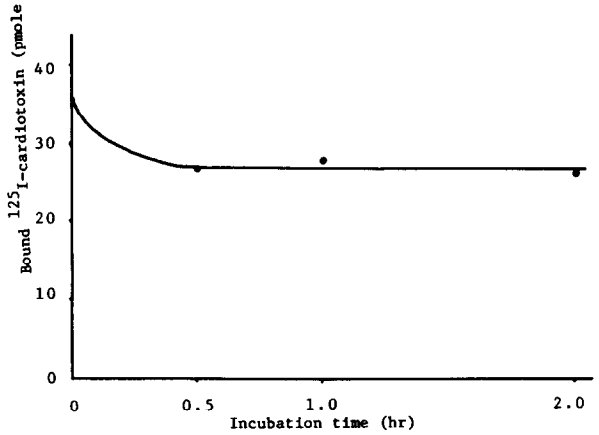

Fig.2. Time course of dissociation of ^{125}I-cardiotoxin analogue III (CT III) from FL cells at 37°C. FL cells which had been exposed for 1 h to ^{125}I-CT III under the conditions of fig.1 were incubated at 37°C for 0.5, 1 or 2 h in assay buffer containing a 10-fold excess of CT III. The amount of radioactivity which remained on the FL cells after each incubation period was measured in an Alloka autogamma counter.

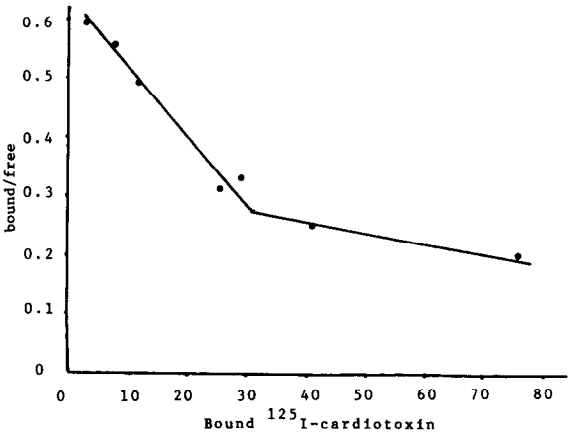

Fig.3. Scatchard plots of ^{125}I-cardiotoxin analogue III (CT III) binding to FL cells at 37°C. Aliquots (0.5 ml) of FL cell suspensions (2.5×10^7 cells/ml) were incubated in assay buffer with various amounts (0.06–3.0 μg) of ^{125}I-cardiotoxin for 1 h at 37°C. After the incubation, the radioactivity of ^{125}I-cardiotoxin bound to FL cells in each tube was counted in an Alloka autogamma counter, and the amount of free ^{125}I-CT III was derived by subtraction of the value of the bound ^{125}I-CT III from that of the total ^{125}I-CT III.

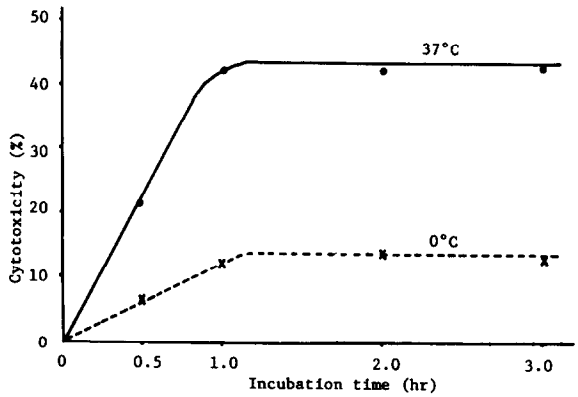

Fig.4. Time course of cytotoxicity of I-cardiotoxin analogue III (CT III) against FL cells at 37 or 0°C. I-CT III was added at 3 μg/ml to 0.5 ml aliquots of FL cell suspensions (2.5×10^7 cells/ml) in assay buffer, and then incubation was carried out at 37 or 0°C for 0.5, 1, 2 or 3 h. The cytotoxicity (%) was measured by the trypan blue exclusion test.

cells at 37 or 0°C is illustrated in fig.1. At both 37 and at 0°C, the binding reached its plateau at 1 h, however, the amount of binding at 37°C was greater than that at 0°C. The time course of dissociation of ^{125}I-CT III from FL cells at 37°C is displayed in fig.2. After 2 h incubation, little ^{125}I-CT III was released from the cells. The Scatchard plots gave a biphasic pattern as shown in fig.3, indicating the existence at 37°C of two types of binding sites with different association constants. The association constant and the number of high-affinity sites was 1.1×10^{10} mol^{-1} and 2.8×10^{6} per FL cell, respectively. The time course of cytotoxicity of I-CT III toward FL cells at 37 or 0°C is shown in fig.4. At either temperature, the cytotoxicity plateaued at 1 h, and that at 37°C was higher than that at 0°C.

4. DISCUSSION

As shown in table 1, because of their low inhibitory concentrations, CL and especially PS and PI appear to be targets of CT III on the FL cell membrane, and this speculation is consistent with the sequence data [2,3], which indicate that positive charge and hydrophobicity of the cytotoxin are supposedly essential for the cytotoxicity. But since conformation is also important [6], cytotoxins cannot be viewed as merely positive detergents. Braganca et al. [4] reported that phosphatidic acid or PS was able to prevent the lytic action of P_6 protein on Yoshida sarcoma cells, but our data (table 1) showed that phosphatidic acid had no effect on the cytotoxicity of CT III.

On the other hand, Condrea et al. [7] investigated the binding of direct hemolytic factor (DLF) in Ringhals venom to erythrocytes. Their data were partly different from ours, but this may be due to the species difference in the cells used. As shown in figs 1 and 4, the amount of binding of ^{125}I-CT III to FL cells was proportional to the cytotoxicity; therefore, the difference in level of cytotoxicity seen among various cells may be explained by variation in the amount of cytotoxin bound, as reported by Condrea et al. [7]. The difference between the degree of cytotoxicity at 37°C and that at 0°C may be due to the change in the state of the FL cell membrane, by which more ^{125}I-CT III was bound to the membrane at 37°C than at 0°C. PS and PI are thought to exist on the inner side of the cell membrane and CL, on the mitochondria inner membrane. This may explain the long lag time (1 h) required for the binding equilibrium to be reached, which is consistent with the report [7] that the binding of DLF to erythrocytes did not reach a plateau even after 2 h, but that to ghosts did within 10 min. Because little ^{125}I-CT III was released from FL cells even with a 10-fold excess of unlabeled CT III, this binding appears to be irreversible. As shown in fig.3, the Scatchard pattern shows an inflection at approx. 30 pmol ^{125}I-CT III bound to 1.25×10^{7} FL cells, an amount which caused about 40% of the cytotoxicity as calculated from figs 1 and 4. Therefore, this curve may reflect a change in the state of the FL cell membrane caused by the cytotoxicity.

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