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Quantitation of Binding and Subcellular Distribution of *Clostridium perfringens* Enterotoxin in Rat Liver Cells

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Binding of enterotoxin from *Clostridium perfringens* type A was studied in suspensions of parenchymal and nonparenchymal cells from rat liver. In hepatocytes, 1.5×10^6 specific binding sites per cell with an association constant of 3.2×10^6 M^{-1} were found. About 1% of the added toxin was nonspecifically bound to the hepatocytes. At concentrations of toxin below 0.1 μg/ml, 80% of the toxin added to a suspension of hepatocytes at 37°C was bound to the cells at a cell density of 7×10^6 cells per ml. Binding did not increase after the cells became permeable to the toxin. Subcellular fractionation in a sucrose gradient produced no evidence for binding to parts of the cell other than the plasma membrane. The degree of binding to nonparenchymal cells was less than 10% of the binding to hepatocytes.

Classical *Clostridium perfringens* food poisoning is caused by certain type A strains which produce enterotoxin. The predominant symptoms are diarrhea and abdominal cramps (15). The enterotoxin is a protein with molecular weight of 34,000, and it has been shown to contain only one polypeptide chain (13). No enzymatic activity has been detected so far.

The enterotoxin is lethal to experimental animals. Hyperemic small intestinal mucosa and congestion in the liver, lungs, spleen, and kidneys are characteristic pathological findings (23). The exact relationships between membrane transport alterations, metabolic disturbances, and tissue damage caused by the enterotoxin are not known (19).

It has been shown that the enterotoxin can inhibit amino acid transport in cultured hepatocytes (10). In Vero cells (from African green monkey kidney), the toxin causes morphological alterations, inhibition of macromolecular synthesis, and increased permeability (20). In experiments with isolated hepatocytes, enterotoxin concentrations above 2 μg/ml caused gross membrane damage with complete release of lactate dehydrogenase (32). The increased membrane permeability is probably related to the decreased amino acid transport in hepatocytes (11).

If mice or rats are injected intravenously with ^{125}I-labeled enterotoxin, most of the acid-precipitable radioactivity is recovered in the liver and kidneys. Concentrations of the toxin that kill parenchymal cells within 20 to 30 min are without effect on nonparenchymal cells (5). This paper presents studies on the binding of *C. perfringens* enterotoxin by parenchymal as well as nonparenchymal cells from rat liver and its subcellular distribution in parenchymal cells.

MATERIALS AND METHODS

Purification and labeling of enterotoxin. Purified enterotoxin was prepared from extracts of *C. perfringens* type A strain NCTC 8239 by a modification of the method of Sakaguchi et al. (26) as reported previously (13). The preparation gave one band on polyacrylamide gel electrophoresis. The toxin was labeled with ^{125}I by a modification (31) of the chloramine T method (14). The iodination was performed at room temperature, and the labeled protein fraction was separated from the unreacted iodine by gel filtration of the reaction mixture through a Sephadex G-50 column (0.9 by 15 cm). The degree of labeling was approximately 0.5 atoms of ^{125}I per molecule of enterotoxin, or 1 MBq/μg. The labeled toxin retained its biological activity as tested in the guinea pig skin test (33). It migrated slightly faster than the unlabeled toxin on polyacrylamide gel electrophoresis and was homogeneous on crossed immunoelectrophoresis (31). In certain experiments that did not require a high specific activity, toxin labeled with ^{125}I by the lactoperoxidase method (9) was used as indicated. This preparation had about one ^{125}I atom per 100 molecules of enterotoxin. No difference was detected between the binding properties of the two preparations.

Preparation of liver cells. Rat liver cells were prepared by a modification (29) of the collagenase perfusion method of Berry and Friend (6). Parenchymal and nonparenchymal liver cells were separated by differential centrifugation (24). Between 95 and 99% of the hepatocytes excluded trypan blue. The purified hepatocytes were suspended in a minimal incubation medium containing essential salts and 1% charcoal-treated bovine serum albumin (37).

Binding studies. Binding to cells was accomplished by mixing different amounts of ^{125}I-labeled enterotoxin with purified liver cells suspended in the minimal medium, either hepatocytes (7×10^6/ml) or nonparenchymal liver cells (2×10^6/ml). The cell suspensions were incubated at 37°C or 4°C in shaking water baths. Portions of the cell suspensions were removed at different time intervals, and the cells were separated from the medium by centrifugation through dibutyl phthalate (parenchymal cells) or a mixture of dibutyl and dinonyl phthalate (nonparenchymal cells) as described previously (24). Enterotoxin in concentrations above 0.5 μg/ml reduced viability of hepatocytes drastically. To include nonviable cells in the binding studies, the cells were centrifuged through a mixture of dinonyl and dibutyl phthalate. This mixture allows sedimentation of dead cells, whereas dibutyl phthalate does not. Cell-associated radioactivity was determined by counting the pellets in an LKB-Wallac 1280 automatic gamma counter. Scatchard (28) plots were analyzed by the SCAFIT computer program written by Munson and Rodbard (22).

Degradation of enterotoxin was followed by determining the amount of radioactivity which remained soluble after mixing an aliquot of the cell suspension with an equal volume of 4% phosphotungstic acid in 2 N HCl (17).

Isopycnic centrifugation. Hepatocytes incubated with ^{125}I-labeled enterotoxin for 60 min at 37°C and 4°C, respectively, were homogenized (35), and the nuclear fraction was centrifuged and washed once with 0.25 M sucrose. An aliquot of the combined supernatants (cytoplasmic extract) was layered on top of a sucrose gradient in which the sucrose concentration varied linearly from 20 to 53% (wt/wt). The gradients were centrifuged for 4 h at 4°C in a Beckman SW27 rotor at 25,000 rpm. The procedure has been described in detail (35).

Biochemical determinations. 5'-Nucleotidase (EC 3.1.3.5) was determined by the method of El-Aaser and Reid (8), and acid phosphatase was determined by the method of Barrett (4) with glycero-2-phosphate as a substrate. In both of these assays, liberated inorganic phosphate was determined spectrophotometrically by means of the molybdate/ascorbic acid reagent of Ames (2).

Materials. Various chemicals were tested for interference with the binding of ^{125}I-labeled enterotoxin to hepatocytes. Ethyleneglycol-bis-(2-aminoetyl ether)-*N,N'*tetraacetic acid (EGTA), methyl β-galactoside, methyl α-mannoside, *N*-acetylglucosamine, and neuraminidase (sialidase, EC 3.2.1.18) were obtained from Sigma Chemical Co., St. Louis, Mo. Units of neuraminidase activity are standard units as determined by the manufacturer without regard to deviation from optimum assay conditions in our experiments.

RESULTS

Binding of enterotoxin to parenchymal liver cells. The time course of increase of cell-associated enterotoxin at 37°C is shown in Fig. 1. Experiments with initial concentrations of 0.5, 2, and 5 μg/ml of enterotoxin are shown. When 2- and 5-μg/ml amounts of enterotoxin were added, all cells became permeable to trypan blue during the experiment. Only at the lowest concentration used (0.5 μg/ml) was the percentage of intact cells higher than 90 at the end of the incubation period. In Fig. 1, solid lines are drawn to a point where more than 50% of the cells were intact. Dashed lines indicate that less than 50% of the cells in the suspension excluded trypan blue.

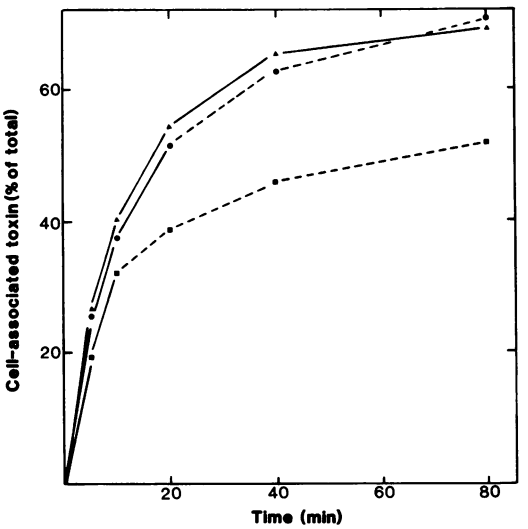

FIG. 1. Binding of labeled enterotoxin by hepatocytes at 37°C. The cell density was 7×10^6 cells per ml. Viability of the cells was monitored by the trypan blue exclusion test. To include the dead cells in the binding studies, a mixture of dinonyl and dibutyl phthalate was used in the centrifuge tubes (see the text). Fully drawn lines indicate that more than 50% of the cells in the suspension were viable, and dashed lines indicate that more than 50% of the cells were dead (see the text). Initial concentrations of enterotoxin were: ▲, 0,5 μg/ml; ●, 2 μg/ml; ■, 5 μg/ml.

Binding of labeled enterotoxin was also measured at five different initial concentrations from 1.6 to 200 ng/ml at 37°C. At these low concentrations of enterotoxin, the cells were still intact at the end of the incubation. All of these experiments gave binding curves which were very similar to the curves obtained with enterotoxin concentrations of 0.5 and 2 μg/ml (Fig. 1). The similarity between these curves means that a concentration of 2 μg/ml is not nearly high enough to saturate all of the binding sites on the cells. The system seemed to approach saturation when the initial concentration of enterotoxin was 5 μg/ml. The interpretation of the binding curves at 2 and 5 μg/ml is made more complicated by the fact that the dead cells are permeable to macromolecules, including the enterotoxin. However, the similarity between the binding curves at 0.5 and 2 μg/ml points toward the conclusion that the binding properties of the

dead cells are not radically different from those of the intact cells.

From the data presented in Fig. 1, estimates may be made of the number of toxin molecules bound when the percentage of intact cells falls below 50. These numbers are 2.8×10^6 molecules per cell at an initial concentration of 2 μg/ml and 4.4×10^6 molecules per cell at 5 μg/ml. These numbers should be interpreted in conjunction with estimates of the total binding capacity of the cells (see below).

No alteration in the rate of the binding reaction was observed on addition of 3 mM EGTA 5 min before the addition of ligand. This concentration of EGTA is sufficient to bind more than 99% of the calcium ions in the suspension. This observation excludes the possibility that the asialo-glycoprotein receptor (3) is involved in the uptake process since the affinity of this receptor is very low at concentrations of calcium ions below 0.1 mM (37). Hepatic uptake of enterotoxin through the asialo-glycoprotein receptor is also unlikely since binding is not increased in dead cells. In dead cells the large amount of intracellular asialo-glycoprotein receptors would probably be available for binding. As the results reported above point toward a large number of binding sites on the cell surface, it was desirable to test whether the toxin was bound to one of the sugar residues in the cell surface glycoproteins and glycolipids in the same way as, for instance, ricin binds to β-galactoside residues (25) and concanavalin A binds to α-mannoside residues (30). Concentrations (10 mM) of either methyl β-galactoside, methyl α-mannoside, or *N*-acetylglucosamine did not, however, affect the binding. In an attempt to see whether an increased number of exposed hexose residues would lead to an increase in binding (or, alternatively, whether the toxin bound to sialic acid residues) (21), the cells were preincubated with 100 mU of neuraminidase per ml for 30 min at 37°C and pH 7.4. The percentage of binding was increased approximately 10% over untreated cells. A possible explanation for this small increase may be the altered surface charge of the cells.

Binding of labeled enterotoxin at 4°C is shown in Fig. 2. At this temperature, the reaction approaches equilibrium more slowly than at 37°C. The initial rate of binding is only about 1/10 of the rate at 37°C, and the percentage bound at near equilibrium is 1/5 of the percentage at 37°C. After the cells were incubated with a trace amount of labeled toxin for 80 min and washed, dissociation of toxin from the cells was not detectable. Addition of a 100-fold excess of unlabeled toxin caused the dissociation of only 15% of the initial amount of cell-associated toxin during 20 min at 4°C, indicating that the binding

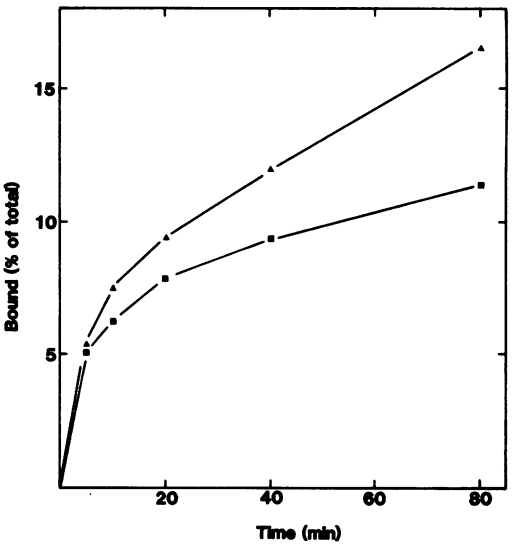

FIG. 2. Binding of labeled enterotoxin to hepatocytes at 4°C. The cell density was 10×10^6/ml. Initial concentrations of enterotoxin were: ▲, 0.1 μg/ml; ■, 10 μg/ml. The ligand was labeled by the lactoperoxidase method.

reaction is practically irreversible. From the experiments shown in Fig. 1 and Fig. 2, we cannot conclude that the higher uptake at 37°C is necessarily due to internalization of enterotoxin by the cells; an equally plausible explanation is an increase in the forward rate constant of the binding reaction of the toxin to its binding sites.

Binding of enterotoxin to nonparenchymal liver cells. To a suspension of nonparenchymal cells (mainly Kuppfer cells and endothelial cells) containing 2×10^6 cells per ml was added 0.08 μg of labeled enterotoxin per ml. The amount of cell-associated enterotoxin increased with time, reaching 2% of the added enterotoxin after 40 min at 37°C (data not shown). This is less than 10% of the amount bound by parenchymal cells under analogous conditions. Since binding could be abolished by adding 1 μg of cold enterotoxin per ml simultaneously with the labeled enterotoxin, it is likely that specific binding to a limited number of sites was involved in the uptake of labeled enterotoxin by the cells in the suspension. Nonparenchymal cells which were carefully purified by the differential centrifugation method still contain approximately 1% parenchymal cells. Therefore, the binding capacity of nonparenchymal cells relative to parenchymal cells is even smaller than that indicated by these figures.

Estimation of association constants and the number of binding sites. Binding of labeled enterotoxin at various concentrations was studied at 4°C, and the measurements were plotted

according to the method of Scatchard (28) (Fig. 3). Total concentrations of enterotoxin added to the cells ranged from 1 to 120 μg/ml. A trace amount (4 ng/ml) of labeled enterotoxin was added first, and then cold enterotoxin was added to give the concentrations required. The suspensions were permitted to come to equilibrium by incubation on a shaking water bath for 1 h, when the initial rapid phase of the uptake reaction (Fig. 2) was over.

The shape of the Scatchard (28) plot indicates the presence of either more than one class of binding sites or of nonspecific binding. From analysis of the data by means of the SCAFIT computer program (22), the best statistical fit was obtained by assuming one class of specific binding sites possessing an association constant of 0.14 ± 0.02 $(\mu g/ml)^{-1}$ and maximum specific binding capacity of 0.084 ± 0.009 $\mu g/10^6$ cells in the presence of $1.00 \pm 0.07\%$ nonspecific binding. The value of the association constant corresponds to 3.2×10^6 M^{-1}, and the maximum specific binding capacity corresponds to 2.5 pmol/10^6 cells or 1.5×10^6 sites per cell. As true equilibrium conditions could not be attained, these values are only approximations.

No binding of labeled toxin to glass flasks or plastic tubes was detected. About 30% of the nonspecific binding represented radioactivity in medium which was trapped between the cells in the pellet after centrifugation through oil; the rest of the nonspecifically bound toxin (about 0.7% of the added toxin) was bound to the hepatocytes.

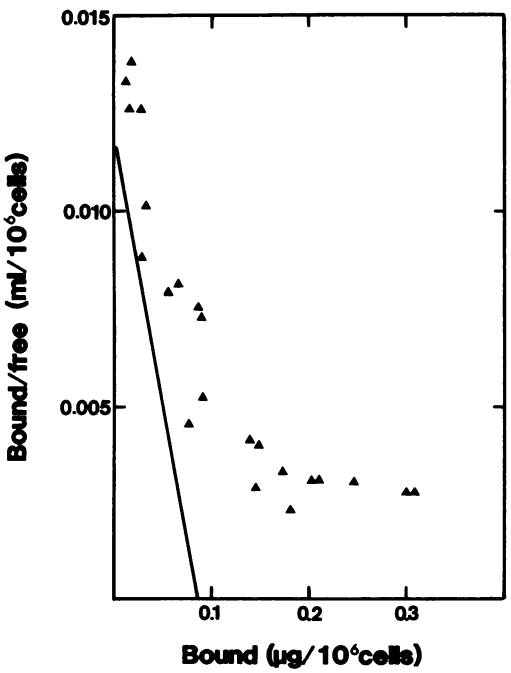

FIG. 3. Scatchard plots of binding of ^{125}I-labeled enterotoxin to hepatocytes at 4°C. The line represents binding to the high-affinity sites corrected for nonspecific binding (22).

Subcellular distribution of cell-associated enterotoxin. Cells that had been incubated with a trace amount (4 ng/ml) of labeled enterotoxin at 37°C were homogenized, the nuclei were removed by centrifugation, and the remaining cytoplasmic extract was fractionated by isopycnic centrifugation in a sucrose gradient (see above). In the gradient (Fig. 4), there is a striking similarity between the distribution of radioactivity and the distribution of the marker enzyme for the plasma membrane (5'-nucleotidase). A large amount of radioactivity is found at the very top of the gradient. This does not represent free enterotoxin as the cells were washed several times before homogenization.

During a 60-min incubation period, no more than 2 to 5% of the added enterotoxin was degraded. In principle, 2% of the radioactivity should be enough to produce a detectable lysosome-associated peak of radioactivity in the gradient. However, there is evidence that the transport of material into the lysosomes of isolated hepatocytes is relatively slow and that degradation in the lysosomes is rapid, so that little intact labeled protein is actually found within the lysosomes at any given time (3).

When the cells were incubated with labeled enterotoxin at 4°C for 60 min before homogenization, essentially the same distribution as that in Fig. 4 was found, but the peak of labeled enterotoxin in the middle of the gradient was smaller (data not shown). The total amount of

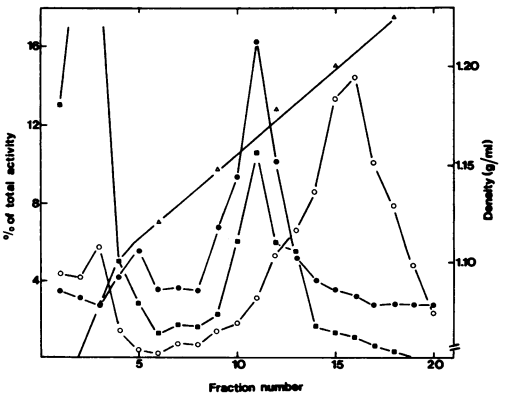

FIG. 4. Distributions of labeled enterotoxin and marker enzymes in a sucrose density gradient. ■, radioactivity; ●, 5'-nucleotidase (plasma membrane); ○, acid phosphatase (lysosomes); and △, density (scale on righthand side). Recoveries of radioactivity and enzyme activities were 90 to 105%.

cell-associated enterotoxin was also much smaller. The similarity of the distributions of radioactivity in cells incubated at 4°C and 37°C points toward the conclusion that the major part of the enterotoxin remains on the outside of the cell. At 4°C, the cell membrane is rigid (12), so that little endocytosis occurs (7).

DISCUSSION

By analysis of binding data, one class of high-affinity binding site was detected, along with a substantial amount of nonspecific binding to the cells. The number of high-affinity binding sites is close to the total binding capacity of Vero cells (20), in which binding occurs to a high-affinity and a low-affinity binding site. The binding data for the Vero cells were obtained at 37°C and may not be comparable to the corresponding values for hepatocytes, which were obtained at 4°C. This low temperature was chosen to avoid errors due to cell death and internalization of toxin.

In Fig. 3, the corrected line which shows specific binding falls outside all of the data points. The reasons for this apparent discrepancy may be outlined as follows, starting with a qualitative assessment of the influence of nonspecific binding on the points which reflect mainly specific binding. Nonspecific binding increases the amount of bound ligand. This is the predominant effect of nonspecific binding on the "specific" points, and it moves these points away from the origin. After correction for nonspecific binding, the line showing specific binding will, accordingly, always fall below the uncorrected points.

At the low initial concentration of 0.5 μg/ml (15 nM), the number of toxin molecules that was bound to the hepatocytes at 37°C was comparable to the number of specific binding sites (Fig. 1). As this concentration had only a small effect on the viability of the cells (Fig. 1) (29), the nonspecific binding sites may also be involved in the mechanism of action of the toxin. The nonspecific binding of *C. perfringens* enterotoxin by hepatocytes may be similar to the binding of concanavalin A by these cells. In the latter case, the number of binding sites is at least several hundred million (34). Binding of toxin by the cells is very similar whether or not the plasma membrane is permeable to macromolecules (including the toxin), indicating that the plasma membrane possesses most of the binding sites for the toxin. This conclusion is also supported by the results of the cell fractionation experiments (Fig. 4).

It was not possible in this investigation to obtain unequivocal evidence that nonparenchymal cells do not bind the toxin as nonparenchymal liver cells cannot be obtained completely free from parenchymal cells by differential centrifugation. All hepatocytes may be removed by treatment of the cell suspension with pronase; however, this treatment alters the surface of the remaining nonparenchymal cells. In this connection, it might be mentioned that the selective killing of parenchymal cells by enterotoxin is a very promising method for producing unaltered nonparenchymal liver cells in high yield (5).

It does seem possible that the lower binding capacity of nonparenchymal cells as compared with hepatocytes is the reason for the increased resistance of the nonparenchymal cells to the toxin. A relatively resistant Vero cell line had approximately 10% of the binding capacity of the sensitive cells (20).

When enterotoxin is added to the cells, there is a lag period, followed by rapid increase in the permeability of the cell membrane, as shown by the release of lactate dehydrogenase (31). The binding of labeled toxin was shown to be calcium independent. However, it has recently been shown (18) that Ca^{2+} is necessary to induce morphological alterations in Vero cells. This means that there are calcium-independent and calcium-dependent steps in the action of *C. perfringens* enterotoxin. From the present results, it appears that the increase in permeability occurs when 3×10^6 to 4×10^6 molecules of enterotoxin are bound per cell, which corresponds to somewhat more than the number of high-affinity binding sites.

No evidence was found for binding to organelles other than the plasma membrane. Binding by the plasma membrane is indicated by the similarity of the distributions of labeled enterotoxin and the plasma membrane marker enzyme 5'-nucleotidase in the gradient (Fig. 4) and the nearly identical distribution of the toxin in sucrose gradients, whether the cells had been incubated at 4°C or at 37°C.

The amount of radioactivity at the top of the gradient is much greater than that which is observed with other ligands that have been shown to be internalized (34, 35). The labeled toxin at the top of the gradient may originate from portions of the plasma membrane that do not contain 5'-nucleotidase.

Portions of the plasma membrane enter a sucrose gradient either as closed vesicles or as fragments that adhere to vesicles formed from the endoplasmic reticulum (1). Thus, the amount of toxin that enters the gradient is not a measure of the amount of toxin that has been internalized by the cells. The low rate of degradation of toxin provides indirect evidence that only a small fraction of the cell-associated toxin is located inside the cell, because the rate of degradation of internalized asialo-fetuin is 40 to 50%/h (37), whereas the rate of degradation of cell-associated toxin is less than 1/10 of this, indicating that

the toxin is internalized to a much smaller degree. (An alternative explanation is that the toxin is exceptionally resistant to proteolysis.)

We are not in a position to measure the amount of internalized toxin, and we cannot exclude the possibility that a tiny amount of internalized toxin is responsible for damage to the cells as seen for other toxins (27). In the case of the *C. perfringens* enterotoxin, however, solid evidence points to the plasma membrane as the site of damage (11, 32). Further investigation has to be undertaken to identify the binding site and establish the mechanism of membrane damage.

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