Pathogenesis of Shigella Diarrhea: Rabbit Intestinal Cell Microvillus Membrane Binding Site for Shigella Toxin

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This study examined the binding of purified 125I-labeled shigella toxin to rabbit jejunal microvillus membranes (MVMs). Toxin binding was concentration dependent, saturable, reversible, and specifically inhibited by unlabeled toxin. The calculated number of toxin molecules bound at 4°C was 7.9 × 1010 (3 × 1010 to 2 × 1011)/μg of MVM protein or 1.2 × 109 per enterocyte. Scatchard analysis showed the binding site to be of a single class with an equilibrium association constant, K, of 4.7 × 1010 M−1 at 4°C. Binding was inversely related to the temperature of incubation. A total of 80% of the labeled toxin binding at 4°C dissociated from MVM when the temperature was raised to 37°C, but reassociated when the temperature was again brought to 4°C. There was no structural or functional change of MVM due to toxin as monitored by electron microscopy or assay of MVM sucrase activity. These studies demonstrate a specific binding site for shigella toxin on rabbit MVMs. The physiological relevance of this receptor remains to be determined.

The role of the cell-free toxin produced by Shigella species in the pathogenesis of the intestinal manifestations of clinical shigellosis has been controversial (19). This toxin has been purified and shown to be a multimeric protein of Mr, 64,000, consisting of one biologically active A subunit (Mr, 32,000) which inhibits cytoplasmic protein synthesis at the ribosomal level, and five B subunits (Mr, 6,500) which mediate the binding of toxin to cell receptors (6). A role for toxin in pathogenesis is suggested by several observations: all Shigella species produce the toxin (20, 30; F. Auclair, A. Donohue-Rolfe, and G. T. Keusch, unpublished data); the purified protein causes fluid secretion by rabbit small intestine as well as the previously described neurotoxic and cytotoxic manifestations associated with the toxin (6); and patients make a serum toxin-neutralizing antibody during infection, indirectly demonstrating the production of toxin antigen in vivo (20, 23). In addition, by using a new enzyme-linked immunofluorescence assay method, we recently found toxin antigen in stools of patients with acute Shigella dysenteriae 1 infection (A. Donohue-Rolfe, M. Kelley, M. Bennish, and G. T. Keusch, submitted for publication).

Controversy has persisted, however, because of the known pathogenic importance of the invasive capacity of the organism and the failure of noninvasive but toxigenic Shigella variants to cause disease in either experimental animal models or human volunteers (11, 24). The latter argument is mitigated, however, because noninvasive strains fail to colonize the host. Even more importantly, it has recently been learned that noninvasive but colonizing enteric pathogens such as enteropathogenic serotypes of Escherichia coli (which do not produce either heat-labile or heat-stable E. coli toxins) and strains of Vibrio cholerae deleted of the genes for both the A and B subunits of cholera toxin, do cause diarrhea and produce a toxin (Shiga-like toxin) apparently identical or closely related to shigella toxin (25, 29).

Because the rabbit has been used as an experimental model to detect the enterotoxic activity of shigella toxin (19), we examined rabbit enterocyte microvillus membranes (MVMs) for the presence of a toxin receptor. In this paper we report the characteristics of this receptor.

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MATERIALS AND METHODS

Purification and assay of Shigella toxin. Toxin was produced from S. dysenteriae 1, strain 60R, and purified by our published methods (6). Bacterial cell lysates of broth-grown organisms were chromatographed on Blue Sepharose (Pharmacia, Inc., Piscataway, N.J.), eluted in 0.5 M NaCl, and subjected to chromatofocusing (LKB, Uppsala, Sweden). The toxin was recovered in the pH 7.0 to 7.1 fractions and passed over a Bio-Gel P-60 column (Bio-Rad Laboratoires, Richmond, Calif.) to remove amphotelytes. Purified toxin was lyophilized in NH4HCO3 buffer and stored at −70°C until reconstituted for use.

Toxin was assayed by cytotoxicity in HeLa cell monolayers and by enterotoxicity in ligated rabbit ileal loops as previously described (22, 23). Toxin protein was measured with the Bio-Rad Assay Kit II (Bio-Rad), with bovine serum albumin as standard.

Radiolabeling of toxin. Purified shigella toxin was iodinated by a modification of the chloramine T method (15). A 1-mCi amount of carrier-free 125I-labeled sodium iodide was added to 10 to 20 μg of toxin in 150 μl of sodium phosphate (0.1 M, pH 7.4). Next, 20 μl of chloramine T (2.5 mg/ml) was added, followed in 30 s by 20 μl of sodium metabisulfite (5 mg/ml). Finally, 100 μg of rabbit hemoglobin was added as a carrier protein, and the unbound iodine was separated on a 10-ml Sephadex G-25 column. Multiple iodinated toxin preparations were used for the different experiments; specific activity of these preparations ranged from 16,000 to 30,000 cpm per ng of toxin protein. There were no differences detected between native toxin and iodinated toxin when
jejunal loop enterotoxicity assays. In some experiments toxin was also labeled by the Bolton-Hunter method (1). Rabbits were sacrificed by using an overdose of nembutal, and the entire small bowel was removed, washed thoroughly for 5 min by vibrating at high frequency and low amplitude for 1 min. This pellet, containing the final MVM preparation, was chilled. After this, villus epithelial cells were released by vibrating on a metal rod in 0.1 M phosphate buffered saline (PBS), (pH 7.4) at 4°C. Cells were recovered by centrifugation, suspended with an equal volume of 0.1% sodium phosphate buffer (pH 7.4) and homogenized in a Waring blender at high speed for 2 min. CaCl2 was added to homogenates to give a final concentration of 10 mM. After incubation for 15 min, the homogenate was centrifuged at 3,000 × g for 15 min. The pellet containing cellular debris and organelles was discarded, and the supernatant was centrifuged at 27,000 × g for 30 min. The resulting pellet, which contained the crude MVMs, was suspended by homogenizing with a Potter-Elvejem homogenizer in 50 mM mannitol-10 mM Tris hydrochloride buffer (pH 7.1) and then was centrifuged at 27,000 × g for 30 min. This pellet, containing the final MVM preparation, was suspended in 0.01 M K2PO4 (pH 6.8) and stored in aliquots at −70°C for subsequent use. Unless otherwise indicated, all data were obtained by using this method of MVM preparation.

In one experiment, MVMs were also prepared by the same technique from a known number of isolated intestinal epithelial cells recovered by a modification of the method of Harrison and Webster (14). Everted jejunal segments were incubated at 4°C with a metal rod in 0.1 M phosphate buffered saline (PBS), (pH 7.4) at 4°C. Mucus and debris were first removed by vibrating at high frequency and low amplitude for 1 min. After this, villus epithelial cells were released by vibrating for 5 min. Cells were recovered by centrifugation, suspended in PBS (pH 7.4), and counted in a Neubauer hemocytometer chamber. When examined by phase contrast microscopy, isolated villus epithelial cells represented more than 90% of the population, as determined by their columnar morphology and the presence of a distinct apical brush border.

Protein content was measured by the method of Lowry et al. (26). The purification of the MVMs was assessed by the method of Grand and colleagues (12, 13, 16). The purified MVMs were enriched by 15- to 20-fold for sucrase activity. Where required, percent recovery of MVM protein was corrected on the basis of sucrase-specific activity.

Binding of 125I-labeled shigella toxin to MVMs. A modification of the membrane filter technique of Cuatrecasas was used for all binding studies (5). Triplicate reaction mixtures were prepared which contained various amounts of MVM protein (0.1 to 100 µg) in PBS (pH 7.4) containing 0.1% bovine serum albumin (PBS-BSA) and 125I-labeled shigella toxin, in a final volume of 100 µL. All experiments included duplicate controls not containing MVM protein. Although less than 1% of the added radioactivity was bound to MVMs in the absence of MVM, all data were corrected for background radioactivity determined from these controls. The mix was incubated at 4°C for 20 min, unless otherwise noted. Labeled shigella toxin bound to MVMs was then separated from free toxin by rapid filtration under vacuum through a 22-µm polyvinylidene fluoride Durapore filter (Millipore Corp., Bedford, Mass.). Filters were then washed with 10 ml of PBS-BSA, air dried, and counted in a gamma scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). Specific binding of shigella toxin to MVMs was determined by the simultaneous addition of labeled toxin plus a 1,000-fold excess of unlabeled toxin to duplicate sets of reaction mixtures. Residual counts under these conditions were subtracted from total counts in the absence of added cold toxin. Non-specific binding was never more than 2% of total binding. Values for the equilibrium association constant (K) and the number of receptors per microgram of MVM protein (n) were obtained by a least-squares fit of the data to a one-ligand, one-receptor Scatchard model (8), using the plot of the bound counts versus toxin concentration. The data were corrected for the binding efficiency of toxin experimentally measured in this study; that is, calculations assumed that the maximum binding of toxin was 50% of the input. Data were weighted in inverse proportion to the number of bound counts, as described by Rodbard (31).

Dissociation of 125I-labeled shigella toxin from MVMs was determined as follows. Labeled toxin was incubated with MVM at 4°C for 20 min, when a 1,000-fold excess of cold toxin was added. Portions were removed before and at various time intervals after the addition of the cold toxin, filtered, and washed as above, and the radioactivity bound to the filters was determined as described above.

To determine temperature dependence of binding, a 1-ml sample of reaction mixture containing 75 µg of MVMs and 50 ng of 125I-labeled shigella toxin was brought to equilibrium at 4°C, then warmed to 37°C, and finally chilled to 4°C again. Samples were removed after 20 min at each temperature during the experiment and were rapidly filtered and counted as above.

Electron microscopy. MVM (0.5 µg of protein) was incubated for 20 min in the presence or absence of 20 ng of shigella toxin in 100 µl of PBS-BSA. MVMs were fixed by the addition of an equal volume of ice-cold 8% gluteraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 2 h, the fixed vesicles were collected for processing by centrifugation, then dehydrated through graded ethanols, embedded in Epon, and sectioned with a diamond knife. Thin sections were examined with a JEOL electron microscope at 60 kV.

Statistical evaluation. All experiments were performed in triplicate and were repeated at least three times with different MVM preparations. Data are reported as the mean ± one standard deviation or standard error.

RESULTS

Binding of purified shigella toxin to MVMs. When 125I-labeled shigella toxin (3 to 4 ng in different experiments) was added to increasing amounts of MVM protein, toxin binding was directly related to the concentration of MVM present and was linear up to a concentration of 5 µg of MVM per 100 µl when the amount of labeled toxin became limiting (Fig. 1). The binding efficiency of iodinated toxin in the presence of excess MVM was 45 ± 5% (mean ± standard deviation). Labeling with the Bolton-Hunter reagent did not improve binding efficiency. Toxin that failed to bind to an excess of MVM was recovered in the filtrate. This material was devoid of cytotoxic activity in the HeLa cell system and did not bind to fresh MVM (data not shown). Iodinated toxin stock (i.e.,
not reacted with MVM) and toxin bound to MVM or recovered in the filtrate were solubilized in sodium dodecyl sulfate and subjected to polyacrylamide gel electrophoresis. Autoradiography of these three labeled toxin preparations revealed neither differences in relative density of the radioactive bands representing the two toxin subunits nor the presence of contaminants (data not shown). We excluded the possibility that toxin bound to MVM vesicles might escape capture by the membrane filters by measuring protein content and sucrase activity in filtrates of control reaction mixtures containing only MVM and buffer. No protein and only trace sucrase activity (<1% of the total applied to the filter) was found.

Binding was saturable when the concentration of MVM was fixed and increasing amounts of 125I-labeled toxin were added (Fig. 2). Until saturation was achieved, binding was directly proportional to the amount of labeled toxin that was added. Based on a molecular weight of 64,000 for the holotoxin (6), we calculated that 7.9 x 10^10 (3 x 10^10 to 2 x 10^11) molecules of toxin were bound per µg of MVM protein in different MVM preparations from individual rabbits. Analysis of equilibrium binding data according to the Scatchard model (as described in Materials and Methods) revealed a single class of binding sites with a K of 4.7 x 10^9 M^-1, determined in six separate MVM preparations (Fig. 3).

MVMs were also simultaneously obtained from isolated enterocytes and from mucosal scrapings in the same animal in order to determine the number of binding sites per enterocyte. In this experiment, 1 µg of MVM was calculated to represent 6.8 x 10^6 cells after correction for the actual recovery of MVMs based on sucrase-specific activity. By using this value to convert our data for number of binding sites per microgram of MVM protein to number of binding sites per cell, we calculate a value of 1.2 x 10^6 binding sites per enterocyte.

Specificity of binding. Heat-denatured toxin (90°C, 30 min) failed to bind to MVM (data not shown). In contrast, when various amounts of undenatured unlabeled shigella toxin were added simultaneously with a fixed amount of 125I-labeled native toxin to the reaction mix, competitive inhibition of binding occurred in a dose-related fashion (Fig. 4). Unrelated proteins, including 10 µg of purified cholera toxin per ml (a gift of D. Michael Gill), Concanavalin A, and wheat germ agglutinin, or 0.1% BSA or 0.01% hemoglobin, all failed to inhibit binding of 125I-labeled shigella toxin to MVM.

Dissociation of 125I-labeled shigella toxin. When an excess of cold toxin was added to MVM after the binding of labeled toxin at 4°C, dissociation of toxin was rapid, and 52 to 63% of specific counts were displaced within 5 min of the addition of the cold toxin, increasing to 75% by 1 h. However, dissociation was never complete, and even after 48 h, 8 to 9% of the label remained associated with MVM.

Effects of temperature on toxin binding. Toxin binding to MVMs was directly related to the incubation temperature (Fig. 5A). Binding at 4°C or 20°C reached equilibrium by 20 min; binding to MVM incubated at 37°C was detectable immediately upon the addition of toxin and did not increase with time. The level of binding at 37°C was approximately 1/30 of that observed at 4°C. Binding at all temperatures was competitively inhibited by excess unlabeled toxin. When binding was allowed to proceed to equilibrium at 4°C (20 min) and the temperature was then shifted to 37°C for another 20 min, approximately 80% of the bound toxin dissociated (Fig. 5B). However, shifting the temperature back to 4°C for 20 min resulted in reassociation of the toxin with the MVM to the same extent as originally observed at 4°C.

Effects of shigella toxin on integrity of MVM. Examination of electron photomicrographs of MVM incubated in the presence or absence of toxin revealed homogenous membrane vesicles of uniform size. There was no evidence of structural damage and no increase in the number of visible membrane fragments, and the vesicles remained intact and
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FIG. 3. Plot of the binding data (Fig. 2) calculated by the method
of Scatchard. The equilibrium binding constant, K, and the number
of receptors per microgram of MVM protein, n, were obtained by a
least-squares fit of the data to a one-ligand, one-receptor Scatchard
model in which maximum binding was assumed to be 50%.

closed. Sucrase activity of the MVM was unchanged when
vesicles were exposed to toxin (data not shown).

DISCUSSION

These results demonstrate a binding site for shigella toxin
on isolated MVM from adult rabbit jejunal enterocytes. This
binding site has the characteristics of a true receptor (18):
binding is rapid, dose dependent, reversible, and it exhibit:
saturability. Moreover, binding is specific, is competitively
inhibited by unlabeled native shigella toxin, is negligible
when toxin is heat denatured, and is unaltered in the
presence of a large excess of cholera toxin or other proteins
unrelated to shigella toxin. Analysis of the binding data by
the Scatchard method revealed a single class of binding site,
with K = 4.7 × 10^5 M^-1.

There was no evidence that toxin binding altered MVM
structural integrity or reduced sucrase activity. Although the
efficiency of binding of toxin to MVM (that is, the percentage
of the total labeled toxin capable of binding to an excess of
MVM) was only 45%, the nonbinding portion was devoid of
cytotoxic effects in HeLa cells. Sodium dodecyl sulfate-
polyacrylamide gel electrophoresis and autoradiography of
the separated labeled peptides showed only the two bands
found in unlabeled pure toxin, thus ruling out the possibility
that fragmented toxin subunits or contaminating proteins
were present in the toxin preparation used. We do not have
an explanation for these findings at the present time.
However, it should be pointed out that the analysis of binding by
the Scatchard method took these results into account by use
of a model in which maximum binding capacity of toxin was
assumed to be 50%.

The extent of toxin binding to MVM was dramatically
affected by changes in the temperature of incubation. Toxin
rapidly dissociated when the temperature was shifted from
4°C to 37°C and rapidly reassocated when the temperature
was again brought to 4°C. A similar temperature-dependent
change in cholera toxin binding has been observed, and it
has been speculated by Fishman and Atikkan (9) that
multivalent binding of the multimeric toxin B subunit to
the GM_1 ganglioside occurs at the higher temperature, thus
explaining the reduced binding of cholera toxin at 37°C
compared with that obtained at 4°C. This phenomenon alone
would not account for the 30-fold decrease in shigella toxin
binding at 37°C compared with that at 4°C. Temperature
dependence of the binding of biologically active molecules
may be a general phenomenon, since a number of other
examples have been reported, although the postulated
mechanism may be different in other cases. For example, Morris
et al. (29) have demonstrated by electron microscopy that
binding of diptheria toxin to Vero cells rapidly diminishes
after a shift of temperature from 4 to 37°C. They suggest that
the shift induces a conformational change involving the
receptor that does not favor toxin binding. Similar tempera-
ture effects have been reported by Critchley and co-
workers for tetanus toxin binding to cultured neurons (4) and
by Ciechanover et al. (2) for insulin binding to human
hepatoma cells. The physiological significance of this phe-
omenon is not clear. However, it should be pointed out that
whereas binding studies are generally performed at 4°C,
biological systems generally function at 37°C.

The calculated number of toxin molecules per
enterocyte at equilibrium at 4°C, 1.2 × 10^6 per cell, is similar
to the data reported by Eiklid and Olsnes (7) for the number
of shigella toxin binding sites on HeLa cells determined at
the same temperature. Studies of toxin binding to entero-
cytes carried out at 24 to 35°C with E. coli ST (10) or
Clostridium perfringens enterotoxin (27) report 5 × 10^4 and
2 × 10^5 binding sites per cell in rat and rabbit intestine,
respectively. Other studies of cholera toxin binding to iso-
lated rat MVM demonstrate approximately 10^10 receptors
per μg of MVM protein at 25°C (3). Thus, the characteristics

FIG. 4. Competitive inhibition of binding of 125I-labeled shigella
toxin to MVM. Increasing concentrations of unlabeled native toxin
were simultaneously added to a fixed amount of iodinated toxin (2.5
ng) and MVM protein (0.75 μg). Total binding was determined as
described in Materials and Methods. Brackets indicate one standard
deviation.
of the rabbit MVM shigella toxin binding site are at least appropriate for it to be a functional toxin receptor.

However, the physiological relevance of a receptor cannot be ascertained from binding parameters alone. There is substantial evidence that the receptor on HeLa cells mediating the cytotoxic effect of shigella toxin is a tunicamycin-inhibitable N-linked glycoprotein. For example, treatment of HeLa cells with the antibiotic tunicamycin, a specific inhibitor of N-linked glycoprotein synthesis, renders the cells resistant to toxin effects (21). More recently, we found evidence of a different nonglycoprotein receptor on both HeLa cells and rabbit intestinal MVM (15a). This second receptor is a glycolipid extractable in chloroform-methanol, is tunicamycin resistant, and does not mediate toxin cytotoxicity to HeLa cells (15a). Because there is no evidence of cytotoxicity in rabbit (33) or monkey (32) jejunum exposed to shigella toxin or viable organisms, respectively, and because our data demonstrate a single class of toxin-binding site on jejunal MVMs, we propose that this binding site is different from the glycoprotein receptor on HeLa cells and that it may mediate the in vivo secretory response of the jejunum to the toxin. Although further investigation will be required to determine whether or not this MVM shigella toxin binding site is, in fact, a functional receptor, the present study clearly identifies for the first time the presence of a specific shigella toxin binding site on rabbit jejunal enterocyte MVMs.

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LITERATURE CITED


