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Binding of Class II *Escherichia coli* Enterotoxins to Mouse Y1 and Intestinal Cells

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The binding of class II *Escherichia coli* heat-labile enterotoxins (LT) to Y1 tissue-cultured cells and mouse intestinal cells was studied and compared with that of class I toxins, including cholera enterotoxin. All radioiodinated (^{125}I) toxins retained their biological activities in both model systems, but only LTIIb could be shown to bind specifically to target cells. LTIIa could inhibit the binding of both class I and LTIIb toxins, a finding which correlates with its ability to bind to multiple gangliosides. LTIIb could not inhibit the binding of the other enterotoxins. The binding and activity of class II toxins could not be modulated by prior exposure of target cells to the B subunit of LTI.

Escherichia coli can cause diarrheal disease by a variety of mechanisms. Some strains (enteroadherent *E. coli*) are better able than others to adhere to target tissues, some (enteroinvasive *E. coli*) are capable of invasion, and some (enterotoxigenic *E. coli*) produce one or more toxins (5, 15, 19–21). The toxigenic *E. coli* can be further divided into strains that produce choleralike, heat-labile enterotoxins (LTs), those that produce heat-stable toxins, and those that produce shigellalike toxins associated with hemorrhagic colitis and hemolytic-uremic syndrome (enterohemorrhagic *E. coli*) (6, 7, 13, 17, 18, 22).

Strains of *E. coli* that produce LT have been classically linked to choleralike syndromes and traveller's diarrhea because of the similarities in immunochemical properties and mechanisms of action of LT and cholera toxin (CT) (4, 6). Both toxins bind to specific GM1 ganglioside receptors in target cell membranes, via similar B subunits, and activate adenylate cyclase via their A subunits, enzymatically ADP-ribosylating the G_s component of adenylate cyclase (4, 9, 16). In recent years, a second class of *E. coli* LTs (LTII) that also activate adenylate cyclase has been discovered, and these toxins have been further subdivided into LTIIa and LTIIb on the basis of immunochemical differences (8, 10, 11, 14). In contrast to classical LT (LTI), which binds with greatest specificity to GM1 ganglioside, LTIIa binds best to GD1b ganglioside and LTIIb binds best to GD1a ganglioside (8).

While some of the ganglioside specificities and immunochemical reactivities of representative type I and type II LTs have been elucidated, their comparative binding properties on target cells have not been determined. For this purpose, Y1 mouse adrenal cells in tissue culture and isolated mouse intestinal cells were used. In addition, the ability of the B subunit of LTI to modulate receptor binding and activity of the two LTII toxins, as it does for CT and LT (2), was studied.

MATERIALS AND METHODS

Toxins. Purified CT was purchased from Sigma (St. Louis). Purified *E. coli* LTI enterotoxin and its B subunit (LT-B), made by recombinant *E. coli* devoid of A subunit genes, were gifts from J. Clements (Tulane University, New Orleans, La.) (2). *E. coli* enterotoxins LTIIa and LTIIb were purified to homogeneity as previously described (10, 11). All toxin preparations were stored at 4°C in 0.01 M Tris-HCl–0.005 M EDTA (pH 7.4). Dilutions of the toxin were made with this buffer or with phosphate-buffered saline (PBS; 0.05 M phosphate and 0.15 M NaCl, pH 7.4).

Target cells. Adrenal cells (Y1) were propagated and maintained in Ham's nutrient mixture F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum in a humidified atmosphere of 5% CO_2 at 37°C (4).

Intestinal epithelial cells were harvested from BALB/c mice by rinsing the intestine with PBS and then treating it with 1 mg of trypsin (Sigma) per ml for 10 min. The dislodged cells were then collected and washed in Ham's nutrient mixture F10 medium supplemented with 10% horse serum. (Toxin binding and adenylate cyclase activity in intestinal cells are unaffected by the trypsin treatment [2].) The cells were resuspended at 10^5/ml for use in the toxin-binding assay.

Binding assay. The toxins were iodinated, as previously described, to a specific activity of 15 to 30 μCi/μg (3). To assess toxin binding, 5 ng of ^{125}I-labelled toxin (~100,000 dpm) was incubated with 10^5 cells in F10 medium in a total volume of 1.0 ml under steady-state conditions (37°C for 30 to 45 min) in the presence or absence of unlabelled toxin or potential inhibitor (3). After incubation, the mixture was poured over EHW filters (Millipore, Bedford, Mass.), the filters were washed with cold PBS, and the number of filter-bound counts was determined with a Beckman 4000 gamma counter (Beckman Instruments, Palo Alto, Calif.) with a counting efficiency of 75%. Corrections were made for nonspecific binding (binding in the presence of excess unlabelled toxin), and the results were analyzed in terms of total bound counts (B_T) and specific bound counts (B_{SP}) (3). Statistical analyses employed the Student *t* test.

RESULTS

Specific binding of LTII toxins. As with radioiodinated CT and LTI toxins (3), radioiodinated LTIIa and LTIIb retained virtually all (>90%) of their activities (i.e., cell rounding) on

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TABLE 1. Binding of ^{125}I-LTIIa and ^{125}I-LTIIb to Y1 and mouse intestinal cells

Inhibitor and concn (ng/ml)	% Binding to[a]:			
	Y1 cells		Intestine cells	
	^{125}I-LTIIa	^{125}I-LTIIb	^{125}I-LTIIa	^{125}I-LTIIb
None	1.94	38.8	4.24	6.23
LTIIa				
50	2.02	41.5	4.15	6.38
100	2.10	41.2	5.05	8.28
500	1.98	36.5[b]	4.97	4.26[b]
LTIIb				
50	2.71	37.2	5.78	5.09[b]
100	2.92	37.0	4.40	4.02[b]
500	2.56	23.4[b]	5.30	2.65[b]
LT-B				
50	2.68	46.7	4.97	6.53
100	2.56	45.8	4.72	5.96
500	2.78	55.4	5.11	7.01
CT				
50	2.17	45.3	4.41	5.97
100	2.38	46.0	4.31	5.94
500	2.41	46.7	4.42	5.54

[a] Percentage of total ^{125}I-LTIIa or ^{125}I-LTIIb bound to cells after incubation for 45 min at 37°C. The values represent the means of triplicate determinations.
[b] $P < 0.05$ compared with respective controls.

Y1 cells. In contrast to class I enterotoxins, however, LTIIa could not be shown to specifically bind to Y1 or mouse intestinal cells; i.e., any binding of ^{125}I-LTIIa to either cell type could not be inhibited by homologous toxin. Manipulations of conditions of temperature, cell numbers, and ^{125}I-LTIIa concentrations, as well as preincubation (30 min) of target cells with unlabelled LTIIa, were all unsuccessful attempts to demonstrate specific binding. Concentrations of the heterologous toxins (LTIIb, CT, and LT-B) 10- to 100-fold that of ^{125}I-LTIIa used in the binding assay were also unable to inhibit the binding of ^{125}I-LTIIa to Y1 or mouse intestinal cells (Table 1).

In contrast to the lack of demonstrable specific binding of LTIIa to Y1 or mouse intestinal cells, LTIIb bound specifically to both of these cell types (Fig. 1 and Table 1). Binding was rapid at 37°C, reaching saturating levels by 20 min. Binding of ^{125}I-LTIIb to Y1 cells was greater than ^{125}I-LTIIb binding to mouse intestinal cells. With neither cell type, however, could binding be shown to be reversible (data not shown), as it is with the class I enterotoxins (3). In addition to homologous inhibition, the binding of LTIIb to both cell types could also be inhibited by LTIIa (although to lesser degrees than by LTIIb), but not by CT or LT-B (Table 1). In some experiments, binding of ^{125}I-LTIIb seemed to be increased over that of controls in the presence of class I enterotoxins.

Inhibition of binding of class I enterotoxins by class II enterotoxins. The binding of either CT or LTI to Y1 cells or mouse intestinal cells can be inhibited equally well by either holotoxin or its B subunit (2, 3). In competition experiments with ^{125}I-CT, LTIIa, but not LTIIb, could be shown to inhibit CT binding to either cell type. The results for mouse intestinal cells are shown in Fig. 2. LTIIa was essentially as

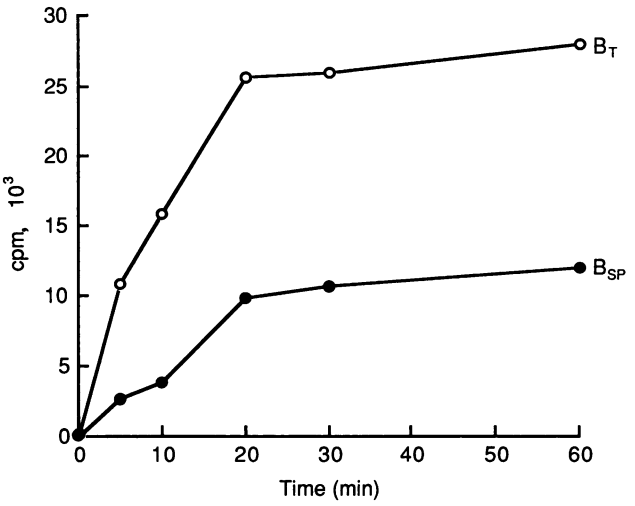

FIG. 1. Binding of ^{125}I-LTIIb to Y1 cells at 37°C over time. Symbols: ○, total binding (B_T); ●, specific binding (B_{SP}).

effective an inhibitor as LTB. In the presence of LTIIb, the binding of ^{125}I-CT may have been increased.

Modulation of class I toxin binding by class II toxins. With Y1 cells, preincubation with class I holotoxins or their B subunits leads to down-regulation of toxin binding, a phenomenon associated with a decrease in the numbers of receptors without a change in the binding affinity (2). Experiments in which a 20-fold excess of either class II toxin was preincubated with Y1 cells showed essentially no change in ^{125}I-CT binding (Table 2).

Modulation of toxin-induced morphologic effects by LT-B. Both class I and class II enterotoxins induce similar morphologic changes in Y1 cells, i.e., cell rounding. Preincubation of Y1 cells with LT-B inhibits cell rounding induced by

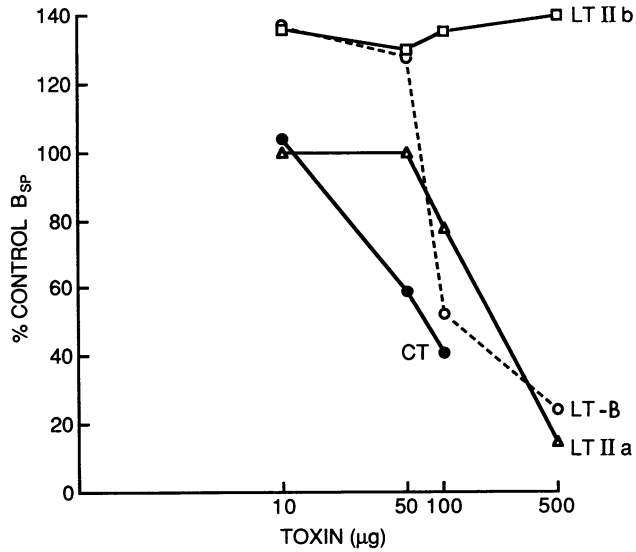

FIG. 2. Inhibition of binding of ^{125}I-CT to mouse intestinal cells by type I and type II enterotoxins. ^{125}I-CT (5 ng) was incubated, with or without the concentrations of unlabelled toxins or subunits indicated, for 45 min prior to the harvesting of cells and the determination of cell-bound ^{125}I-CT counts. Symbols: ●, CT; ○, LT-B; △, LTIIa; □, LTIIb.

TABLE 2. Modulation of CT binding to Y1 cells by *E. coli* toxins[a]

Toxin	% Binding at[b]:			
	0 h		1 h	
	B_T	B_{SP}	B_T	B_{SP}
None	5.15	2.25	3.92	1.64
LTIIa	4.05	1.94	3.95	1.83
LTIIb	3.69	1.48	3.65	2.07
LT-B	3.11[c]	0.48[c]	2.63[c]	0.47[c]

[a] Y1 cells were preincubated with 100 ng of toxins per ml at 37°C for 1 h prior to extensive washing of cells; they were then assayed for ^{125}I-CT binding 0 and 1 h later.

[b] Results are expressed as percentage of total bound toxin (B_T) and percentage of specifically bound toxin (B_{SP}) at the indicated time postincubation and are the means of triplicate determinations.

[c] $P < 0.05$ compared with respective controls.

either LTI or CT, concomitant with its down-regulating effects on LTI and CT binding (2). On the other hand, LT-B had no effect on the cell rounding activities of either LTIIa or LTIIb (data not shown).

DISCUSSION

CT and the LT of *E. coli* (LTI) have been firmly established as virulence factors in diarrheal diseases associated with these organisms. Such a role for the new class of *E. coli* LTs (LTIIa and LTIIb) has yet to be delineated. The class II enterotoxins have been shown to also activate adenylate cyclase, as do class I toxins, and bear some immunochemical and genetic similarity to class I toxins (8, 10, 14). The A subunits of class I and class II enterotoxins are highly homologous, whereas their B subunits show poor homology (1). Consistent with these differences in their B subunits are differences between the class I and II enterotoxins in their specificities for ganglioside receptors. Whereas class I enterotoxins bind with highest affinities to GM1 ganglioside, the class II enterotoxins bind best to GD1 gangliosides (8). LTIIa binds best to GD1b ganglioside, but it can also bind to GM1 ganglioside. LTIIb binds best to GD1a ganglioside and demonstrates virtually no binding to GM1 ganglioside.

The results of our studies extend previous observations on the relationships between the class I and class II enterotoxins (8, 10). Of the class II enterotoxins, only LTIIa could inhibit the binding of CT. This is consistent with the fact that LTIIa, but not LTIIb, can bind to GM1 ganglioside (8). The lack of converse inhibition (i.e., inhibition of LTIIa binding by CT) is difficult to interpret because of the absence of demonstrable specific binding of LTIIa. LTIIa binds to multiple gangliosides to which CT fails to bind, and this multireceptor binding capability of LTIIa could explain both its ability to inhibit class I and class II toxin binding and the lack of inhibition of its binding by other class I and class II toxins. This ability, therefore, of LTIIa to bind to multiple receptors may technically obfuscate our ability to demonstrate specific binding. It might be possible to demonstrate specific binding of LTIIa if a different system was found that consisted of only one receptor for the toxin. Indeed, mutations in LTIIa that affect its binding to GD1b ganglioside may not affect its binding to GM1 ganglioside (1). Alternatively, the multireceptor binding of LTIIa may lead to a "bivalent," irreversible linkage that, while technically a nonspecific binding process, is capable of transmitting or transducing responses.

LTIIb did demonstrate specific binding to both Y1 and mouse intestinal cells, although this binding was irreversible. The binding of LTIIb could also be inhibited, although to lesser degrees, by large concentrations of LTIIa, but not by class I enterotoxins. In some experiments, binding of LTIIb may have even been increased in the presence of class I enterotoxins. Of particular note as well is the large percentage of LTIIb toxin bound to Y1 cells, implying a large number of receptors, both specific and nonspecific. The ganglioside composition of Y1 cells is known to contain GD1a, in addition to GM1 and other gangliosides (8). Further experiments, utilizing sialidases and various proteases, would be of interest in determining the effects of these agents on toxin binding and modulation of toxin receptors. Not considered here is the potential influence and involvement of any glycoprotein receptor(s) in the binding of class II or class I enterotoxins (12).

Exposure of target cells to the B subunits of class I enterotoxins has been shown to down-regulate binding of class I toxins and modulation of the toxins' effects in vitro and in vivo (2). The results of our experiments using LT-B did not show any effects on binding or activities of either class II enterotoxin. Conversely, the LTII toxins had no demonstrable effects on the regulation of CT binding. Despite the absence of cross-regulatory effects, in vitro effects of one class of toxin on the other's binding and action in vivo have not yet been evaluated. Since the class II enterotoxins are as active as class I toxins in vivo, by using the SAM (sealed adult mouse) model (reference 2 and unpublished results), the interactions of the two types of toxins in vivo may lead to valuable insights into the pathogenesis of the toxigenic diarrheas.

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