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K88-Mediated Binding of *Escherichia coli* Outer Membrane Fragments to Porcine Intestinal Epithelial Cell Brush Borders

JAAP M. MIDDELDORP AND BERNARD WITHOLT*

Department of Biochemistry, University of Groningen, 9747 AG Groningen, The Netherlands

We have examined the interactions between various radiolabeled membrane fractions obtained from an enterotoxigenic *Escherichia coli* strain and brush borders isolated from porcine intestinal epithelial cells. Outer membrane fragments containing the K88 attachment factor bound tightly to brush borders, whereas cytoplasmic membrane vesicles did not. Three different types of outer membrane preparations were tested: (i) cellular outer membranes isolated from lysozyme spheroplasts, (ii) medium vesicles, or outer membrane fragments released into the medium during growth, and (iii) periplasmic vesicles, or outer membrane fragments which were released from the cells during spheroplast formation and were therefore isolated in the periplasmic fraction. Of these fractions, which were heterogeneous, it was always the outer membrane subfraction which bound tightly to brush borders. This binding, which was K88 dependent, may have some physiological significance in view of the association between outer membrane fragments and enterotoxin. Thus, released outer membrane fragments equipped with attachment factors may function as enterotoxin carriers which increase the efficiency with which enterotoxin can be delivered to intestinal epithelial cells.

Enterotoxigenic *Escherichia coli* produce a heat-labile enterotoxin (LT) which resembles cholera toxin with respect to function and structure (1, 15). Despite their overall resemblance, however, LT and cholera toxin are not made and released to the same extent by *E. coli* and *Vibrio cholerae*, respectively. Whereas *V. cholerae* may release up to 40 mg of toxin per liter of medium (10, 17), the total amount of LT produced intra- and extracellularly by the best LT producers does not exceed 1 mg (7, 30) per liter of medium and is often one or two orders of magnitude less (15); of this, at least 90% is associated with the cellular fraction (31). In addition, LT is usually produced in a rather inactive form and requires proteolytic treatment for activation (1, 23).

As a result, the enterotoxin activity detected in enterotoxigenic *E. coli* supernatants is between three and five orders of magnitude lower than that found in *V. cholerae* supernatants. Nevertheless, enterotoxigenic *E. coli* strains can cause significant diarrhea in animals (7), suggesting that they may have evolved mechanisms to do one or more of the following after being introduced into an animal gut: (i) colonize the gut very effectively (19, 22); (ii) synthesize more LT than under typical laboratory conditions; (iii) release more of their intracellular LT; (iv) activate LT before or during release; or (v) direct the available LT to epithelial cells with high efficiency.

In this paper we will focus on the last mechanism, which is hinted at by the results and considerations listed below. Most of the intracellular LT is associated either with the outer membrane or the periplasmic fraction (31). *E. coli* strains generally release some outer membrane material during normal growth (11) and in the case of enterotoxigenic strains of *E. coli*, these fragments contain higher LT activities than found in any of the other cell compartments (9). If these fragments (which are derived from the outer cell layer) contain attachment factors, they could bind to epithelial cells, thereby effectively delivering a packet of LT to the surface of the epithelial cell.

To test this hypothesis, we have examined the interaction of various bacterial cell components with isolated epithelial cell brush borders. In this paper we report that there is a tight and specific interaction between bacterial outer membrane material and isolated brush borders, which is mediated by an attachment factor. The implications of this finding with respect to the transfer of LT from bacteria to host epithelial cells are discussed.

MATERIALS AND METHODS

Bacterial strain, growth, and labeling procedures. *E. coli* 2100 (serotype O8:K87:K88ab), enterotoxigenic for pigs, was kindly provided by P. A. M. Guinée (Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands). It was grown in a mini-

mal medium containing E-salts (29), 0.5% dextrose, and 1% BME vitamins (Flow Laboratories) at 37 or 18°C on a rotary shaker at 200 rpm (32). [^{35}S]methionine (770 Ci/mmol; Radiochemical Centre, Amersham, England) was added during the early exponential phase. The total amount of radioactivity incorporated varied in different experiments but was typically about 2×10^8 cpm/mg of cell protein. Bacteria were grown for 16 h to the stationary phase and harvested by centrifugation at 5,000 × *g* for 10 min (32).

Isolation of membrane fragments released into the growth medium (medium vesicles). Membranous material released during growth (11) was sedimented from the medium at 190,000 × *g* for 120 min and suspended in 10 mM sodium phosphate (pH 7.4)-buffered Ringer's solution (PBR).

Isolation of bacterial fractions. After harvesting, bacteria were spheroplasted with the tris(hydroxymethyl)aminomethane-ethylenediaminetetraacetic acid-sucrose-lysozyme procedure (32) using 2.5 mM ethylenediaminetetraacetic acid and 120 μg of lysozyme per ml at 0°C. Spheroplasts were collected at 5,000 × *g* for 10 min.

The periplasmic fraction (i.e., the supernatant of the spheroplast pellet) was recentrifuged at 190,000 × *g* for 120 min to give a pellet containing "periplasmic membrane vesicles." Spheroplasts, suspended in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) containing 50 μg of deoxyribonuclease and ribonuclease (4), were disrupted at 0°C either by sonication (seven bursts of 30 s at 50 W, using a Branson sonifier) or in a French press at 7,000 to 8,000 lb/in^2. Large cell fragments were removed by centrifugation at 5,000 × *g* for 10 min. Crude outer membrane was obtained by further centrifugation at 30,000 × *g* for 15 min, and crude cytoplasmic membrane was isolated from the resulting supernatant at 190,000 × *g* for 120 min. Purified outer and cytoplasmic membranes were obtained by sucrose density centrifugation (4). All bacterial fractions were suspended in PBR as described above.

Protein was determined by the method of Lowry et al. (16), using bovine serum albumin as a standard.

Isolation of epithelial cells and brush borders. Epithelial cells and brush borders were isolated by a modification of several procedures described elsewhere (6, 8, 14, 24). Briefly, segments from the middle of the small intestine were obtained from freshly killed pigs, flushed with ice-cold PBR, everted (8), and incubated in PBR containing 0.05 mg of trypsin inhibitor (Sigma) and 1 mg of glucose per ml on a rotary shaker for 5 min. Epithelial cells detached as large sheets (6) and were isolated by centrifugation (200 × *g*, 5 min), followed by one wash in cold PBR. Cells were carefully suspended in PBR for further use; their viability was better than 90% as tested by trypan blue exclusion. Brush borders were isolated from these cells by osmotic shock in 5 mM $NaHCO_3$ (pH 8.2) at 0°C and gentle homogenization in a Dounce homogenizer. Intact brush borders were separated from small cellular fragments by repeated centrifugation (450 × *g*, 10 min) and gentle homogenization. Nuclei were removed by a Mg^{2+} sedimentation step (14, 24) (using 10 mM $MgCl_2$ in PBR to clump the nuclei), followed by filtration over glass wool (8). The brush border preparations obtained by this procedure maintained their typical morphology (8, 25), contained less than 5% nuclear contamination, similar to the results of Forstner et al. (8), and were free from other cellular fragments as determined by phase-contrast microscopy and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). They banded at a density of 1.19 g/cm^3 on discontinuous sucrose gradients.

Isolated brush borders were sedimented at 450 × *g* for 10 min and suspended in PBR to 5 (experiments II and III) and 18 (experiment I) mg of protein per ml.

Adhesion experiments. A 100-μl sample of bacterial fragments was added to 200 μl of brush borders (both in PBR). Each mixture contained 3.3 mg (experiment I) or 1 mg (experiments II and III) of brush border protein and from 3 to 267 μg of bacterial protein (specific radioactivity of about 2×10^5 cpm/μg of bacterial protein). There were no saturation effects under these conditions, as discussed below.

Based on the finding that, under incubation conditions similar to those used for the brush border adhesion experiments, binding of whole bacteria to isolated epithelial cells was maximal within 30 min at 37°C and that 80% of the bacteria bound to the epithelial cells within 15 min, bacterial fragments were allowed to bind to brush borders for 45 min at 37°C in polyvinylchloride tubes (diameter, 0.6 cm) on a rotary shaker (100 rpm). As shown below (Table 1), bacterial binding to brush borders was complete within this incubation time.

The incubation was stopped by adding 1 ml of cold (0°C) PBR. Brush borders were sedimented by centrifugation at 800 × *g* for 3 to 5 min, washed in 1 ml of PBR (0°C), and centrifuged as described above. This treatment was repeated three to five times (see Results). Supernatants (washes) and the final washed brush border pellet were analyzed by scintillation counting, sucrose density analysis, SDS-PAGE, and fluorography.

Sucrose density gradient analysis. Samples (about 10^6 cpm) were suspended in 20% (wt/wt) sucrose in 10 mM tris(hydroxymethyl)aminomethane–1 mM ethylenediaminetetraacetic acid (pH 8.0) and layered on top of a 25 to 60% discontinuous sucrose gradient. Gradients were run for 60 h at 30,000 rpm in an SW41 rotor in a Beckman L5-65B ultracentrifuge and removed by extrusion with 65% sucrose (4). Sucrose was removed from the fractions by fourfold dilution in PBR and recentrifugation at 190,000 × *g* for 120 min.

SDS-PAGE and fluorography. Protein samples (about 10^4 cpm per slot) were analyzed in 12.5% polyacrylamide slab gels and stained with fast green as described (4). Radioactive protein bands were detected by fluorography of the dried slab gels as described (3).

RESULTS

Experimental approach. To carry out the experiments described here it was necessary to develop a medium in which enterotoxigenic *E. coli* could be labeled with radioactive amino acids (thus precluding rich protein-based media) while at the same time allowing the expression

TABLE 1. *Binding of bacterial membrane fractions to isolated brush borders*[a]

Bacterial fraction	Prepn[b]		Contents of incubation mix			% of radioactivity recovered in:						
	No.	Method	Radioactivity (cpm × 10^{-6})	Bacterial protein[c] (μg)	Brush border protein (mg)	Washes					Washed brush borders	Total recovery (%)
						1	2	3	4	5		
Whole cells												
1	I	37°C	58	240	3.3	2.6	0.7	0.4	0.4	0.4	95	99.5
2	II	37°C	11.1	46	1	3.7	0.5	0.6			97.8	102.6
3	III	18°C	11.1	62	1	33.4	18.9	6.1			41.4	99.8
Total sonic extract												
4	II	37°C	28.2	117	1	71.6	4.5	1.6			18.2	95.9
5	III	18°C	26.5	147	1	84.4	5.9	1.1			10.2	101.6
Cytoplasmic membrane												
6	I	37°C, s, c	3.1	13	3.3	76	6.4	1.7	1.5	0.9	12.2	97.7
7	I	37°C, Fp, c	10.4	43	3.3	77	4.8	1	0.6	0.7	10.4	94.5
8	II	37°C, Fp, c	49.3	204	1	82.5	4.7	1.2			10.3	98.7
9	II	37°C, Fp, g	4.7	20	1	80	6	2.1			12.5	100.6
10	III	18°C, Fp, c	48	267	1	90.7	3.1	0.4			2.3	96.5
Outer membrane												
11	I	37°C, s, c	11	46	3.3	72	3.6	1.2	0.9	0.5	17.4	95.6
12	I	37°C, Fp, c	10	42	3.3	70	5	1.6	0.7	0.3	17	94.6
13	II	37°C, Fp, c	33	136	1	40.9	2.8	1.6			40.5	85.8
14	II	37°C, Fp, g	10.7	45	1	56	9.9	2.8			32	100.7
15	III	18°C, Fp, c	33	183	1	72.7	3.0	0.6			2.8	79.1
Periplasmic vesicles												
16	I	37°C	3.4	14	3.3	51	6	1	1.5	1.4	27.4	88.3
17	II	37°C	25.1	105	1	44	1.7	0.5			57.0	103.2
18	III	18°C	3.8	21	1	76.5	3.9	1.2			4.9	86.5
Medium vesicles												
19	I	37°C	4.7	20	3.3	55	6.8	1.5	1.3	1.3	24.3	90.2
20	II	37°C	34.4	143	1	63.3	3.4	0.6			32.1	99.4
21	III	18°C	0.6	3	1	93.8	5.6	3.4			10.3	113.1

[a] Bacterial membrane fragments labeled with [^{35}S]methionine (from 3 to 267 μg of protein and 0.6 × 10^6 to 58 × 10^6 cpm) were added to brush borders (1 or 3.3 mg of protein) and incubated in a total reaction mixture of 300 μl.

[b] Membranes prepared by: s, sonication; Fp, French press; c, crude; g, gradient.

[c] Bacterial protein was estimated based on the known radioactivity added to the brush borders and the known specific activity of total bacterial protein (240,000 cpm/μg of protein in preparations I and II and 180,000 cpm/μg of protein in preparation III).

of attachment factors. Briefly, we searched for minimal media which would still allow for considerable adhesion of various *E. coli* strains to isolated porcine intestinal epithelial cells as determined by phase-contrast microscopy. In addition, promising media were further tested by agglutinating presumed K88ab-carrying bacteria with monospecific anti-K88ab antiserum (a kind gift of P. A. M. Guinée).

The minimal medium used here meets the criteria set out above and was in fact better than rich media (nutrient broth and brain heart infusion, both from Difco) in eliciting the synthesis of the K88ab attachment factor. We confirmed that we were dealing with the K88ab attachment factor by isolating it from *E. coli* 2100 grown on minimal medium, according to the method of Stirm et al. (28), and comparing it (by SDS-PAGE and immunodiffusion) with the K88 attachment factors K88ab, K88ac, K88ad, and K88ad(e) recently isolated and characterized by Mooi and De Graaf (18); the K88 preparation isolated by us banded with the K88ab of Mooi and De Graaf at 25,000 daltons and showed complete identity with K88ab in immunodiffusion (Ouchterlony) against anti-K88ab antiserum. The other K88 preparations of Mooi and De Graaf did not react with the anti-K88ab antiserum.

When *E. coli* 2100 was grown in minimal medium at 18°C, the bacteria could no longer be agglutinated by anti-K88ab antiserum, in agree-

ment with the results of Jones and Rutter (13).

The amount of K88ab antigen obtained from *E. coli* 2100 grown in minimal medium in large-scale cultures (6 to 8 liters) was about 1% of the total cell protein. In addition, the cell membranes and the periplasmic fraction were isolated from small non-radiolabeled cultures and analyzed by SDS-PAGE. The 25,000-dalton band, which was not present in the membranes and periplasm of cells grown at 18°C, was a major outer membrane component, equal in staining intensity (fast green) to the matrix protein and the *tolG* protein, each of which occurs in about 1×10^5 to 2×10^5 copies per cell (5). The 25,000-dalton band also accounted for about 5% of the total periplasmic fraction and about 30% of the periplasmic vesicles. Based on these results, it was estimated that on the order of 1 to 2% of the total cell protein was K88ab antigen, in agreement with the preparative yields described above.

Given the results obtained with the minimal medium, *E. coli* 2100 was grown and labeled with [^{35}S]methionine in the minimal medium, various bacterial membrane fractions were isolated, and these were added to brush borders. After incubation, the brush borders were washed several times to remove unbound or loosely bound bacterial material. These washes and the material that remained bound to brush borders were examined by a combination of SDS-PAGE and sucrose gradient analysis.

SDS-PAGE was used to detect cross-contamination of outer and cytoplasmic membranes, which differ substantially with respect to protein composition (Fig. 1, lanes 4 and 5, respectively). Prominent outer membrane bands include the free lipoprotein (molecular weight 7,500), various forms of the bound lipoprotein containing from one to seven murein subunits (molecular weights from 9,000 to 18,000; Wensink and Witholt, Eur. J. Biochem., in press), protein III (17,000), the *tolG* or II* protein (33,000), the matrix proteins between 36,000 and 38,000, and several proteins related to enterochelin binding at about 75,000 molecular weight (for review see reference 5). In addition, the outer membrane of K88 strains also shows the K88 attachment factor at 25 kilodaltons (18). In contrast to the outer membrane, the cytoplasmic membrane typically shows many faint and only a few stronger bands, including a band at the dye front (molecular weight 6,500 in high-resolution gels [2]), the K88 attachment factor, and a prominent band at molecular weight 58,000.

Sucrose gradient analysis was used to determine which membrane subfractions of the various bacterial membrane preparations bound

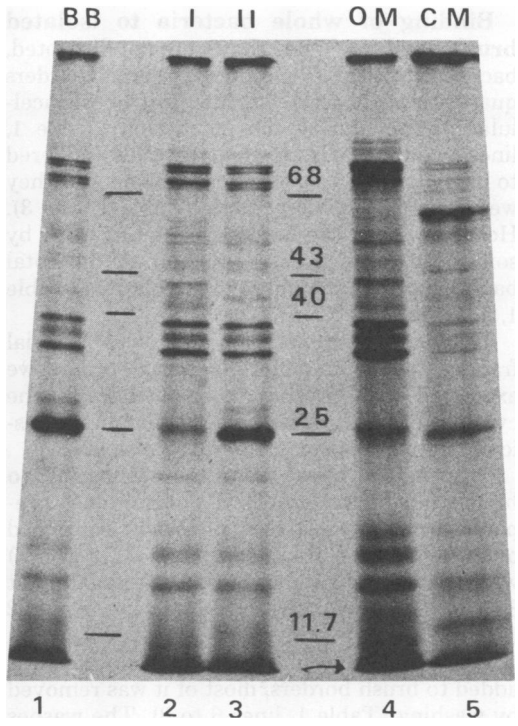

FIG. 1. *SDS-PAGE of radiolabeled cytoplasmic membrane fractions before and after interaction with brush borders. Crude cytoplasmic membrane, prepared by differential centrifugation, was added to brush borders. After incubation, the brush borders were washed. Lane 1, Washed brush borders (BB). The washed brush borders were applied to a sucrose gradient (Fig. 2, solid line) which resulted in peaks I (lane 2) and II (lane 3). For comparison, lanes 4 and 5 show outer and cytoplasmic membranes, respectively, obtained by sucrose density gradient centrifugation. Radioactive bands were detected by fluorography (3). Standards indicated by markers are, from top to bottom: bovine serum albumin (molecular weight 68,000), ovalbumin (43,000), aldolase (40,000), chymotrypsinogen (25,000), and cytochrome c (11,700).*

tightly to brush borders, based on the fact that the original density of such fractions was shifted to the brush border density of 1.19 g/cm^3.

Initially the brush borders were washed five times. Table 1 shows, however, that washes 4 and 5 removed very little radioactivity from the brush borders. Furthermore, SDS-PAGE of these washes showed them to closely resemble the material which remained bound to the brush borders (data not shown). Accordingly, only three washes were used in all subsequent experiments. Experiment III differs from experiments I and II in that bacteria were labeled at 18°C to repress the synthesis of the K88 attachment factor (13).

Binding of whole bacteria to isolated brush borders. When they were not disrupted, bacteria grown at 37°C adhered to brush borders quantitatively; moreover, they lost no intracellular protein during the incubation (Table 1, lines 1 and 2). Cells grown at 18°C also adhered to brush borders to a significant extent, but they were washed off more easily (Table 1, line 3). However, when the bacteria were disrupted by sonication, only a small portion of the total bacterial protein bound to brush borders (Table 1, lines 4 and 5).

To gain more information about the bacterial fractions which do adhere to brush borders, we examined the bacterial outer membrane, the cytoplasmic membrane, medium membrane vesicles, and periplasmic membrane vesicles.

Binding of cytoplasmic membranes to brush borders. Cytoplasmic membranes prepared by differential centrifugation contained outer membrane fragments (ρ = 1.24 g/cm^3) which failed to be removed by centrifugation at 30,000 × *g* for 15 min, as can be seen in Fig. 2 (dashed line, fractions 10 to 14). When such a crude cytoplasmic membrane preparation was added to brush borders, most of it was removed by washing (Table 1, lines 6 to 8). The washes were heterogeneous in that they contained cytoplasmic as well as outer membrane proteins (data not shown). The 10 to 12% of the material which remained associated with the washed brush borders (Fig. 1, lane 1) appeared in two main peaks on sucrose gradient analysis (Fig. 2, solid line). Peak I, which was evidently released from the brush borders during gradient centrifugation, showed little K88 antigen (Fig. 1, lane 2). Interestingly, although the density of peak I (1.153 g/cm^3) was lower than that of cytoplasmic membranes, this peak contained mostly outer membrane proteins (compare Fig. 1, lane 4). This phenomenon, which we have observed for K-12 strains as well, is due to the fact that after spheroplast lysis some of the outer membrane occurs as vesicles with a high phospholipid/protein ratio (see also reference 4). These low-density outer membrane fragments, which contained little K88 attachment factor, apparently bound to brush borders in a nonspecific fashion, since they were washed off rather easily.

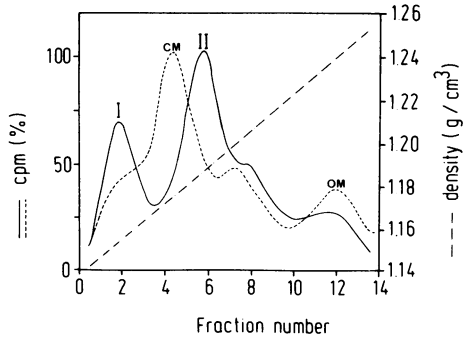

FIG. 2. *Sucrose density gradient centrifugation of a crude cytoplasmic membrane fraction before and after incubation with brush borders. Cytoplasmic membranes prepared by differential centrifugation* (-----); *material adhering to washed brush borders* (——); *density* (-·-·-). *Gradient profiles were normalized to fit within the same figure: 100% equals 10,000 cpm* (-----) *and 12,000 cpm* (——). *CM, Cytoplasmic membrane peak; OM, outer membrane peak.*

The other fraction (peak II) was tightly bound to the brush borders (ρ = 1.19 g/cm^3) and contained predominantly outer membrane proteins and K88 antigen (Fig. 1, lane 3).

Thus, the 10 to 12% of the crude cytoplasmic membrane fraction which bound to the brush borders appeared to be due to a subfraction of contaminating outer membrane enriched in K88 antigen. Table 1 (line 9) shows that similar results were obtained with a cytoplasmic membrane fraction prepared by gradient centrifugation (Fig. 1, lane 5), indicating that this particular preparation may have contained more contaminating low-density outer membrane than usual (4). These results are supported by the finding that a constant proportion of the added cytoplasmic membrane preparation (10 to 12%) adhered to the brush borders when as little as 20 μg (Table 1, line 9) or as much as 204 μg (Table 1, line 8) of bacterial membrane protein was added to 1 mg of brush border protein. This indicates that the brush borders were not saturated by the cytoplasmic membrane proteins used in these experiments, but instead a constant proportion (contaminating outer membrane fragments) of the cytoplasmic membrane preparations bound to the brush borders.

When synthesis of attachment factor was repressed, there was essentially no binding of cytoplasmic membrane to brush borders (Table 1, line 10).

Outer membranes. Outer membranes bound to brush borders to a considerable extent (Table 1, lines 11 to 14). As with the cytoplasmic membranes, the method of membrane preparation had relatively little effect on the adhesion of outer membranes (compare lines 11 versus 12 and 13 versus 14). The variation between experiments I and II was probably due to a variation in the amount of attachment factor produced and present on different outer membrane fragments formed during spheroplast lysis; such fragments are not necessarily identical (4). After incubation with brush borders, the first wash resembled the starting material on a gradient and on SDS-PAGE but contained little K88

attachment factor (data not shown). The brush border-associated material remained tightly bound to washed brush borders and was not released from the brush borders during gradient centrifugation (data not shown), in contrast to the experiment in which cytoplasmic membranes were incubated with brush borders (Fig. 2, solid line).

The fact that after addition to brush borders the density of the outer membranes (ρ = 1.24 g/cm^3) shifted to that of the brush borders (ρ = 1.19 g/cm^3) indicated that the outer membrane fragments did indeed bind to the brush borders and not to each other. Additional proof that there was no mutual aggregation of outer membrane fragments came from control adhesion experiments in which brush borders were omitted; less than 0.02% of the total outer membrane radioactivity was found in the pellet after three washes, as compared to 17 to 40% when brush borders were present in the incubation mixture (Table 1).

The brush borders did not appear to be saturated by outer membrane material in these experiments; 32 and 40.5%, respectively, remained bound when 45 μg (Table 1, line 14) or 136 μg of protein (Table 1, line 13) was added to 1 mg of brush border protein. When synthesis of the attachment factor was repressed, outer membranes adhered no more than did cytoplasmic membranes (compare Table 1, lines 15 and 10).

Thus, when outer membrane was added to brush borders, 45 to 75% of it was washed away easily whereas 20 to 40%, which was enriched with the K88 antigen, bound to the brush borders quite tightly. As much as 55 μg of outer membrane protein bound to 1 mg of brush border protein in these experiments (calculated from Table 1, line 13).

Medium vesicles. Normally growing cells release outer membrane material into the medium (11). *E. coli* 2100 also produced such medium vesicles, which bound to brush borders to a considerable extent (Table 1, lines 19 and 20). These medium vesicles were heterogeneous and consisted of three major peaks on gradient centrifugation (Fig. 3, dashed line). Peak I contained very little outer membrane protein. Peak II appeared to consist mostly of low-density (ρ = 1.175 g/cm^3) outer membrane material; it contained a few outer membrane proteins, considerable K88 antigen, and the 58,000-molecular-weight protein normally associated with the cytoplasmic membrane fraction (Fig. 4, lane 2). Peak III resembled purified outer membrane with respect to density (ρ = 1.235 g/cm^3) and protein composition (compare Fig. 4, lanes 3 and 9).

After the total medium vesicle fraction (Fig. 4, lane 4) was incubated with brush borders, material containing the 58,000-molecular-weight protein was washed away (Fig. 4, lanes 5 and 6), while a fraction containing the K88 antigen remained associated with the brush borders (Fig. 4, lane 7). Sucrose density gradient analysis indicated that almost all of this fraction bound tightly to brush borders (Fig. 3, solid curve); as expected, the tightly bound material (Fig. 4, lane 8) was identical to the total brush border-associated fraction. The tightly brush border-bound material was very similar to authentic outer membrane (Fig. 4, lane 9) except for an enrichment in K88 antigen and a lower content of lipoprotein derivatives with molecular weights below 18,000 (see Discussion). Up to 46 μg of medium vesicle protein remained bound per mg of brush border protein (calculated from Table 1, line 20). Thus, outer membrane fragments released into the medium by cultures grown at 37°C adhered to brush borders to the same extent as the corresponding cellular outer membranes (Table 1, lines 19 and 20). In contrast, cultures grown at 18°C released virtually no outer membrane or vesicular material; of the small amount isolated, only 10% bound to brush borders (line 21), probably due to aspecific adsorption as indicated by SDS-PAGE of the bound material (data not shown).

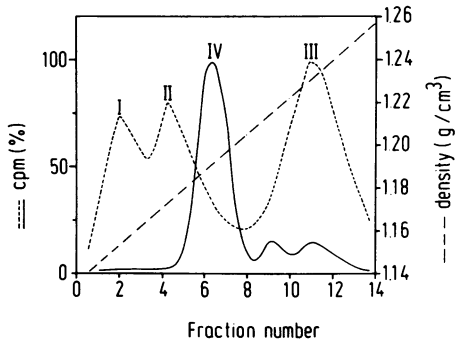

FIG. 3. *Sucrose density gradient centrifugation of medium vesicles before and after incubation with brush borders. Medium vesicles* (----) *(100% = 50,000 cpm); medium vesicles adhering to brush borders after three washes* (——) *(100% = 11,000 cpm); density* (-·-·-).

Periplasmic vesicles. Lysozyme–ethylenediaminetetraacetic acid–osmotic shock treatment of *E. coli* releases a periplasmic fraction which contains a variety of soluble proteins and a heterogeneous collection of membrane fragments or periplasmic vesicles (31). Sucrose density gradient centrifugation of the periplasmic vesicles prepared from *E. coli* 2100 (Fig. 5, dashed line) showed that they were qualitatively similar to medium vesicles (Fig. 3, dashed line),

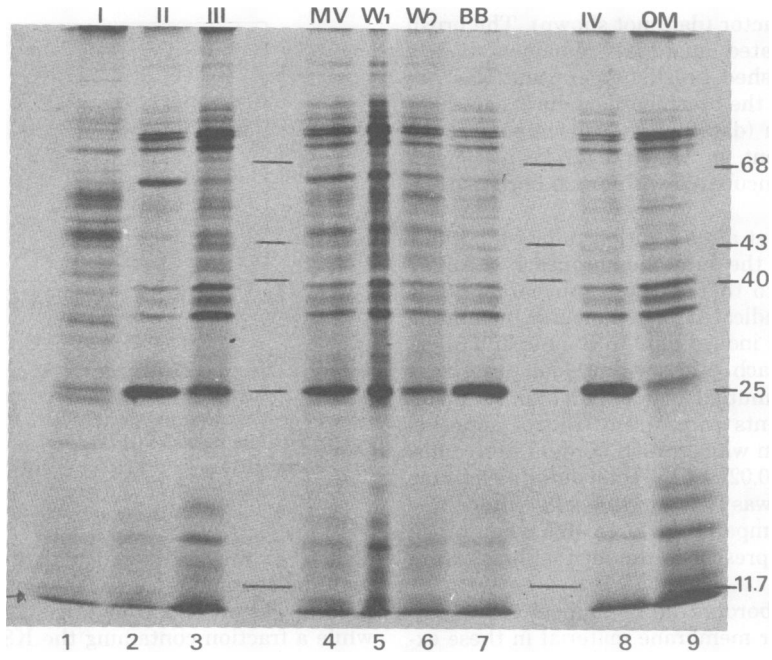

FIG. 4. *SDS-PAGE of radiolabeled medium vesicles before and after binding to brush borders. Lanes 1 to 3, peaks I to III of Fig. 3; lane 4, combined medium vesicles (MV); lanes 5 and 6, washes 1 and 2 (W1, W2); lane 7, washed brush borders (BB); lane 8, peak IV of Fig. 3; lane 9, authentic outer membrane (OM). Standards as in Fig. 1.*

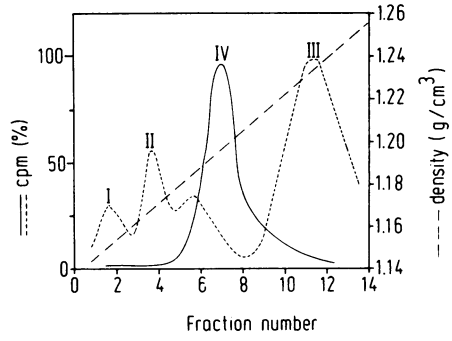

FIG. 5. *Sucrose density gradient centrifugation of periplasmic vesicles before and after incubation with brush borders. Periplasmic vesicles (-----) (100% = 9,400 cpm); periplasmic vesicles adhering to brush borders after three washes (——) (100% = 8,000 cpm); density (-·-·-).*

although quantitatively they contained more outer membrane material (peak III).

Results similar to those seen for medium vesicles were obtained when periplasmic vesicles were added to brush borders. A fraction resembling peak II was washed off. The material which remained associated with the washed brush borders was tightly bound (Fig. 5, peak IV). With respect to protein composition it resembled purified outer membrane, except that it contained considerable amounts of K88 attachment factor. These periplasmic outer membrane fragments adhered to brush borders even more effectively than did the corresponding outer membranes (Table 1, lines 16 and 17 versus 11 to 14), and as much as 60 μg of periplasmic vesicle protein remained attached per mg of brush border protein (calculated from the experiment of Table 1, line 17). In contrast, periplasmic vesicles obtained from cells grown at 18°C showed little or no adherence (Table 1, line 18). Thus, brush borders appeared to selectively bind those periplasmic vesicles which contained K88 attachment factor and most closely resembled outer membranes.

Differences between preparations I and II (Table 1) were reflected in all of the outer membrane-containing fractions; preparation I (lines 11, 12, 16, and 19) bound to a lesser extent than did preparation II (lines 13, 14, 17, and 20), suggesting differences in the amount or activity of the K88 attachment factor in these preparations.

DISCUSSION

Binding of different bacterial membrane fractions to brush borders. Cellular outer membranes obtained from lysed spheroplasts

bound to brush borders, whereas the corresponding cellular cytoplasmic membranes did not. The limited binding that did occur when cytoplasmic membrane preparations were added to brush borders was due to contaminating outer membrane material in the cytoplasmic membrane preparations. Outer membrane fragments released by whole cells during growth (medium vesicles) or lysozyme–ethylenediaminetetraacetic acid–osmotic shock treatment (periplasmic vesicles) bound to isolated brush borders as well as or better than the cellular outer membrane fragments.

Thus, brush borders selectively bound the outer membrane fragments present in each of the membrane fractions tested. This binding was abolished when bacteria were grown at 18°C to repress synthesis of the attachment factor K88. This indicates that the binding of bacterial material to brush borders is not due to aspecific aggregation with the external or internal surfaces or the contents of the open brush borders used in these experiments.

The most likely explanation for the systematic binding of outer membrane fragments in the presence of the K88 factor is that such outer membrane fragments contain pilus (K88) remnants which link them to a brush border, to the extent of at least 60 μg of bacterial membrane protein per mg of brush border protein.

Physiological significance of outer membrane-brush border interactions. The enterotoxin (LT) activity of the porcine strain AP1 is localized mostly in the outer membrane and in periplasmic outer membrane fragments such as those described in this paper (31). We have extended these observations to several other porcine strains, including the strain used in this paper, and to *E. coli* 711(P307), a K-12 strain into which an LT plasmid has been transferred (27); in all cases we found LT to be localized predominantly in the periplasmic fraction and in the outer membrane (J. P. Dekker and B. Witholt, Soc. Gen. Microbiol. Q. **6**:146, 1979). Similarly, although we only find a few percent of the total cellular LT in the medium, the medium LT is associated with released outer membrane fragments (9). We have argued that this binding is not likely to be an artifact (31); moreover, the association of LT and outer membrane fragments is at least as great as that of intrinsic major outer membrane proteins and the outer membrane, (9). Thus, although LT resembles cholera toxin with respect to structure and function (1, 15), its synthesis and release differ significantly from those of cholera toxin, which appears in the medium as a soluble protein (10, 30).

In principle, LT might be released in the same manner (Fig. 6A). However, it is more likely that the LT A and B subunits are synthesized on ribosomes bound to the cytoplasmic membrane (5), that the A and B chains are extruded through the cytoplasmic membrane in analogy with outer membrane (2) and periplasmic proteins (26) (Fig. 6B), and that the subunits combine into an AB_4 or AB_5 molecule (1, 15) which binds to the outer membrane. Outer membrane fragments containing LT occasionally bud off (11, 20).

We have studied this budding process and have found that released outer membrane fragments contain newly synthesized proteins (20). We have suggested therefore that budding may occur because newly synthesized outer membrane sometimes fails to be linked to the underlying peptidoglycan layer via covalent peptidoglycan-lipoprotein linkages (5). As the insertion

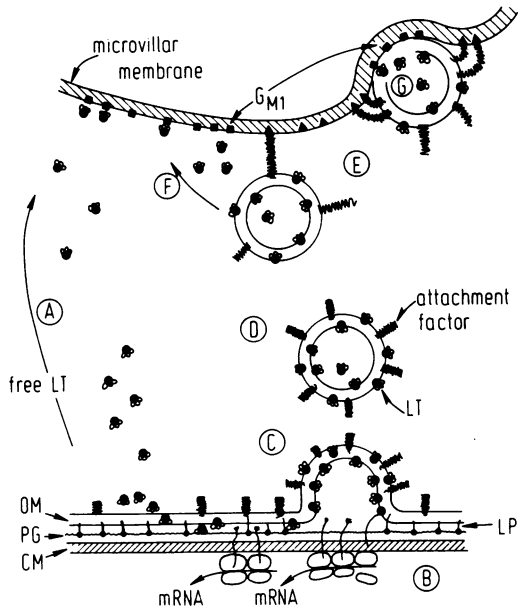

FIG. 6. *Model for the transfer of LT and attachment factor-bearing outer membrane fragments from E. coli to an epithelial cell. The bacterial envelope (lower portion) consists of a cytoplasmic membrane (CM), a peptidoglycan layer (PG), and an outer membrane (OM). The outer membrane and peptidoglycan layer are linked covalently by bound lipoprotein (LP). Ribosomes bound to the cytoplasmic membrane synthesize LT-A (●) and LT-B subunits (not shown) which associate ( ) as they enter the periplasmic space or bind to the outer membrane. The attachment factor ( ) is located externally to the outer membrane. The microvillar membrane (upper portion) contains receptors for the attachment factor (▲) and LT (■). See text for further details.*

of outer membrane components (proteins, phospholipids, lipopolysaccharides) continues, such a local patch grows (Fig. 6C), buds out, and is eventually released. Released outer membrane fragments may have a variable composition: if a given fragment contains more lipid than the average outer membrane, it may band at a lower density (e.g., Fig. 3, peak II). In addition, such fragments should not contain peptidoglycan or peptidoglycan-linked lipoprotein (Fig. 6D), which could explain why the bound lipoprotein bands seen in authentic outer membrane (Fig. 4, lane 9) are not seen in medium vesicles (Fig. 4, lane 8) and periplasmic vesicles.

If a released outer membrane fragment contains an attachment factor, it may bind to host cells (Fig. 6E). LT may come off outer membrane fragments by one of several mechanisms (Fig. 6F and G) and bind to G_{M1} in analogy with soluble cholera toxin (12).

The model shown in Fig. 6 is applicable to whole bacteria as well. In that case, the cellular outer membrane, rather than small outer membrane fragments, interacts with the host cells. It is clear that only part of the cellular outer membrane can interact directly with host cells. It is possible, therefore, that contact between enterotoxigenic bacteria and host cells promotes the release of outer membrane fragments from the bound bacteria; these fragments may then also bind directly to the epithelial cells. It is interesting that surface granules (outer membrane blebs) were in fact seen by scanning electron microscopy when enterotoxigenic bacteria were added to intestinal segments in vivo (21).

Thus, although *V. cholerae* excretes three to five orders of magnitude more enterotoxin activity than does *E. coli*, the latter may have evolved mechanisms to optimize delivery of toxin to its target. Specifically, outer membrane fragments may well function as generalized vehicles which can be endowed with specific plasmid-encoded attachment factors and toxins.

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