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Effects of enteropathogenic *Escherichia coli* on microvillar membrane proteins during organ culture of rabbit intestinal mucosa

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Abstract

This study examines the effects of an enteropathogenic *Escherichia coli* on microvillar membrane proteins during organ culture of rabbit ileal explants. Explants maintained with enteropathogenic *E coli* showed brush border effacement affecting approximately 50% of enterocytes, and where enteropathogenic *E coli* were closely adherent to the enterocyte surface microvilli were apparently being shed as vesicles. The microvillar membrane enzymes alkaline phosphatase, aminopeptidase N and α-glucosidase were released into the culture medium during organ culture, and this process was significantly enhanced by enteropathogenic *E coli*. This increased loss of microvillar membrane enzymes into the culture medium was associated with decreased tissue activities of microvillar membrane enzymes in enteropathogenic *E coli* infected ileal explants compared with control. For aminopeptidase N in particular, however, total enzyme activities in the tissue plus culture medium were increased comparing enteropathogenic *E coli* with control, suggesting that there might be an increase in the rate of synthesis of certain microvillar membrane proteins. Reorientating sucrose density gradient centrifugation of culture medium showed that alkaline phosphatase, aminopeptidase N and α-glucosidase were predominantly associated with particles of peak modal density 1·19 g/ml in both groups, confirming that enteropathogenic *E coli* accelerate release of microvillar membrane enzymes as vesicles. Analytical fractionation of ileal explants showed that enteropathogenic *E coli* resulted in a loss of microvillar membrane enzyme activities from the main brush border peak of modal density 1·21 g/ml present in controls. The density of the remaining smaller and lighter peak increased from 1·19 g/ml to 1·23 g/ml after homogenisation in digitonin, confirming association of these proteins with cholesterol containing membranes and not endoplasmic reticulum. These findings suggest that enteropathogenic *E coli* accelerate the normal shedding of microvillar membrane proteins as vesicles, and may stimulate a compensatory increase in microvillar membrane protein synthesis.

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The strains of *Escherichia coli* that produce diarrhoea do so by one of at least five distinct mechanisms.[1] Enteropathogenic *E coli* were originally delineated as those strains that were neither enterotoxigenic nor enteroinvasive, but it is now known that enteropathogenic *E coli* produce distinctive ultrastructural damage to the brush border that can seriously interfere with enterocyte function.[2] This has been described as an attaching effacing lesion which is characterised by close apposition of bacteria to the enterocyte surface associated with a considerable loss of microvilli with cup and pedestal formation at the sites of bacterial attachment. Such damage has been shown to occur during natural and experimentally induced infection of man,[3-5] rabbits,[6] pigs,[7] calves,[8,9] and cats.[9]

Examination of duodenal mucosa from a child with enteropathogenic *E coli* 0111 showed that attaching effacement was accompanied by a selective loss of microvillar membrane enzymes,[10] and comparable changes were observed when the same enteropathogenic *E coli* 0111 was used to infect rabbit ileal explants in organ culture.[11,12] The latter technique has facilitated sequential electron microscopic studies which have provided some evidence that microvilli are lost by a process of vesiculation.[11-14] These findings have now been pursued by examining the effects of enteropathogenic *E coli* on microvillar membrane proteins during organ culture of rabbit ileal explants in order to explore the mechanism of damage and response of enterocytes to the loss of microvillar membrane proteins.

Methods

BACTERIA

The strain of enteropathogenic *E coli* (K798) used in these experiments was isolated from a child with gastroenteritis,[10] and had been stored at −70°C in glycerol broth. This enteropathogenic *E coli* of serogroup 0111 a, b, H^-, did not produce heat labile (LT), heat stable (ST), or vero-cytotoxin (VT), but did exhibit localised adhesion to HEp-2 cells. It did not contain DNA sequences homologous to the LT, STa, STb, VT1, VT2, and enterohaemorrhagic *E coli* probes, but did hybridise with the enteropathogenic *E coli* adherence factor probe[15] and the *E coli* attaching effacing (eae) probe.[16] The enteropathogenic *E coli* was cultured in brain heart infusion broth (Oxoid Ltd, Basingstoke, UK) at 37°C for 18 hours. The culture was then centrifuged and the bacteria washed in sterile saline. The enteropathogenic *E coli* were resuspended to give a final concentration of 10^8 cfu/ml in organ culture medium consisting by volume of 65% Trowells T8 culture medium, 20% NCTC 135 and 15% fetal calf serum (Gibco,

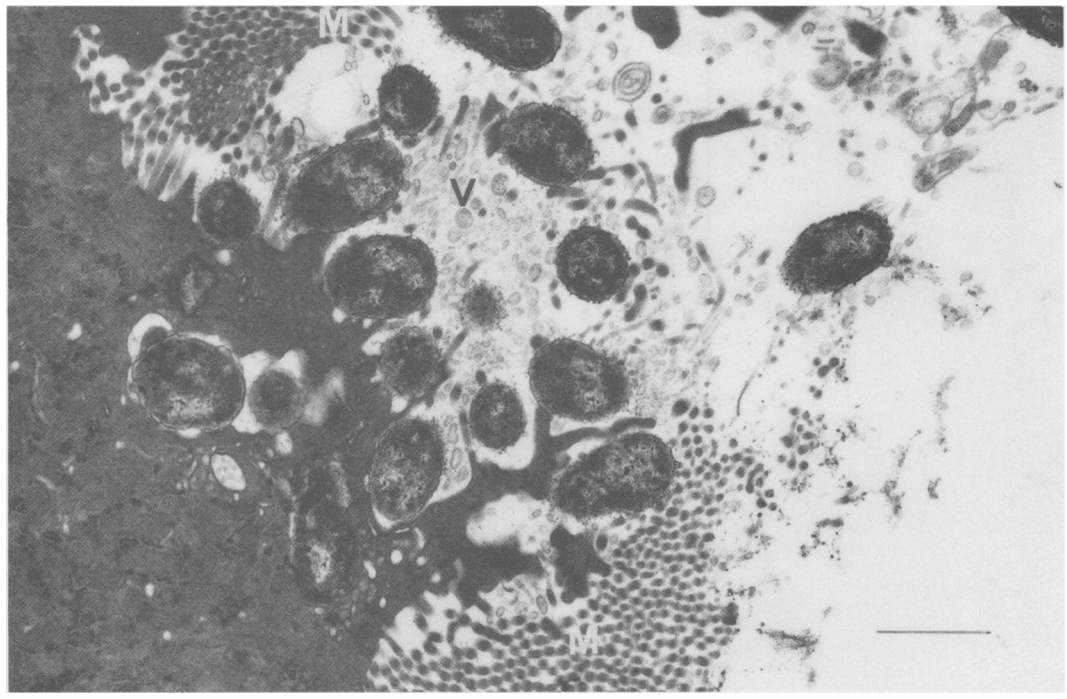

Figure 1: Electron microscopic appearance of rabbit ileal explant maintained with enteropathogenic E coli *for eight hours showing vesiculation (V) of microvilli in areas where bacteria are closely adherent to the enterocyte surface, and some microvilli (M) cut in section in adjacent areas with a normal brush border. Bar represents 1 μm.*

Paisley, UK) containing benzyl penicillin (60 mg/l) and L-glutamine (2 mmol/l).

ORGAN CULTURE

Ileal mucosal fragments obtained from adult Dutch rabbits (average weight 2·5 kg) were maintained in organ culture as described previously.[11] The rabbits were fasted overnight and anaesthetised with Valium (Roche Ltd, Welwyn, UK) and Hypnorm (Crown Chemical Co, Ltd, Lamberhurst, UK). The ileum was exteriorised through a vertical midline incision and the mucosa was flushed with prewarmed (37°C) iso-osmotic saline. Mucosal fragments were taken randomly from a 5 cm region extending from 5 cm to 10 cm proximal to the ileocaecal valve using iridectomy scissors. Each fragment was washed in pregassed (95% O_2:5% CO_2) prewarmed (37°C) organ culture medium and trimmed into small fragments (approximately 2 mm^3). The fragments were suspended for 10 min either in organ culture medium containing enteropathogenic *E coli* (10^8 cfu/ml) or in organ culture medium alone (control). Then the fragments were removed, separated and incubated for 24 hours at 37°C in an atmosphere of 95% O_2 and 5% CO_2 on stainless steel grids in contact with organ culture medium in sterile dishes.

ULTRASTRUCTURAL STUDIES

The ileal fragments were fixed for one hour in cacodylate buffered glutaraldehyde (2·5% v/v), washed three times in cacodylate buffer and refixed with osmium tetroxide (1% v/v). They were then washed three times in distilled water, embedded in araldite, thin sectioned and stained with Reynold's lead citrate and uranyl acetate (1% v/v). Thin sections were mounted on 200 mesh copper grids and examined using a Philips 301 electron microscope.

BIOCHEMICAL STUDIES

In each experiment, explants and culture medium from pairs of organ culture dishes, each pair containing three control or enteropathogenic *E coli* infected mucosal fragments, were pooled separately. Pooled explants were homogenised in 5 ml sucrose medium (0·3 mmol/l sucrose, 22 mmol/l ethanol, 1 mmol Na_2 EDTA, pH 7·4) with or without digitonin (0·12 mmol/l), and a postnuclear supernatant was prepared by low speed centrifugation (800 *g*) in a refrigerated centrifuge (Sorvall RT 6000).[17] The nuclear pellet was further homogenised in 2 ml sucrose medium and stored at −20°C for enzyme assays. An aliquot (3 ml) of each postnuclear supernatant or pooled organ culture medium (diluted 1:1 in sucrose medium) was subjected to reorientating sucrose density gradient centrifugation by layering onto a sucrose gradient which ranged in density from 1·088–1·280 g/ml with a cushion of density 1·32 g/ml.[17] Each gradient was centrifuged at 163 000 *g* for 45 minutes in a Sorvall TV-850 vertical tube rotor using a Sorvall OTD-65 ultracentrifuge. After centrifugation, 17 fractions of approximately 2 ml were collected, and alkaline phosphatase activity was measured. The remainder of the fractions were stored at −20°C before further assay. Fractionation results were averaged by computer[18] and are presented in the form of frequency density histograms. Frequency is defined as that portion of total recovered activity present in an individual fraction divided by the density span covered by that fraction. Relative frequency was derived by multiplying the frequency data for enteropathogenic *E coli* by the relative enzyme activity (mU/mg DNA) comparing enteropathogenic *E coli* with control explants.

Brush border enzymes were assayed in the tissue, the gradient fractions and organ culture medium; alkaline phosphatase and zinc-resistant α-glucosidase were assayed as described pre-

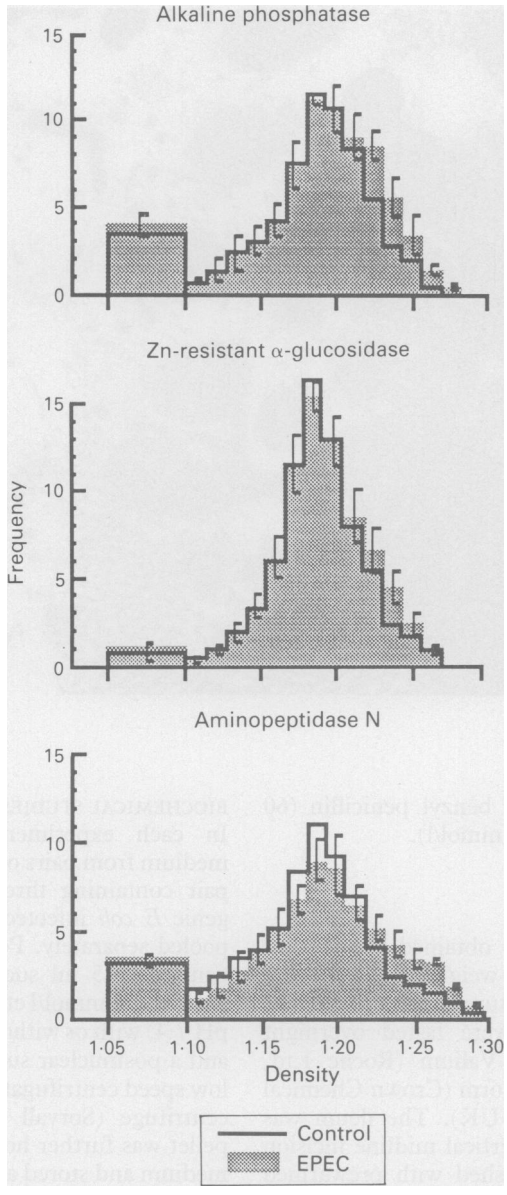

Figure 2: Reorientating sucrose density gradient centrfugation of culture medium (six) following maintenance of explants for 24 hours in the absence (control: solid line) or presence of enteropathogenic E coli *(shaded, mean (SEM)). For each enzyme, areas are normalised for enzyme activity and density span 1·05-1·10 g/ml represents soluble enzyme activity.*

viously,[19] and aminopeptidase N was assayed by a modification of the method of Saifu *et al*[20] with 0·2 mmol/l (final concentration) L-alanine 7-amido-4-methyl coumarin (Sigma) as substrate in 0·1 mol/l Tris HCl pH 7·2 containing 1 mmol/l Co^{2+} and 0·1% Triton X-100. Tissue DNA was assayed by the method of Labarca and Paigen[21] with calf thymus DNA as standard (Sigma Type V, Poole, UK). Statistical analyses were by Student's *t* test for paired data.

Results

ULTRASTRUCTURAL STUDIES

In each of six experiments, electron microscopic examination of control tissue maintained in organ culture for 24 hours provided no evidence of ultrastructural damage to enterocytes, and brush borders remained intact. In contrast, explants maintained with enteropathogenic *E coli* for eight hours showed vesiculation of microvilli where bacteria were closely adherent to the enterocyte surface (Fig 1), and by 24 hours there were large areas of brush border effacement with approximately 50% of enterocytes examined being affected. Other parts of the enterocytes appeared unaffected and there was no evidence of invasion by enteropathogenic *E coli*.

Effects of enteropathogenic E coli *on release and activities of brush border enzymes*

Group	Alkaline phosphatase	Aminopeptidase N	Zinc resistant α-glucosidase
Release of enzymes (% medium/total)			
Control	36·3 (2·9)	39·8 (2·0)	24·6 (1·1)
Enteropathogenic *E coli*	72·8 (3·6) [p<0·01]	70·8 (2·9) [p<0·01]	63 3 (2·8) [p<0·001]
Specific activity (mU/mg tissue DNA)			
Control	267 (51)	794 (115)	142 (29)
Enteropathogenic *E coli*	145 (27) [p<0·05]	560 (104) [p<0·01]	77 (16) [p<0·01]
Total activity (total mU/mg tissue DNA)			
Control	426 (81)	1310 (180)	190 (41)
Enteropathogenic *E coli*	577 (12) [NS]	1920 (330) [p<0·05]	210 (39) [NS]

Data are shown as mean (SEM) (six) with statistical significance in square brackets.
NS: not significant.
Data show release, specific activities, and total activities of brush border enzymes after maintenance of ileal explants for 24 hours either in the absence (control) or presence of enteropathogenic *E coli*. Release represents enzyme activity in organ culture medium as a percentage of total activity in tissue plus medium; specific activity represents enzyme activity in explant expressed as tissue mU/mg tissue DNA; total activity represents enzyme activity in tissue plus organ culture medium expressed as total mU/mg tissue DNA.

BIOCHEMICAL STUDIES

The brush border enzymes alkaline phosphatase, aminopeptidase N and zinc-resistant α-glucosidase were released into the medium during organ culture of control rabbit ileal explants, and this process was significantly enhanced in the presence of enteropathogenic *E coli* (Table). Increased loss was associated with decreased tissue activities of these enzymes in enteropathogenic *E coli* infected ileal explants, while total enzyme activities in the tissue and culture medium were increased comparing enteropathogenic *E coli* with control, although this increase only reached statistical significance for aminopeptidase N.

Reorientating sucrose density gradient centrifugation of culture medium (Fig 2) showed that released brush border enzymes were predominantly associated with particles of peak modal density 1·19 g/ml after incubation of explants either alone or in the presence of enteropathogenic *E coli*. Density gradient distributions were broadly similar for all three enzymes comparing control with enteropathogenic *E coli*, although the proportion of soluble enzyme activity (density 1·05-1·10 g/ml) was smaller for zinc resistant α-glucosidase than for the other two enzymes.

Density gradient centrifugation of ileal explants (Fig 3) showed that maintenance in the presence of enteropathogenic *E coli* for 24 hours resulted in a marked loss of alkaline phosphatase and aminopeptidase N activities from the main brush border peak of modal density 1·21 g/ml

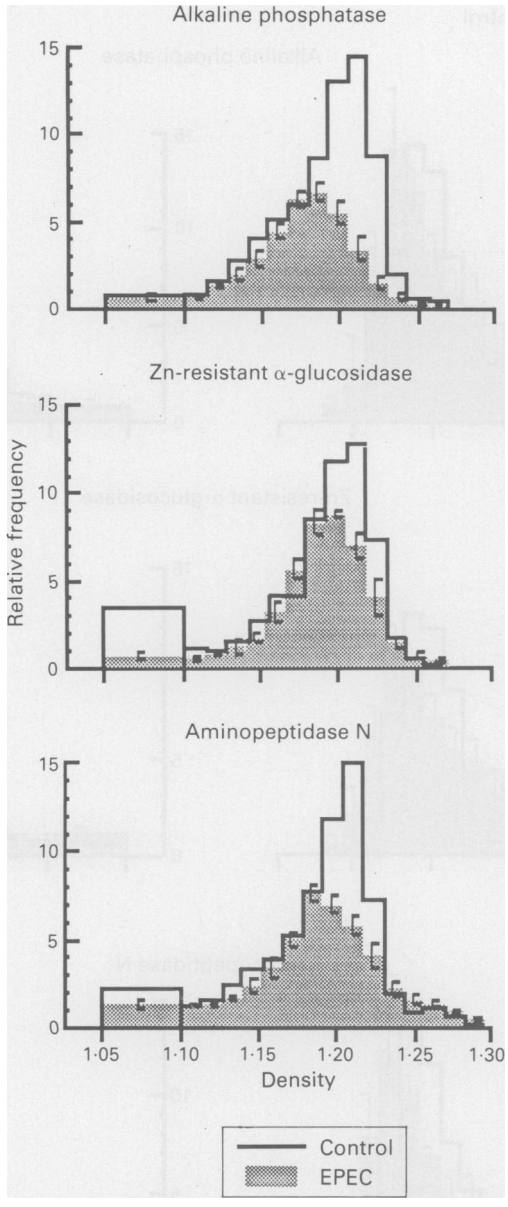

Figure 3: Reorientating sucrose density gradient centrifugation of ileal explants (six) following maintenance for 24 hours either in the absence (control: solid line) or presence of enteropathogenic E coli *(shaded, mean (SEM)). For each enzyme, the areas comparing control and enteropathogenic* E coli *are proportional to tissue enzyme activity (mU/mg tissue DNA). The density span 1·05–1·10 g/ml represents soluble enzyme activity.*

present in controls, leaving a smaller and lighter peak of modal density 1·19 g/ml. In contrast, while there was a loss of brush border zinc resistant α-glucosidase activity, there was also a marked fall in soluble enzyme activity in the enteropathogenic *E coli* infected explants. Homogenisation of control explants in digitonin prior to density gradient centrifugation resulted in the anticipated increase in the modal density of brush borders from 1·21 to 1·23 g/ml (Fig 4), but there was a greater increase in the modal density of brush border enzymes in enteropathogenic *E coli* infected explants from 1·19 g/ml to 1·23 g/ml.

Discussion

Evidence from animal[22 23] and organ culture models[11 13] suggests that enteropathogenic *E coli* induced ultrastructural damage is a two step process. The first stage is non-intimate attachment of enteropathogenic *E coli* particularly to goblet cells and mucin overlying the brush border[12 13] by a process that can involve plasmid encoded factors,[24 25] while the second stage involves adhesions that promote intimate attachment of bacteria to enterocytes resulting in brush border effacement with cup and pedestal formation. Diarrhoea is thought to be caused by defective absorption of osmotically active molecules, fluid and electrolytes after the loss of functional microvillar membrane proteins, but the mechanism of damage to the microvilli is unclear. Electron microscopic studies have previously shown that bacteria penetrate between and attach intimately to the base of microvilli during the second stage of the process, followed by elongation and apparent shedding of microvilli as vesicles.[2 11 13] The present study has extended these observations and provides evidence for acceleration of the normal shedding of functional microvillar membrane proteins as vesicles during the development of enteropathogenic *E coli* mediated damage to the brush border.

Proportions of the total activities of three brush border enzymes present in the culture medium were higher for enteropathogenic *E coli* infected than for control explants, confirming previous observations[11] and indicating that enteropathogenic *E coli* cause enhanced release of functional microvillar membrane proteins during organ culture. Reorientating sucrose density gradient centrifugation of the culture medium showed that released brush border enzymes have relatively large particulate and minor soluble components, with little difference between the normalised profiles for the two groups. The modal density of 1·19 g/ml was lower than for normal brush borders in control explants, and could reflect a loss of cytoskeletal components or a fall in the glycoprotein-to-lipid ratio of the membrane. These findings are compatible with an ability of enteropathogenic *E coli* to cause vesiculation of microvilli containing intrinsic microvillar membrane proteins, and suggest that this may represent acceleration of a normal process.[26] This mechanism contrasts with the loss of intrinsic microvillar membrane enzymes such as disaccharidases documented in small intestinal bacterial overgrowth, where anaerobic bacteria in particular may cause either destruction or solubilisation of these proteins from the microvillar membrane[27–29]; this is thought to be because of direct damage by bacteria or their secreted products, and the metabolism of intraluminal contents to potentially toxic products such as deconjugated bile acids and hydroxy fatty acids.[30] In marked contrast with small intestinal bacterial overgrowth, where intraluminal bacteria can cause solubilisation of proteins from the microvillar membrane, it is now apparent that intimate contact between enteropathogenic *E coli* and the enterocyte surface results in shedding of microvilli as vesicles still associated with intrinsic microvillar membrane proteins. The mechanism is unclear, but it has been suggested that enteropathogenic *E coli* induced damage may be

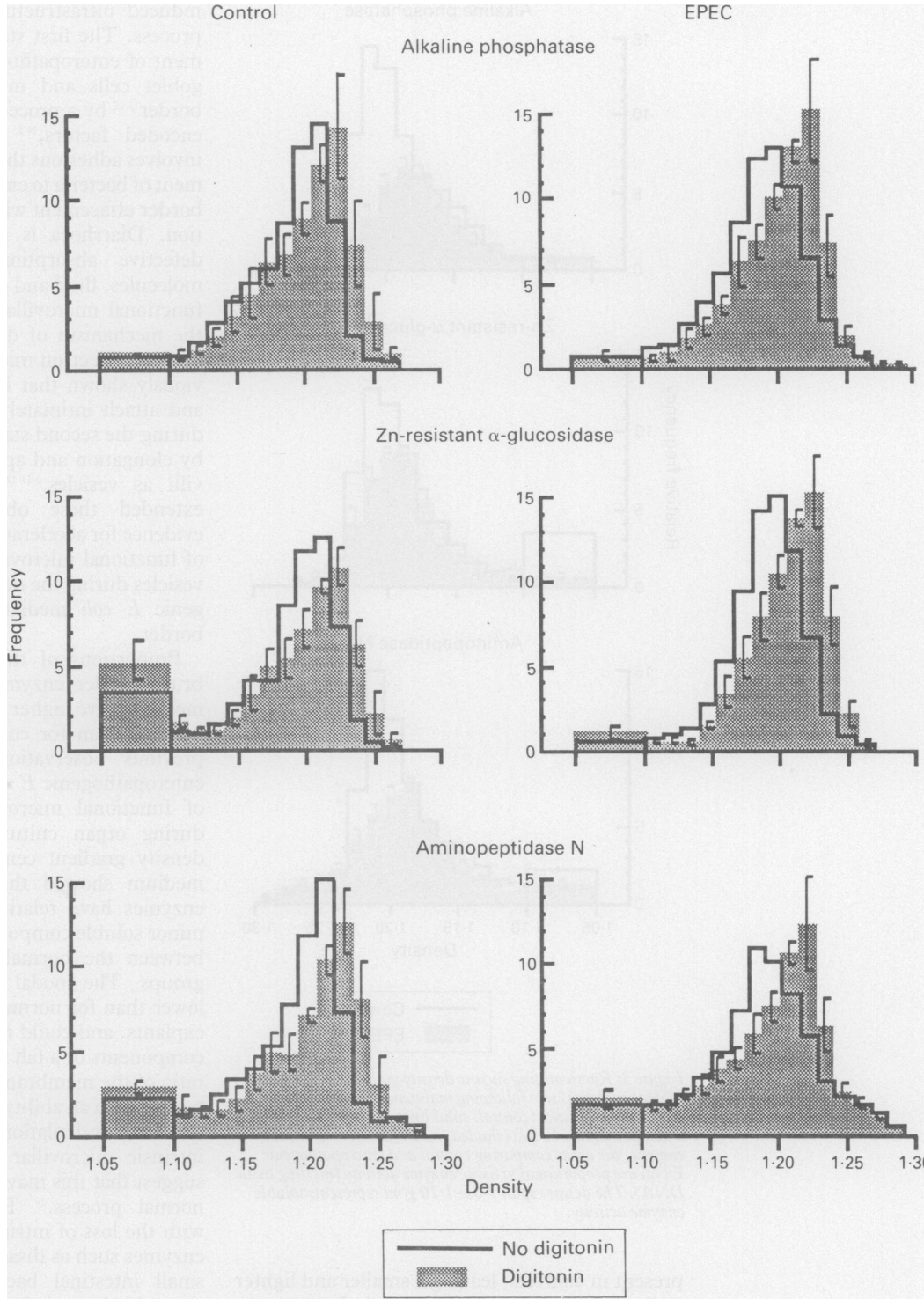

Figure 4: Reorientating sucrose density gradient centrifugation of ileal explants (six) homogenised with (shaded) or without (solid line) digitonin, after maintenance for 24 hours either in the absence (control) or presence of enteropathogenic E coli. *For each enzyme, areas are normalised for enzyme activity and density span 1·05–1·10 g/ml represents soluble enzyme activity.*

mediated by alteration in intracellular concentrations of Ca^{2+} resulting in activation of the calcium sensitive actin severing protein villin and degradation of the actin filaments of the microvillous cytoskeleton.[13] The inability to reproduce vesiculation and effacement of microvilli by incubation of ileal explants in the presence of Ca^{2+} and the divalent cation ionophore A23187[31] indicates that enteropathogenic *E coli* does not act by allowing gross influx of extracellular Ca^{2+}, but there is now some evidence that enteropathogenic *E coli* may cause redistribution of intracellular Ca^{2+}.[32] It is difficult to reconcile such intracellular changes with the localised effects of enteropathogenic *E coli*, however, which affect microvilli in the close vicinity of these bacteria, while more distant microvilli of the same enterocyte remain intact.[11,12] A lectin like property of enteropathogenic *E coli* causing damage to microvilli by interfering with the mobility of intrinsic microvillar membrane proteins is an alternative possibility, but while certain lectins can damage microvilli it has not yet proved possible to reproduce all the ultrastructural changes associated with enteropathogenic *E coli*.[31]

Specific activities of brush border enzymes were decreased in enteropathogenic *E coli* infected explants, consistent with increased shedding of microvillar membrane proteins

as vesicles and compatible with findings in naturally occurring infection with enteropathogenic *E coli*.[10] In a previous study, the decline in tissue activities of certain brush border enzymes in rabbit explants during culture for 24 hours was considered less than anticipated in relation to the extent of microvillar membrane damage, and it was suggested that this might indicate enhanced synthesis of these enzymes in response to increased loss from enterocytes.[11] In which case the proportion of tissue enzyme activity in transit to the microvillar membrane might be expected to be relatively high compared with controls. In the present study, further supportive evidence for increased synthesis of brush border enzymes was provided by finding that for aminopeptidase N in particular there was a marked increase in total enzyme activity in the tissue and culture medium comparing enteropathogenic *E coli* with control. In addition, analytical fractionation of ileal explants showed that enteropathogenic *E coli* resulted in a loss of microvillar membrane enzyme activities from the main brush border peak of modal density 1·21 g/ml present in controls, leaving a smaller and lighter peak of modal ensity 1·19 g/ml. Density increased to 1·23 g/ml after homogenisation in digitonin indicating that this residual peak represented cholesterol containing membranes and not endoplasmic reticulum. These findings could indicate association of these enzymes with membranes other than the microvillar membrane which could potentially be in transit to the enterocyte surface. An alternative explanation, however, is that remaining brush borders have a lower glycoprotein-to-lipid ratio or cytoskeletal content than normal, perhaps because of secreted products of enteropathogenic *E coli* causing additional but more subtle damage at sites distant to bacterial attachment or because of the relative immaturity of the enterocytes containing residual enzyme activity.

In conclusion, we have provided physical evidence to complement electron microscopic studies showing that enteropathogenic *E coli* induce brush border effacement by a process of vesiculation. Furthermore, our findings suggest that enteropathogenic *E coli* induced shedding of microvillar membrane proteins as vesicles may represent acceleration of a normal process, and provide supportive evidence for compensatory stimulation of microvillar membrane protein synthesis. The possibility that such compensatory mechanisms act to repair enterocyte damage is currently being investigated.

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