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Interactions of Enteric Pathogens and the Intestinal Mucosa [and Discussion]

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Interactions of enteric pathogens and the intestinal mucosa†

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[Plate 1]

To cause disease in a mammalian host, enteric bacterial pathogens must overcome a variety of non-specific defence mechanisms in the host which include gastric acidity, intestinal motility, the normal intestinal microflora, and the mucous layer that coats the intestinal epithelial cells. Some attributes of pathogens that may assist them in combating normal resistance in the host are motility, the elaboration of glycosidases, and chemotactic and attachment factors. As a result of these properties, pathogens interact with the intestinal epithelium. Three different patterns of infection have been observed at the cell surface: attachment to the brush border (*Vibrio cholerae* and enterotoxigenic *Escherichia coli*); disruption of the brush border and attachment to the cell membrane (enteropathogenic *E. coli*); and invasion of the cell (shigellae and salmonellae).

1. Introduction

To induce disease, most enteric pathogens must establish some degree of association with the intestinal mucosa. Before this contact is made, however, the pathogen must initially overcome a variety of defence mechanisms in the host. These include gastric acidity, the motility of the small intestine, and the competition of the resident microbial flora.

The acidity of the stomach is the first major defence that an enteric pathogen encounters. This is reflected in the infectious dose of *Vibrio cholerae* and enterotoxigenic *Escherichia coli*, which can be substantially reduced by neutralizing gastric acidity before oral challenge. Shigellae are apparently more resistant to acidity, for as few as ten bacteria can cause clinical disease without bicarbonate pretreatment. After passage through the stomach, pathogens are rapidly propelled through the lumen of the small intestine in a bolus of digesta. Pathogens that colonize this organ are usually motile, and a rapid movement of bacteria across fluid flow planes and into intervillous spaces is probably a necessary step in the colonization of the small intestine. Organisms that do not colonize the proximal small intestine pass into the ileum and the large bowel, where they come into contact with a stable microbial flora that may limit the multiplication of exogenous organisms. Two mechanisms for limiting multiplication have been suggested. Short-chain fatty acids produced by the normal flora have an inhibitory effect on the growth of both salmonellae and shigellae under conditions of acid pH and low *Eh* (Meynell 1962; Hentges 1975). In addition, Freter *et al.* (1983) have presented convincing evidence that the inhibitory effect of H_2S together with the competition for growth factors such as glucose or pyruvate limits the multiplication of *E. coli*, *Fusobacterium* sp. and *Eubacterium* sp. The protective effect of the gastrointestinal flora also prevents detectable translocation of auto-

† The views of the authors do not purport to reflect the position of the Department or the Department of Defense.

chthonous bacteria across the mucosa and into the mesenteric lymph nodes (Berg & Owens 1979).

In addition to overcoming the hostile environment generated by gastric acidity, intestinal motility and normal bowel flora, pathogens must also cope with a mucous barrier on the intestinal epithelium. It has been suspected for many years that the mucous layer is a non-specific defence mechanism of the intestine. Organisms trapped in this layer are thought to be efficiently cleared from the small intestine by the propulsive motility of this organ. Recently Orksov *et al.* (1980) have observed that *E. coli* with common mannose-sensitive type I fimbriae attach to mucin from the human urinary tract and trachea. The attachment is inhibited by mannose, and attachment did not occur with *E. coli* expressing mannose-resistant fimbriae. Should intestinal mucin be shown to have fimbrial binding characteristics analogous to mucin from the urinary tract and the trachea, the differences in attachment of type 1 and mannose-resistant fimbriae to mucin could be an important factor in pathogenesis in the intestine.

Even though pathogens may be resistant to the cleansing action of mucin, they still must penetrate this layer to reach the glycocalyx of the epithelial cell. Burnet & Stone (1947) were the first to demonstrate that *V. cholerae* produces a mucinase that depolymerizes ovomucin, and similar findings were obtained with some serotypes of *S. flexneri* (Formal & Lowenthal 1956). More recently, Prizont (1982) has observed glycosidase activity in a strain of *S. flexneri* 4b when mouse intestinal glycoproteins were used as a substrate. Using an *in vivo* assay, Schneider & Parker (1978) found that *V. cholerae* mutants that are deficient in proteases have a decrease virulence for infant mice, and Finkelstein *et al.* (1983) have evidence that cholera haemagglutinin has protease activity. These studies imply that some intestinal pathogens use mucous glycoproteins as substrates and that these may facilitate the organisms' ability to penetrate the mucous layer. Chemotaxis may be yet another mechanism that enhances a pathogen's capacity to reach the mucosa. It has been shown that motile strains of *V. cholerae* and *Salmonella typhimurium* are chemotactically attracted to the mucosal surface (Freter *et al.* 1981; Uhlman & Jones 1982) and that non-chemotactic mutants are less able to penetrate the mucous layer (Freter & O'Brien 1981*b*). Taxins generated by intestinal epithelial cells include a variety of amino acids (Freter & O'Brien 1981*a*).

After the initial contact of the bacteria and the mucosa, three different patterns of infection have been observed: colonization of the normal mucosa (*Vibrio cholerae* and enterotoxigenic *Escherichia coli*); disruption of the epithelial cell microvilli with close bacterial attachment and deformation of the plasma membrane (enteropathogenic *E. coli*); and overt invasion of the epithelial cell (shigellae, salmonellae and invasive *E. coli*).

2. Enterotoxigenic organisms

V. cholerae and enterotoxigenic *E. coli* characteristically colonize the surface of the proximal small intestine, but do not cause significant histological damage to the epithelium. Electron microscopic studies on tissues from infected animals indicate that the organisms are characteristically positioned 50 to 100 nm from the intact brush border (microvillus) plasma membrane. Underlying the mucous layer and covering the surface of the epithelium is a well developed filamentous coat or glycocalyx. This carbohydrate-containing coat is resistant to proteolysis and it varies in thickness from species to species (figure 1). Because of difficulties in fixation, the glycocalyx is not seen in most electron micrographs. Thus it is possible that enterotoxigenic pathogens colonize the epithelial cell glycocalyx rather than the plasma membrane itself.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The determinants of adherence (adhesins) that assist *V. cholerae* in attaching to the mucosa have not been precisely identified, although the cholera haemagglutinin may be involved in this process (Finkelstein *et al.* 1983). However, in enterotoxigenic strains of *E. coli*, adhesins occur as specific types of filamentous appendages termed fimbriae (Duguid 1955) or pili (Brinton 1965). Smith & Linggood (1971) provided the classical evidence for the role of these surface antigens in intestinal colonization when they showed that the K88 antigens, previously described by Orskov & Orskov (1966), allowed enterotoxigenic *E. coli* to colonize the small intestine of piglets and produce diarrhoea. These studies were possible because K88 antigen and *E. coli* heat-stable enterotoxin are encoded by separate plasmids, permitting the preparation of strains producing either K88, enterotoxin (ENT), or both. Only the $K88^+$, ENT^+ strain produced diarrhoeal disease comparable with the natural infection, whereas $K88^-$, ENT^+ strains had no effect. The $K88^+$, ENT^- strains colonized the intestine and elicited some diarrhoea, suggesting either a possible role for adherence factors in the secretion of intestinal fluid or the elaboration of an additional enterotoxin. Other fimbrial adherence antigens have been described, and these include colonization factor antigens (e.g. CFA/I) (Evans *et al.* 1975) and fimbrial (F) antigens (Morris *et al.* 1982). Like the K88 antigen, these are helical rods (diameters for different types range from 2 to 7 nm) composed of repeating polypeptide subunits (molecular masses ranging from 3 to 30 kDa). In some cases immunological differences have been confirmed by studies of amino acid composition and sequence. A partial list of these adhesins would include K88, K99, 987P, F41 (which confer adherence in pigs and calves), and CFA/I and CFA/II (which mediate colonization of the human small intestine). All of these structures are immunologically and morphologically distinguishable from the type 1, or common fimbriae.

Although the accumulated evidence for fimbrial antigens as major determinants of host-specific intestinal adherence is compelling, morphological demonstration of such filamentous appendages on organisms attached to the intestinal mucosa has been difficult. However, Chan *et al.* (1982) have recently shown that the fine strands apparently tethering enterotoxigenic organisms to the mucosa of the calf are immunologically identifiable as K99 (figure 2). In addition to filamentous surface structures, the bacterial slime layer or capsule may have an important function in mucosal colonization. This structure is currently considered to be only a low-affinity mucosal adhesin. It may, however, enhance the organisms' ability to interact with one another to form adherent microcolonies or 'consortia' of microorganisms (Costerton *et al.* 1981).

Isolated intestinal epithelial cells, or brush-border membranes from these cells, have been used to study the binding of fimbrial antigens from enterotoxigenic organisms to the intestinal mucosa. Attempts to inhibit binding in these systems with simple sugars have generally been ineffective, whereas more complex carbohydrate structures such as those found on native gangliosides inhibit binding of K88, K99 and CFA/I (Gaastra & de Graaf 1982).

Genetic analysis has identified piglet strains whose brush borders differ in their ability to bind K88-positive enterotoxigenic *E. coli*. Kerns & Gibbons (1979) have suggested that there are differences in the glycolipid composition between brush borders from piglets that bind K88 antigen and those that do not, and these differences may be related to the loss of K88 receptor function. Glycolipids are present in great abundance in the apical plasma membrane of intestinal epithelial cells, but Slomiany *et al.* (1981) have shown that they are also present in the more superficial glycocalyx layers and in the mucous layer. Thus the observed association of enterotoxigenic *E. coli* with the intestinal surface may be the result of interactions of adhesive fimbriae with specific complex carbohydrate receptors in the glycocalyx. Indeed, competition

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

between receptors on the membrane, in the glycocalyx, or in the mucous layer may determine whether organisms expressing adhesins are swept away in the mucus, attach to the glycocalyx (as appears to occur with enterotoxigenic *E. coli*) or achieve a more intimate association with the epithelial cell membrane (as may occur with the enteropathogenic *E. coli* discussed below). below).

3. Enteropathogenic *E. coli*

A group of enteropathogenic *E. coli* (EPEC) serotypes causing outbreaks of diarrhoea in infants, frequently in association with nurseries, has been recognized since the 1950s. Although some of the strains have now been shown to produce cytotoxins, the diarrhoeal mechanism of EPEC infections remains obscure (Wade *et al.* 1979; O'Brien *et al.* 1982). None the less, the continued occurrence of EPEC outbreaks, and challenge studies in human volunteers, have reaffirmed the pathogenic potential of these organisms (Levine *et al.* 1978).

Recently, intestinal biopsies from an infant have revealed a close association of EPEC strain O125ac:H21 with the duodendal mucosa (Ulshen & Rollo 1980). This association is morphologically distinguishable from that described for the enterotoxigenic strains in that the organisms are closely associated with the apical surface of the epithelial cells, i.e. less than 20 nm separates the plasma membrane from the bacterium. Microvilli are characteristically absent in the areas of close adherence, and there is a disruption or absence of the normal cytoskeletal elements that make up the core of the microvillus. The apical plasma membrane partly surrounds the attached organisms in a cup-like or pedestal-like extension (figure 3). Similar observations have also been made on biopsies obtained from outbreaks of EPEC diarrhoea caused by *E. coli* O119 (Rothbaum *et al.* 1982), suggesting that this pathology is characteristic of EPEC infections. The determinants of this interaction are unknown although the morphology suggests some type of adhesin–receptor interaction. Adherence of EPEC strains to human foetal intestinal tissue *in vitro* has been reported, and this has been shown to be plasmid-mediated (Williams *et al.* 1977). It is not known, however, whether the attachment is due to fimbriae or to some other mechanism.

The study of the pathogenesis of EPEC diarrhoea has been hampered by the lack of an

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Description of plate 1

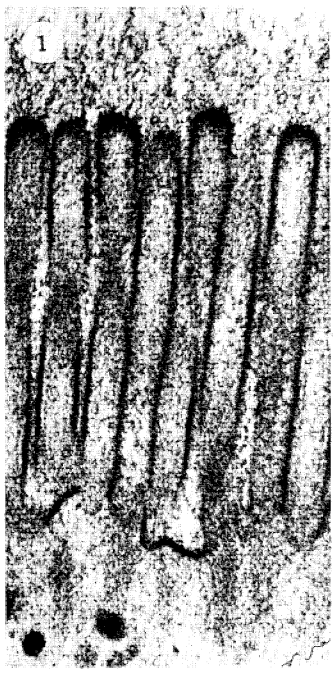
Figure 1. Transmission electron micrograph of the intestinal epithelium stained with ruthenium red. Note electron-dense fibrous glycocalyx extending above the tips of the microvilli. (Photograph courtesy of J. Trier.) (Magn. ×40000.)

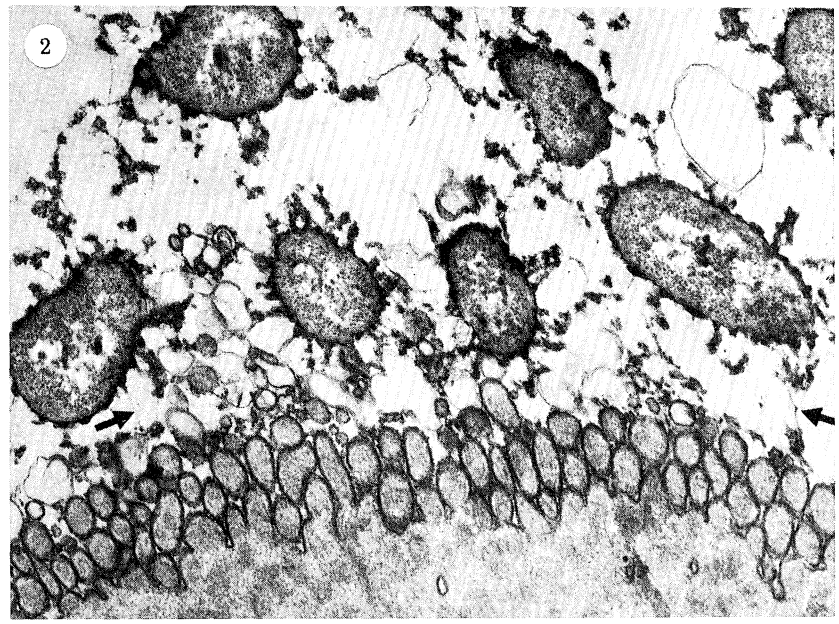
Figure 2. Electron micrograph of ileal mucosa from a calf infected with an enterotoxigenic strain of *E. coli* (ETEC). The tissue was treated with anti-K99 monoclonal antibody before staining with ruthenium red. Note the thickened K99 fimbriae (arrows) that appear as fine electron-dense projections from the bacterial surface. The extracellular polysaccharide capsule became dehydrated during fixation, and collapsed to form amorphous accretions on the bacterial surface and on the fimbriae. (Photograph courtesy of J. W. Costerton.) (Magn. ×20000.)

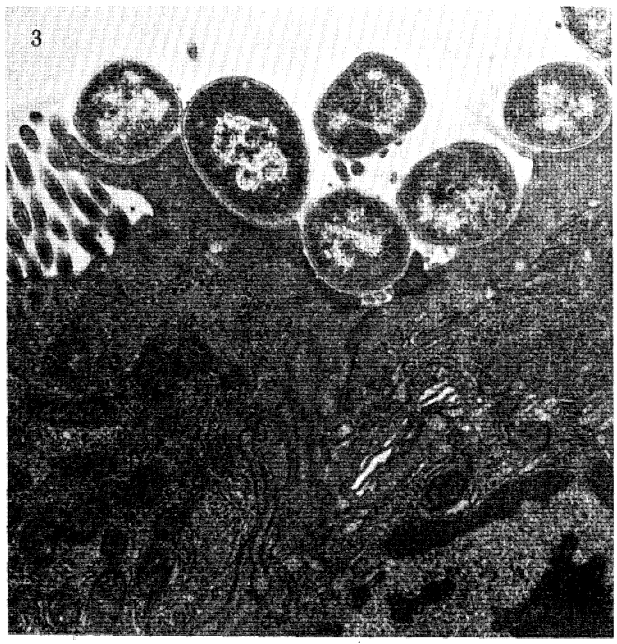
Figure 3. Electron micrograph of an enteropathogenic *E. coli* (EPEC) adhering to the jejunal epithelium of an infected infant. At areas of bacterial contact, the microvilli are obliterated, and the adherent organisms are found in cup-like depressions of the simplified plasma membrane. (Photograph courtesy of R. Giannella.) (Magn. ×18000.)

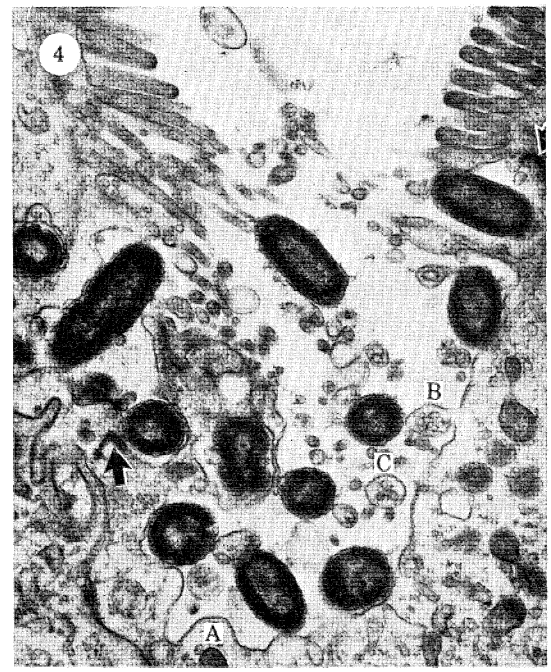
Figure 4. Electron micrograph of *S. typhimurium* invading the ileal epithelium of a guinea pig. The microvilli, terminal web, and apical cytoplasm are replaced by a cavity lined with bleb-like projections (A), some of which contain small vesicles (B, C). An intercellular junctional complex is displaced laterally (arrows) while the bacteria are being internalized. (Photograph courtesy of A. Takeuchi.) (Magn. ×18000.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES









FIGURES 1–4. For description see opposite.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

animal model that reproduces the human syndrome. The discovery of a rabbit diarrhoea *E. coli* (RDEC-I) strain that mimics the pathogenesis of EPEC strains (Cantey & Blake 1977) may have furnished a model of enteropathogenic infection. Like EPEC strains, RDEC-I does not produce detectable amounts of the recognized *E. coli* enterotoxins. Morphologically, the adherence of strain RDEC-I to rabbit intestine (ileum and caecum) as studied by Takeuchi *et al.* (1978) and Cantey *et al.* (1981) is indistinguishable from the adherence of EPEC strains to the human intestinal mucosa. In recent studies, Inman & Canty (1983) have demonstrated that the initial site of attachment of this pathogen is to the membranous (M) cells of the lymphoid follicle rather than to the absorptive epithelial cells. Attachment to M cells was observed a few hours after oral challenge, whereas colonization of the absorptive cells was seen after several days.

Cheney *et al.* (1980) have demonstrated the species-specific adherence of strain RDEC-I to rabbit intestinal brush-border (apical) membranes. Adherence is related to the expression of fimbriae, and the fimbriated phenotype is associated with the presence of a plasmid (Cheney *et al.* 1983). This attachment is not due to type I fimbriae, however, because wild-type RDEC-I does not agglutinate guinea-pig red blood cells; nor is its adherence to isolated brush-border membranes inhibited by mannose or other simple sugars. Receptor activity on rabbit brush borders is an age-related property, first expressed at the time of weaning. Receptor activity (bacterial agglutinins) can be solubilized from brush borders, and it appears to reside in the glycoprotein fraction.

4. Enteroinvasive pathogens

In addition to the enterotoxigenic and enteropathogenic modes of virulence, some organisms cause disease by invading the intestinal mucosa. Invasive pathogens include the salmonellae, shigellae, 'shigella-like' *E. coli*, *V. parahemolyticus*, *Yersinia enterocolitica*, *Campylobacter fetus* (subspecies *jejuni*) and perhaps *Aeromonas hydrophilia*. It should be emphasized that some of these 'invasive' species may also have characteristics of enterotoxigenic and enteropathogenic organisms. For example, *S. dysenteriae* I produces a toxin that is both enterotoxic and cytotoxic (Eiklid & Olsnes 1983), and *S. typhimurium* produces a toxin that is antigenically and biologically similar to choleragen (Peterson & Sandefur 1979). As a result of this redundancy of virulence mechanisms, invasive organisms can elicit a variety of clinical manifestations including gastroenteritis, diarrhoea, dysentery and systemic febrile infections.

Perhaps the most thoroughly studied invasive species are *S. typhimurium* and *S. flexneri*. Ultrastructural studies indicate that these pathogens invade the cells of the intestinal epithelium by an endocytic mechanism (Takeuchi 1967). The organisms initially cause a localized degeneration of the organization of the microvillus at the luminal surface of the epithelial cells, and they are subsequently engulfed in invaginating areas of the apical membrane (figure 4). After internalization, salmonellae are usually found in membrane-bound cytoplasmic vesicles, whereas shigellae apparently digest these vesicles and are found free in the cytoplasm.

The complexity of *in vivo* models of enteric infection has prompted the use of tissue-culture monolayers as simplified models of the intestinal epithelium. Although an endocytic mode of bacterial invasion has been observed in the tissue-culture model (Kihlstrom & Nilsson 1977; Hale *et al.* 1979), the biochemical process that induces uptake of enteroinvasive pathogens remains obscure. It has been found that other invasive agents such as chlamydiae, rickettsiae and many types of protozoans express proteinaceous receptors that recognize glycoconjugate

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

determinants on the surface of cultured mammalian cells. This receptor-mediated interaction plays an integral role in the endocytic uptake of the above organisms (reviewed by Hale *et al.* 1983*a*). By analogy, *S. typhimurium* express mannose-resistant haemagglutinating activities (m.r.h.a.) that may induce the endocytosis of these organisms (Jones & Richardson 1981) by sequentially binding to determinants in or on the glycocalyx of mammalian cells.

No haemagglutinin comparable with the m.r.h.a in salmonellae has been demonstrated in shigellae. However, ultrastructural evidence suggests that *S. flexneri* establish discrete points of close apposition with the plasma membrane of HeLa cells during the infection process (Hale *et al.* 1983*a*). After fixation *en bloc*, colloidal thorium dioxide is excluded from these areas, indicating that there is a gap of less than 10 nm between the bacterium and host cell. The points of close opposition may represent interactions between the surfaces of the bacterium and the host cell that allow the organism to be engulfed by circumferential ligand binding, i.e. the 'zipper mechanism' (Griffin *et al.* 1975). The chemical nature and structural organization of these putative ligands is unknown; however, genetic analysis may provide a clue. Recently it has been shown that a 140 MDa plasmid encodes determinants necessary for the invasive phenotype in *S. flexneri* (Sansonetti *et al.* 1982). Indeed, this plasmid can even confer the invasive phenotype on rough *E. coli* K12 transconjugants (Sansonetti *et al.* 1983). The translation products of the 140 MDa plasmid have been studied in anucleate shigella minicells, and it appears that at least ten polypeptide species are encoded by the plasmid (Hale *et al.* 1983*b*). Because these polypeptides are found in the outer membrane, it is possible that they constitute part of an adhesin that binds to mammalian cells and induces the endocytosis of shigellae.

So far the host-cell determinants recognized by enteroinvasive bacteria have not been identified. None of the monosaccharide moieties that commonly occur in the mammalian glycocalyx inhibit the activity of the *S. typhimurium* m.r.h.a. (G. W. Jones, personal communication), and lectin-resistant Chinese hamster ovary cells with various glycosylation defects remain exquisitely sensitive to invasion by shigellae (T. L. Hale & P. Stanley, unpublished data). These observations suggest that salmonellae do not bind to a single sugar moiety and that shigellae do not recognize the terminal sugars on common glycoproteins. The organisms may adhere to an unidentified portion of the glycocalyx or to an exogenous substance like fibronectin. In addition, it should be remembered that the receptor on intestinal epithelial cells may differ from the receptor on tissue culture cells. None the less, the latter model has yielded some information about the interaction of enteroinvasive pathogens with mammalian cells, and its future use will no doubt yield additional data.

Impressive progress has been made in understanding the initial stages of the pathogenesis of bacterial infections of the bowel. This knowledge is of importance, because interruption of these steps, by whatever means, would offer an excellent approach to prevention of clinical disease in animals and human beings. Encouraging results have already been obtained in preventing diarrhoea caused by *E. coli* enterotoxigenic strains in animals, and attenuated oral vaccines have been shown to protect against shigellosis and typhoid fever. The former vaccines prevent the colonization of the proximal bowel by *E. coli*; the latter presumably prevent the penetration of the intestinal epithelial cell by shigellae or *S. typhi*. As more basic knowledge becomes available about colonization factors and about the mechanisms involved in penetration of epithelial cells, it is certain that additional and improved procedures to confer immunity against enteric diseases will be devised.

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Discussion

C. Lam (*Sandoz Research Institute, Vienna, Austria*). Can the knowledge of bacterial adherence be exploited in the development of novel antimicrobial agents? In other words, can we treat human bacterial infections chemically with drugs that specifically inhibit the biosynthesis of bacterial adhesins?

S. B. Formal. Recognition of the pivotal importance of bacterial adherence in pathogenesis suggests that novel agents could be developed to interfere with the process and thereby prevent disease. Antibiotics at doses below the minimum inhibitory concentration have been shown to suppress the expression of type I fimbriae *in vitro* and similar effects may obtain *in vivo* for adherence fimbriae. This may be a mechanism whereby low-dose antibiotic therapy prevents and treats traveller's diarrhoea caused by an adherent enterotoxigenic *E. coli* strain. Because

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fimbrial expression is usually negatively influenced by high levels of nutrient, it may be possible to limit attachment by controlling the intraluminal environment in the intestine by dietary means. Finally, attachment may be inhibited by introducing analogues of the mucosal receptor, either free in solution or immobilized on gel or fibre matrices, into the human. Such a receptor analogue may be expected to bind organisms and promote their clearance.

J. M. Rutter (*Institute for Research on Animal Diseases, Compton, Berkshire, U.K.*). Have any of the engineered strains containing the large *Shigella* plasmid or the other genes that Dr Formal mentioned have been tested in human volunteers?

S. B. Formal. *E. coli* K12 strains carrying the 140 MDa plasmid from *S. flexneri* have not been tested in volunteers. One hybrid with the plasmid that also inherited the chromosomal genes for O-antigen expression has been fed to monkeys at a dosage of 5×10^{10} cells. An occasional animal experienced a transient diarrhoea. These animals were resistant to subsequent challenge with virulent *S. flexneri* of the homologous serotype.

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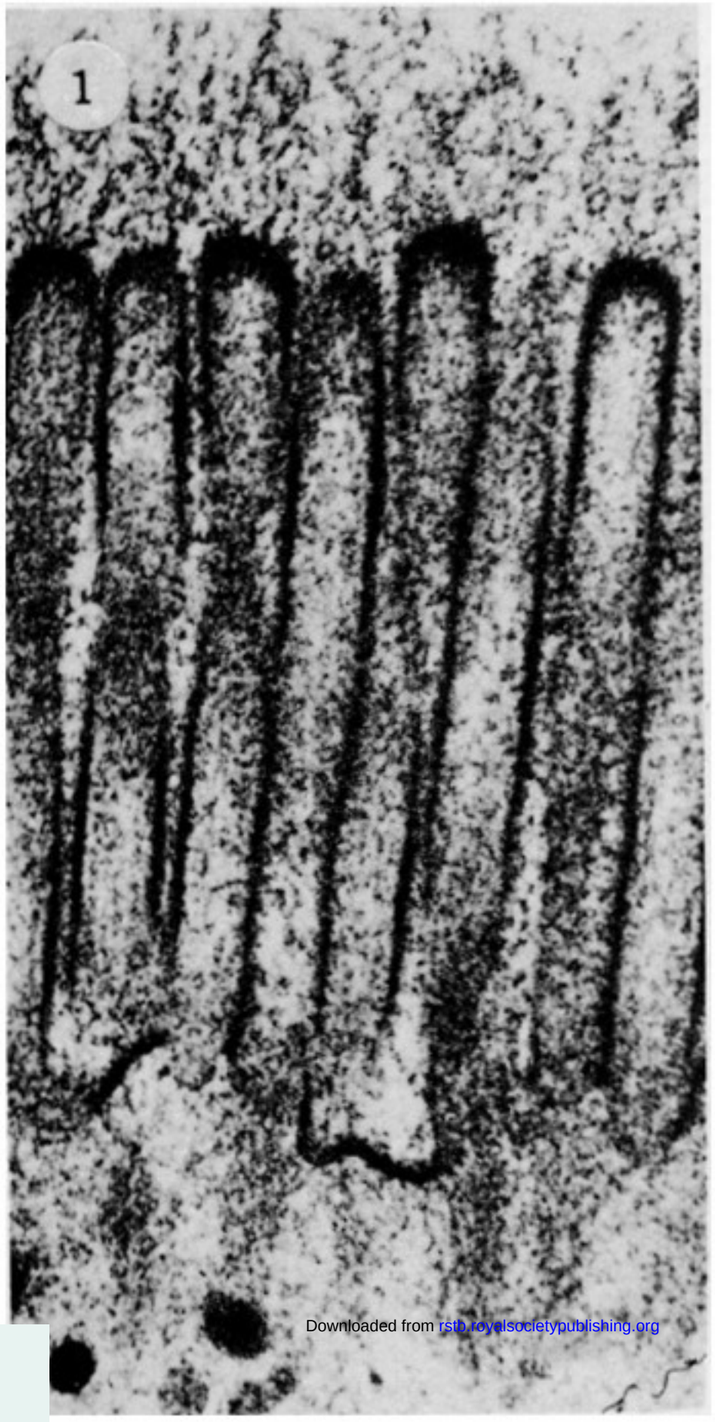

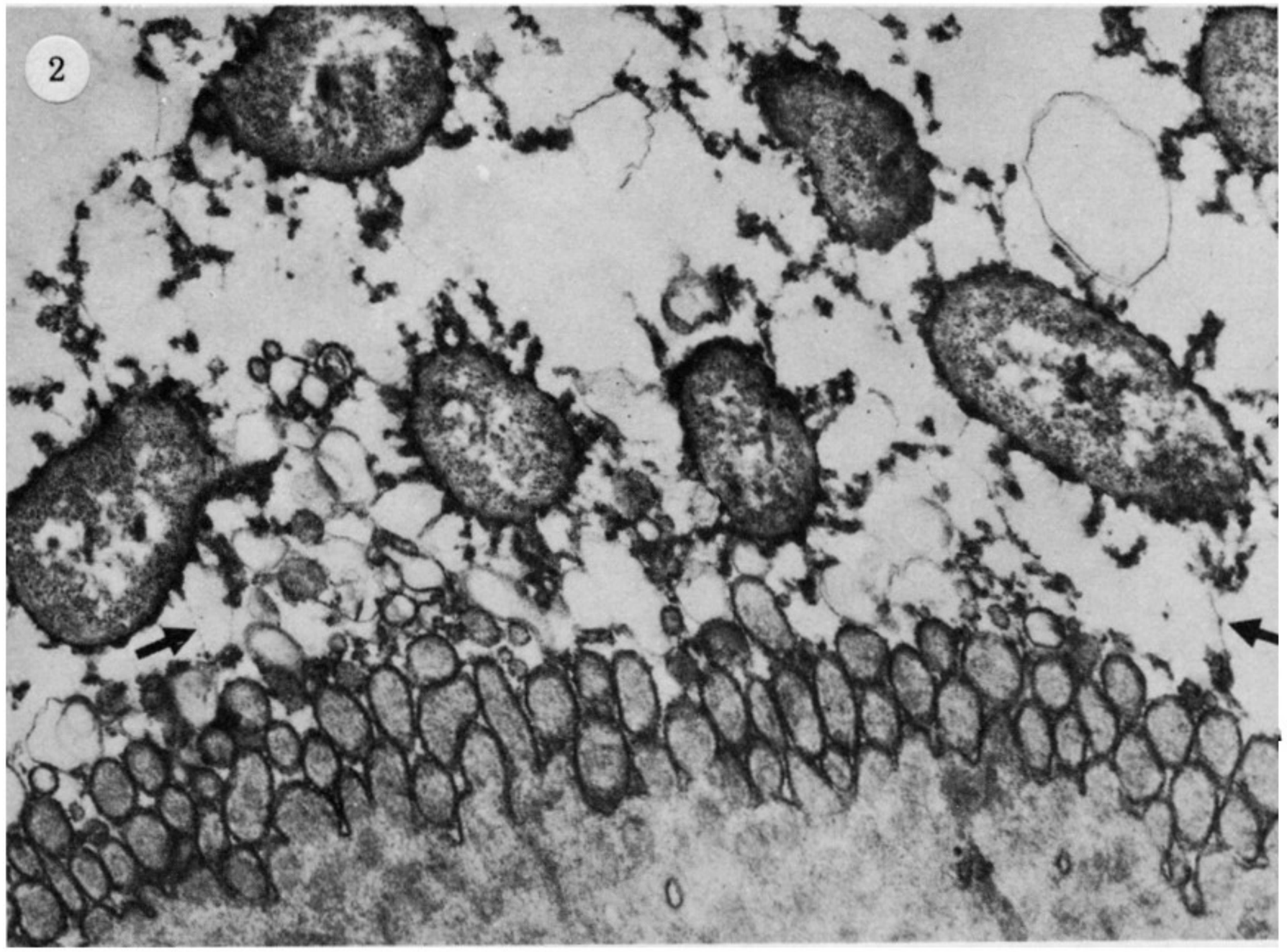

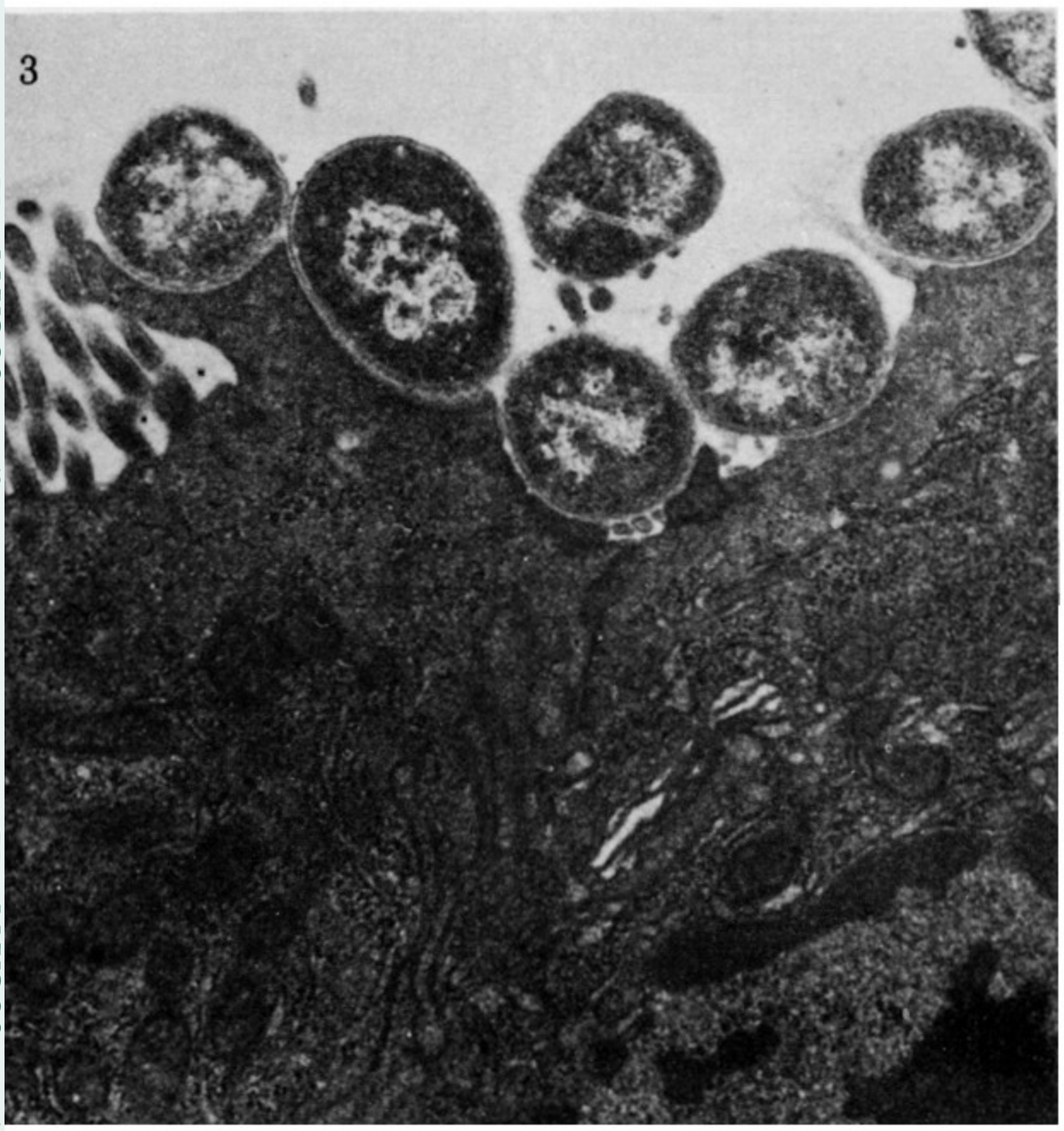

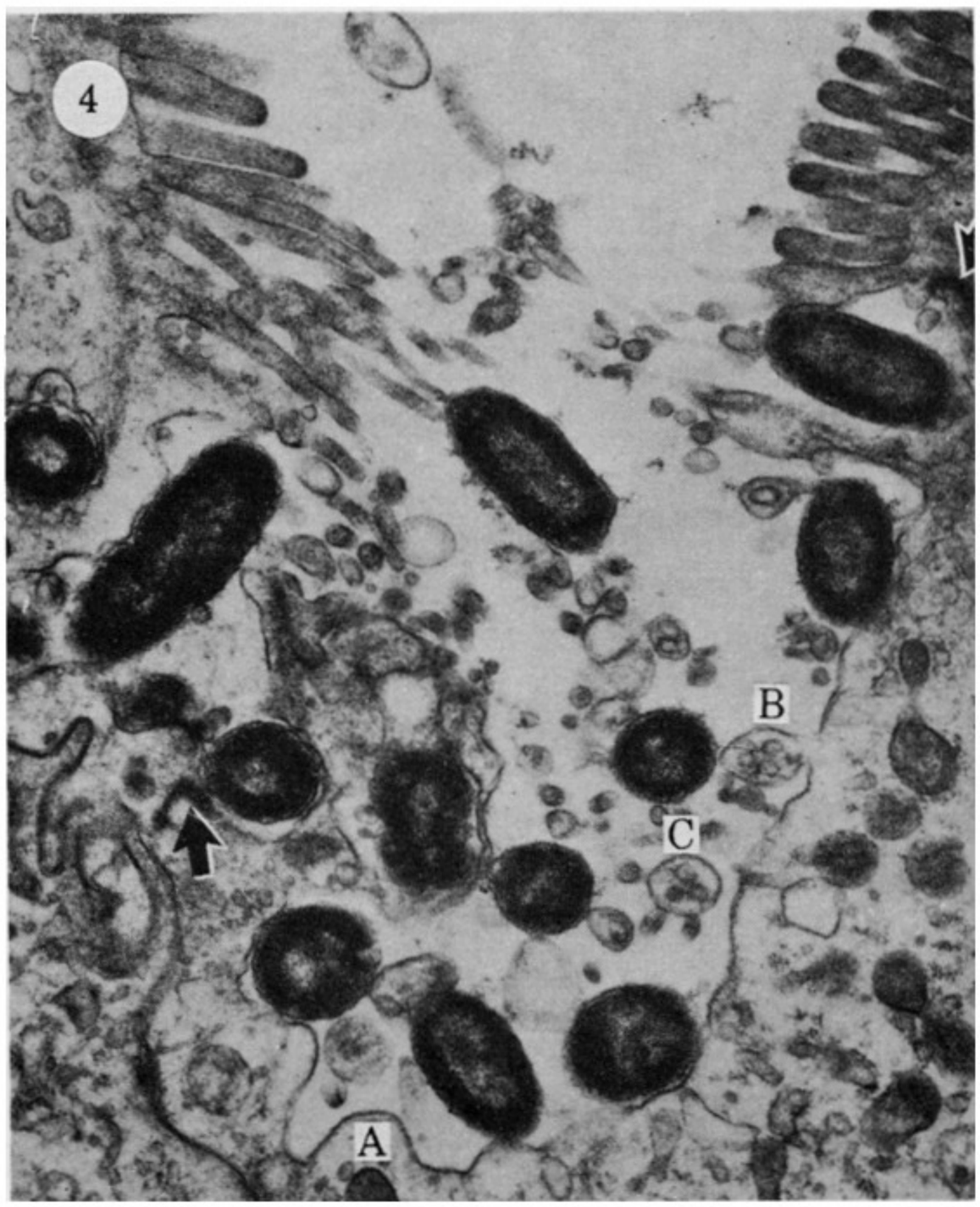

FIGURES 1–4. For description see opposite.