The Use of Specific Antibodies to Demonstrate the Glycocalyx and Spatial Relationships of a K99$^-$, F41$^-$ Enterotoxigenic Strain of *Escherichia coli* Colonizing the Ileum of Colostrum-deprived Calves

R. Chan, C.J. Lian, J.W. Costerton and S.D. Acres*

ABSTRACT

Electron microscopy was used to study the interaction between the glycocalyx of enterotoxigenic *Escherichia coli* strain 210 (09:K30+;K99-;F41-:H-) and the glycocalyx of epithelial cells in the ileum of experimentally infected newborn colostrum-deprived calves. Fixation of tissues in anti-K30 antibody and ruthenium red was used to stabilize the bacterial glycocalyx so that the spatial relationship between the bacteria and the intestinal epithelial cells could be characterized.

When strain 210 was grown *in vitro* and reacted with anti-K30 antibody prior to staining with ruthenium red, the extensive glycocalyx could be clearly visualized surrounding the bacterial cells. By negative staining, an unidentified pilus was also seen. Sections of ileum from infected calves, which were not fixed in antibody nor stained with ruthenium red, revealed attached bacteria which were surrounded by an electron-translucent zone and no visible bacterial glycocalyx. When ruthenium red staining was used, the bacterial glycocalyx partially collapsed during the dehydration steps of fixation, but could be seen as either a fibrous capsule or an electron-dense accretion on the bacterial cell surface. When ileal tissue was reacted for one hour in anti-K30 antibody before staining with ruthenium red, the bacterial glycocalyx was seen as a discrete electron-dense structure up to 1.0 μm thick which was in intimate contact with the glycocalyx of the epithelial cells. The importance of the bacterial exopolysaccharide to microcolony formation on the villi could be clearly visualized.

RÉSUMÉ

Cette expérience consistait à utiliser la microscopie électronique pour étudier l'interaction entre le glycocalice de la souche entérotoxinogène 210 d'*Escherichia coli* (09: K30+; K99-; F41-: H-) et celui des cellules épithéliales de l'iléon de veaux nouveau-nés et privés de colostrum, soumis à une infection expérimentale. On utilisa la fixation des tissus dans des anticorps anti K30 et la coloration au rouge de ruthénium pour stabiliser le glycocalice des colibacilles, afin de pouvoir caractériser la relation spatiale entre ces bactéries et les cellules épithéliales de l'iléon.

Quand on cultiva la souche 210 *in vitro* et qu'on la soumit à l'action des anticorps anti K30, avant de la colorer avec le rouge de ruthénium, on pouvait facilement visualiser le glycocalice extensif qui entourait les cellules bactériennes. La coloration négative permit aussi de voir un filament protoplasmique non encore identifié. Les sections de l'iléon des veaux expérimentaux qu'on ne soumit pas à l'action des anticorps et qu'on ne colora pas avec le rouge de ruthénium, recelaient des bactéries attachées à la muqueuse, entourées d'une zone translucide, à la microscopie électronique, mais dépourvues d'un glycocalice visible. Quand on utilisa la coloration au rouge de ruthénium, le glycocalice bactérien s'affaissa partiellement, au cours des étapes de déshydratation de la fixation; on pouvait cependant le voir, soit comme une capsule fibreuse, soit comme une accrétion dense, à la microscopie électronique, sur la surface des cellules bactériennes. Par ailleurs, lorsqu'on soumit des sections de l'iléon des veaux expérimentaux à l'action des anticorps anti K30, durant une heure, avant de procéder à la coloration au rouge de ruthénium, le glycocalice bactérien se présenta sous la forme d'une structure discrète et dense, au microscope électronique; cette structure mesurait jusqu'à 1 μm d'épaisseur et elle était en contact étroit avec le

*Department of Biology, The University of Calgary, Calgary, Alberta T2N 1N4 (Chan and Costerton) and Veterinary Infectious Disease Organization, 124 Veterinary Road, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0 (Lian and Acres).

Reprint requests to Dr. S.D. Acres.

Submitted October 6, 1982.

glycocalice des cellules épithéliales. On put aussi visualiser clairement l'importance de l'exopolysaccharide bactérien à l'égard de la formation de microcolonies sur les villosités intestinales.

INTRODUCTION

In a previous paper we described the attachment and spatial relationship between enterotoxigenic Escherichia coli (ETEC) strain B44 (09:K30+;K99+;F41+:H-) and the ileal mucosa of colostrum-fed calves (4). Attachment, multiplication and the subsequent formation of microcolonies covering the intestinal epithelial cells are critical steps in the pathogenesis of diarrhea caused by ETEC. Pili have been shown to be an important mechanism of bacterial attachment to the intestinal mucosa in several animal species (12). Most epidemiological studies of bovine strains of *E. coli* have found a high correlation between the presence of K99 pili and enterotoxigenicity (9, 13, 17), suggesting that K99 antigen is the main attachment mechanism in calves. However, there may be other mechanisms of adherence in addition to K99 antigen. Girardeau and co-workers demonstrated the attachment of K99- strains of *E. coli* to calf intestinal villi *in vitro*, and suggested the occurrence of two other pilus attachment factors on calf ETEC which they called F(Y) and F (31a) (8, Girardeau, J.P., H.C. Dubourguier and M. Contrepois. 1979. Attachment des *E. coli* entéropathogènes à la muqueuse intestinale. In gastro-entérites néonatales du veau, Société Française de Buiatrie. pp. 53-66). More recently, Morris et al described a second pilus in addition to K99, which occurs on ETEC strains possessing 0 antigens 9 or 101, and which they have labelled F41 (6, 18, 19). Smith and Huggins were also able to reproduce diarrhea in colostrum-deprived calves by orally inoculating ETEC strains which lacked the K99 antigen (21). Using one of these strains obtained from H.W. Smith (VIDO strain 210, serotype 09:K30+;K99-;F41-:H-), Bellamy and Acres also reproduced diarrhea in colostrum-deprived calves and demonstrated that the organisms attached to intestinal mucosa. However, in contrast to K99+ ETEC which form layers covering intestinal villi in the ileum and jejunum, colonization of the villus surface by strain 210 was much more focal and was confined to the ileum (2). The present study was conducted to determine if the spatial relationship between the glycocalyx of attached bacterial cells and the glycocalyx of the ileal mucosa in colostrum-deprived calves challenged with ETEC strain 210 is as intimate as that seen in calves infected with ETEC strain B44.

MATERIALS AND METHODS

STRAINS OF *E. COLI*

Escherichia coli strains of the serotypes 09:K30+;K99-;F41-;H-(VIDO strain 210) and 09:K30-;K99-(VIDO strain 211) were obtained from Dr. H.W. Smith, Houghton Poultry Research Station, England. Both strains were derived from an 09:K30+:K99+ calf enteropathogenic strain, believed to be reference strain B44, by methods previously described (21). Strains 210 and 211 were previously designated as the 0+K+99- and 0+K-99- variants respectively by Smith and Huggins (21). Both strains produced heat-stable enterotoxin as detected by the infant mouse assay (17). Neither strain agglutinated with standard K99 antiserum when grown on Minca agar containing 1% Isovitalex[1] (Minca-IS) (10). Strain 210, but not strain 211, agglutinated in K30 antiserum. Strain 210 grown on Minca agar was negative when examined for the presence of F41 antigen using an indirect immunofluorescent technique (personal communication, J.A. Morris), and had previously been shown not to possess K88 or 987P pili (2).

PREPARATION OF ANTISERA

The antisera used were those described previously (4). The K99 antiserum, which was prepared using purified K99 antigen (11), had an agglutinating titer of 1/2048 when tested against K99 reference strain B41 (0101:K99) and the K30 antiserum had an agglutinating titer of 1/64 when tested against strain 210 grown on blood agar.

PREPARATION OF CULTURED CELLS FOR ELECTRON MICROSCOPY

Aliquots (4 mL) of cells from experimental cultures of strains 210 and 211 grown on Minca-IS for 18 h at 37°C and washed once in phosphate buffered saline (PBS), were mixed with 0.1 mL of K30 antiserum (diluted 1:2 with PBS) and held for one hour at room temperature. Following this specific antibody stabilization, the cells were washed in PBS and fixed and processed in the presence of ruthenium red by the methods of Mackie *et al* (16). Cells to be examined by negative staining were mixed with an equal volume of 2% (v/v) ZrO_2 (pH 7.0, containing 0.2% sucrose) and a drop of this mixture was placed on polyvinyl Formvar-coated copper grid, blotted and air dried.

INOCULATION OF CALVES

Two newborn Holstein bull calves were removed from their dams immediately after birth before nursing had occurred and were placed in individual isolation rooms. At three hours of age they were challenged orally by slowly inoculating a trypticase soy broth culture of *E. coli* strain 210 into the back of the mouth using a 20 mL syringe as previously described (2).

COLLECTION OF TISSUE SAMPLES

Calves were examined at regular intervals following challenge inoculation for the presence of diarrhea. At 42 (Calf 80-40) and 25 (calf 80-42) hours of age the calves were euthanized by an intravenous

[1]BBL, Cockeysville, Maryland.

inoculation of sodium pentobarbitol (Euthanyl, MTC Pharmaceuticals, Hamilton, Ontario) and tissue sections were taken from five equally spaced sites in the small intestine and processed as described previously (4). For stabilization of the bacterial cell capsule, tissue sections were immersed in a 1:5 dilution of K30 antiserum for one hour at room temperature prior to staining with ruthenium red (15) and fixation.

RESULTS

When strains of ETEC were grown on Minca-IS medium and examined by TEM (as anti-K30 antibody-stabilized, ruthenium red-stained preparations) the K30- strain (211) was seen to lack any discernable structure outside the outer membrane of the cell wall (Fig. 1a), while cells of K30+ strain 210 were seen to be surrounded either by thin radially-structured glycocalyces (Fig. 1b), or by very extensive glycocalyces composed of fibrous exopolysaccharide material up to 1.0 μm thick (Fig. 1c). Negatively-stained preparations of strain 210 showed the presence of variable numbers of very thin flexible pilus-like structures. A comparatively heavily "piliated" cell is shown in Fig. 2.

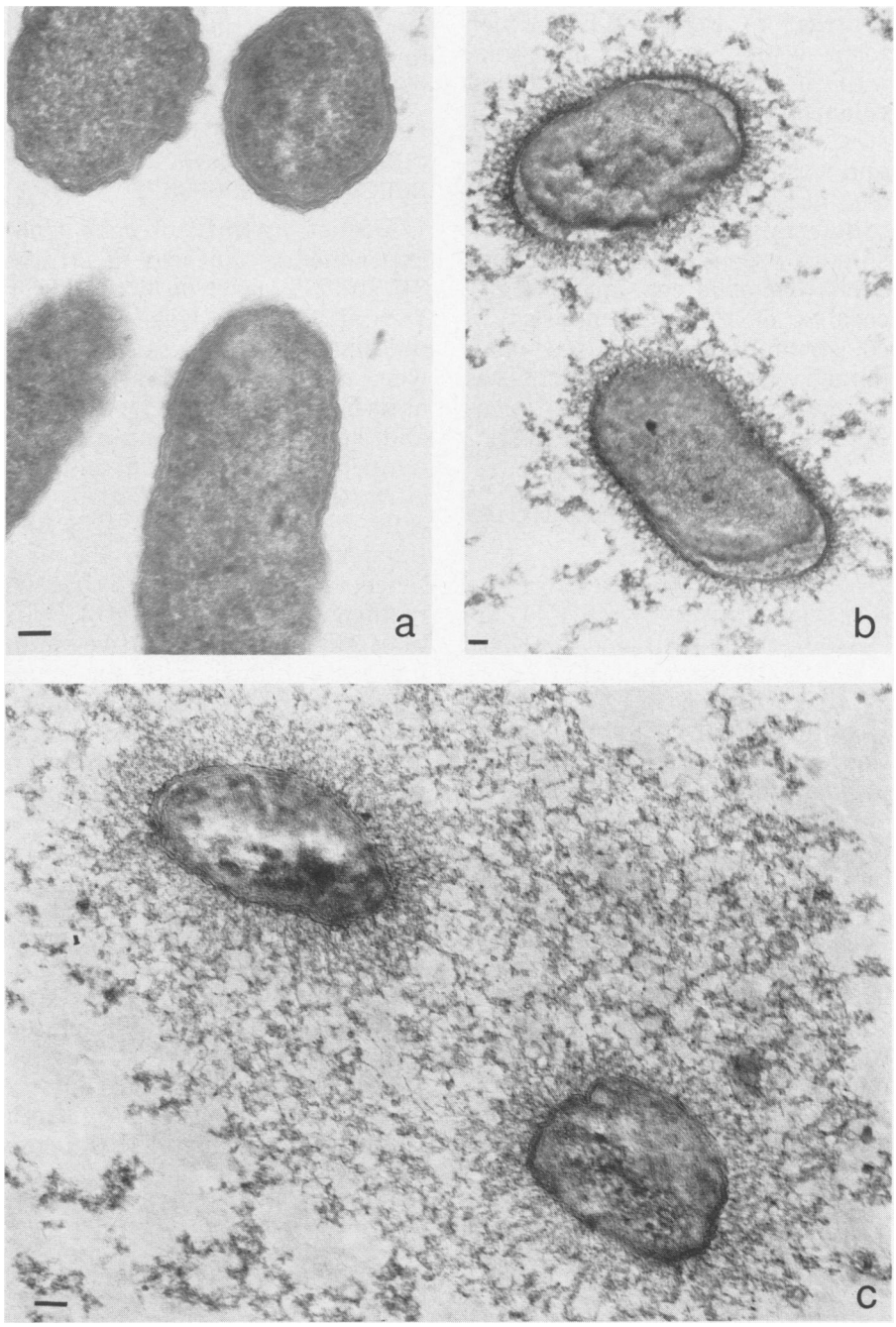

Fig. 1. Electron micrographs of anti-K30 antibody-stabilized, ruthenium red-stained ETEC strains grown on Minca-IS medium. Strain 211 (09:K30-:K99-) shows no glycocalyx and no structures outside the outer membrane of the cell wall (Fig. 1a), whereas strain 210 (09:K30+:K99-;F41-:H-) shows distinct consolidated glycocalyces varying from a relatively thin radially-arranged fibrous matrix (Fig. 1b), to very extensive fibrous masses surrounding the cells in a radial pattern and occupying more than 1 μm of intercellular space (Fig. 1c). The bars in these and all subsequent electron micrographs indicate 0.1 μm.

Electron microscopy of the ileum (intestinal locations 4 and 5) of colostrum-deprived neonatal calves orally infected with strain 210 showed numerous bacteria in immediate proximity to the microvillus border (Figs. 3-7). When both ruthenium red staining and antibody stabilization were omitted the bacterial cells were seen to be separated from the tissue surface, and from adjacent bacteria and debris by an electron-translucent zone of variable dimensions (Fig. 3). When the preparations were stained with ruthenium red, but not stabilized by specific antibody, the exopolysaccharide of the infecting bacterial cells, which had collapsed to a variable extent during dehydration, was seen to form either a fibrous capsule (Fig. 4a) or a very highly condensed electron-dense accretion on the bacterial cell surface (Fig. 4b).

When preparations of infected tissue were treated with specific anti-K30 antibody before fixation and staining with ruthenium red, the bacterial glycocalyces were seen as thick consolidated electron-dense structures surrounding the bacterial cells and in intimate contact with the brush border (Figs. 5, 6 and 7). These very extensive glycocalyces were seen to completely surround the bacteria and to play an important role in the formation of planar microcolonies at the tissue surface. In areas into which

the stabilizing antibodies had penetrated less well (Fig. 5, inset, and the inner glycocalyces in Fig. 6) the bacterial glycocalyx is less completely stabilized and consolidated. The specificity of this stabilization of the bacterial glycocalyx is supported by the total lack of stabilization of the tissue cell glycocalyces at the microvillar borders seen in Fig. 6 and in Fig. 7 (arrows). Well stabilized glycocalyces are seen at high magnification in Fig. 7, and both the fibrous nature of the exopolysaccharide and its role in microcolony formation are clearly illustrated.

DISCUSSION

The ability of K99- strains of encapsulated ETEC to colonize the small intestine and cause diarrhea in colostrum-deprived calves was previously demonstrated by Smith and Huggins (21); however, they did not attempt to visualize attachment of the bacteria to villus epithelial cells. Bellamy and Acres confirmed that strain 210, obtained from H.W. Smith, caused diarrhea in colostrum-deprived calves and also demonstrated focal attachment of this strain to the ileal epithelium (2). The present report confirms that strain 210 attaches to intestinal epithelial cells, and demonstrates that the degree of contact between the glycocalyx of adherent bacteria and the glycocalyx of epithelial cells in the ileum is just as intimate as that seen with strain B44 (4).

The molecular architecture of the cell surface of this strain is of interest because it bears a pilus of unknown function and is surrounded by K30 capsular antigen, an extensive fibrous polyanionic glycocalyx composed of D-glucuronic acid, D-mannose and D-galactose (3). This extensively hydrated structure (22) condenses during the dehydration steps used in routine preparation of tissue for electron microscopy (16), and ruthenium red staining reveals the remnants of this collapsed matrix as an even electron dense "crust" at the cell surface (Fig. 4b). When the collapse of the glycocalyx is less complete (Fig. 4a), or when it is prevented by cross-linking with specific antibodies (16), this complete fibrous surface structure is seen to form a very coherent and highly organized matrix close (ca 0.3 μm) to the cell wall, and a much more diffuse matrix that may

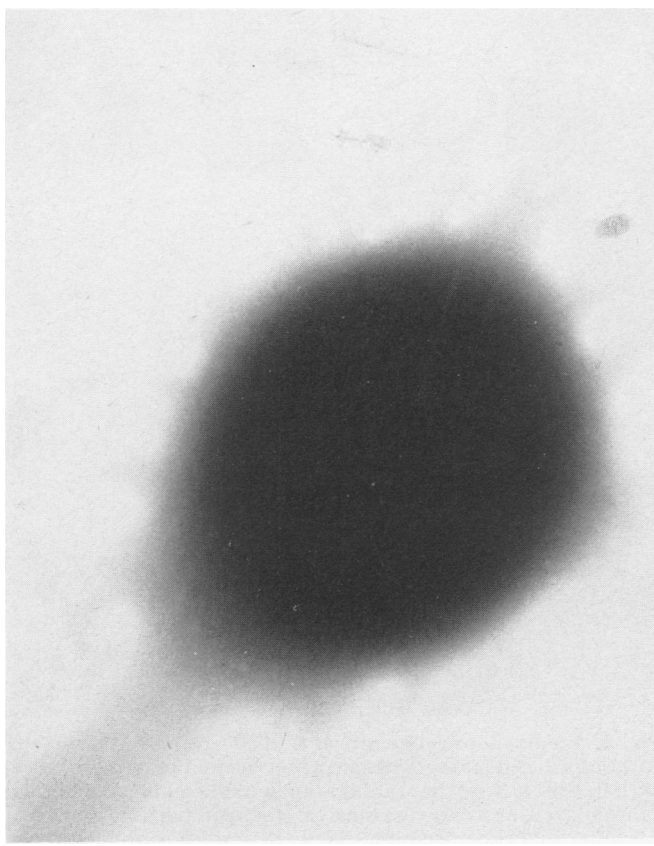

Fig. 2. Negative stain of cells of strain 210 showing very fine flexible pilus-like structures. This strain had previously been shown, by fluorescent antibody staining of intestinal sections, to lack K99, K88, 987P antigens.

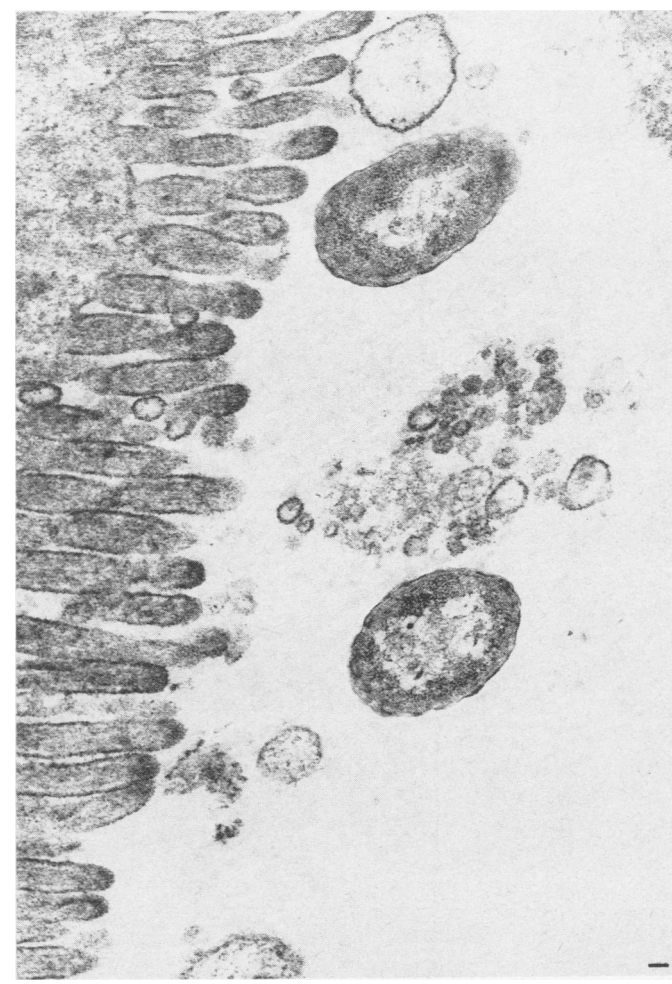

Fig. 3. Electron micrograph of a preparation of the ileum (location 5) of a colostrum-deprived neonatal calf infected with ETEC strain 210 (09:K30+:K99-;F41-:H-). Note the absence of any electron-dense projections from the surfaces of these cells and the extensive electron-translucent zones separating these bacteria from the tissue surface.

extend several μm into the surrounding milieu (Fig. 1c). The glycocalyx is not seen when the K30 antigen is absent (Fig. 1a), or when ruthenium red fixation is not used (Fig. 3). Similar results were observed when the attachment of K99+ strains to the brush border of colostrum-fed calves was examined using the same techniques (4).

The specificity of the stabilization of the glycocalyces by anti-K30 antibodies is attested to by the lack of reaction with K30- cells and by the fact that the chemically similar (20) glycocalyx of the epithelial cells of the ileum is not affected in any way by these sera. Consolidation of the glycocalyx is more complete where this structure is most accessible to stabilizing antibodies (Fig. 6), but this greater access to antibodies changes only the degree of consolidation and not the spatial extent of the glycocalyx.

When the real extent and distribution of the glycocalyces of these bacteria is seen in antibody-stabilized preparations, the role of these fibrous exopolysaccharide structures in the formation of microcolonies is obvious. Stabilization of the bacterial glycocalyx reveals that this exopolysaccharide material connects neighbouring bacterial cells so that planar microcolonies form on the surface of the infected tissue. Similar microcolonies are often seen in natural ecosystems such as streams (7), soils (1), and the bovine rumen (5), and in pathogenic systems such as the infected lung in cystic fibrosis (14).

The mechanism of attachment of strain 210 to the ileal mucosa of calves is unknown. By negative staining, cells of this strain grown on Minca-IS had pili that did not react with antiserum to the known attachment factors (K99, F41, 987P, K88) found on ETEC isolated from calves, sheep or swine. It is possible that adhesion is mediated by this undefined pilus. In addition, this strain possesses a

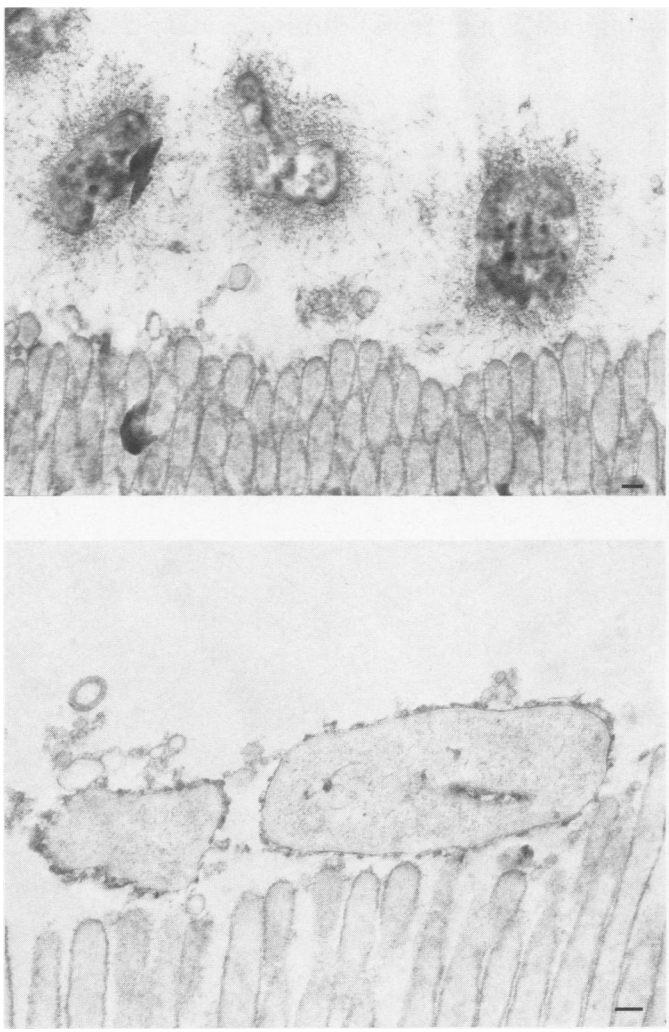

Fig. 4. Electron micrograph of a ruthenium red-stained preparation of the ileum (location 5) of a colostrum-deprived neonatal calf infected with ETEC strain 210. Collapse of the exopolysaccharide glycocalyces of the bacteria produces either a matrix of electron-dense fibres (Fig. 4a) or a very thin electron-dense accretion at the bacterial cell surface (Fig. 4b).

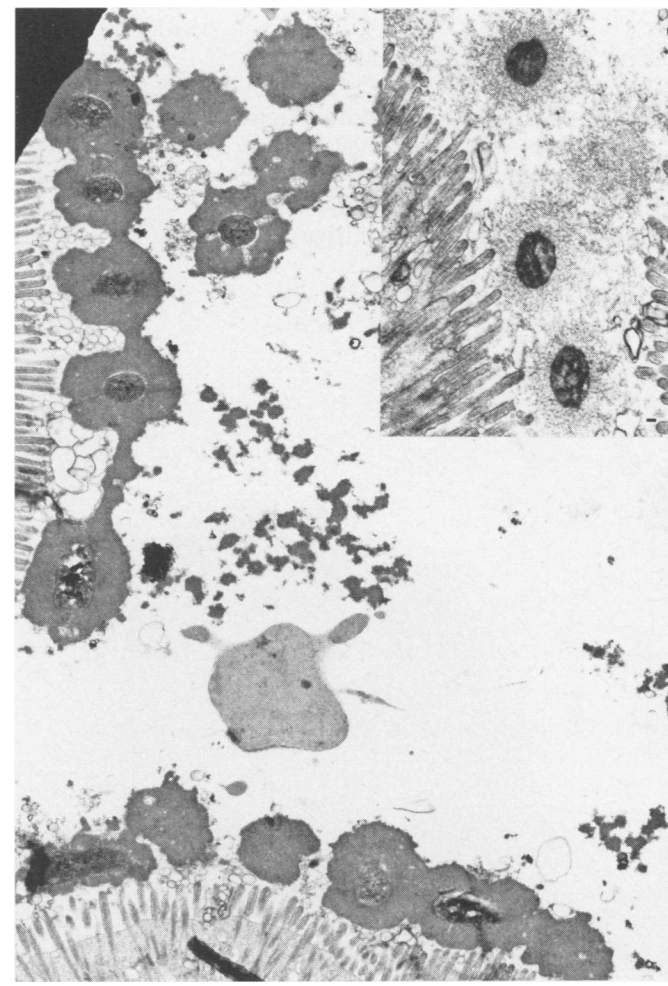

Fig. 5. Electron micrograph of a K30 antibody-stabilized, ruthenium red-stained preparation from the same source used in Fig. 4. Note the stabilization which produced a highly consolidated and very extensive electron-dense glycocalyx surrounding the infecting bacteria and the obvious role played by this structure in the formation of planar microcolonies at the surface of the infected tissue. In the intervillus space of the ileal epithelium, where access of the specific antibody was limited, a less consolidated stabilization of the bacterial glycocalyx is seen (inset).

capsular antigen (K30) and the exact role of this glycocalyx in attachment is not known. From this study, as well as the previous one on strain B44, it is evident that large amounts of this dense fibrous material is formed *in vivo*, encapsulates the attached bacterial cells, and is in intimate contact with the brush border. A clearer definition of the specific role of the pilus and the glycocalyx in colonization of the ileum requires further study.

ACKNOWLEDGMENTS

This study was supported by the Agricultural Research Council of Alberta, Farming for the Future. The technical assistance of Sandra Feschuk, Joyce Nelligan and Sheila Costerton is gratefully acknowledged.

REFERENCES

1. **BAE, H.C., E.H. COTA-ROBLES** and **L.E. CASIDA, Jr.** Microflora of soil as viewed by transmission electron microscopy. Appl. Microbiol. 23:623-648. 1972.
2. **BELLAMY, J.E.C.** and **S.D. ACRES.** A comparison of histopathological changes in calves associated with K99- and K99+ strains of enterotoxigenic *Escherichia coli.* Can. J. comp. Med. 47:143-149.1983.
3. **CHAKRABORTY, A.K., H. FRIEBOLIN** and **S. STIRM.** Primary structure of the Escherichia coli serotype K30 capsular polysaccharide. J. Bact. 141:971-972. 1980.
4. **CHAN, R., S.D. ACRES** and **J.W. COSTERTON.** The use of specific antibody to demonstrate glycocalyx, K99 pili, and the spatial relationships of K99+ enterotoxigenic *E. coli* in the ileum of colostrum-fed calves. Infection & Immunity 37:1170-1180. 1982.
5. **CHENG, J.J., R.P. McCOWAN** and **J.W. COSTERTON.** Adherent epithelial bacteria in ruminants and their roles in digestive tract function. Am. J. clin. Nutr. 32:139-148. 1979.
6. **DE GRAAF, F.K.** and **I. ROORDA.** Production, purification and characterization of the fimbral adhesive antigen F41 isolated from calf enteropathogenic Escherichia coli strain B41 M. Infection & Immunity 36:751-758. 1982.
7. **GEESEY, G.G., W.T. RICHARDSON, H.G. YEOMANS, R.T. IRVIN** and **J.W. COSTERTON.** Microscopic examination of natural sessile bacterial populations from an alpine stream. Can. J. Microbiol. 23:1733-1736. 1977.
8. **GIRARDEAU, J.P.** A new *in vitro* technique for attachment to intestinal villi using enteropathogenic Escherichia coli. Ann. Microbiol. (Inst. Pasteur) 131B:31-37. 1980.
9. **GUINEE, P.A.M.** and **W.H. JANSEN.** Detection of enterotoxigenicity and attachment factors in Escherichia coli strains of human, porcine and bovine origin; a comparative study. Zentbl. Bakt. Hyg., I. Abt. Orig. A 243:245-257. 1979.
10. **GUINEE, P.A.M., J. VELDKEMP** and **W.H. JANSEN.** Improved Minca

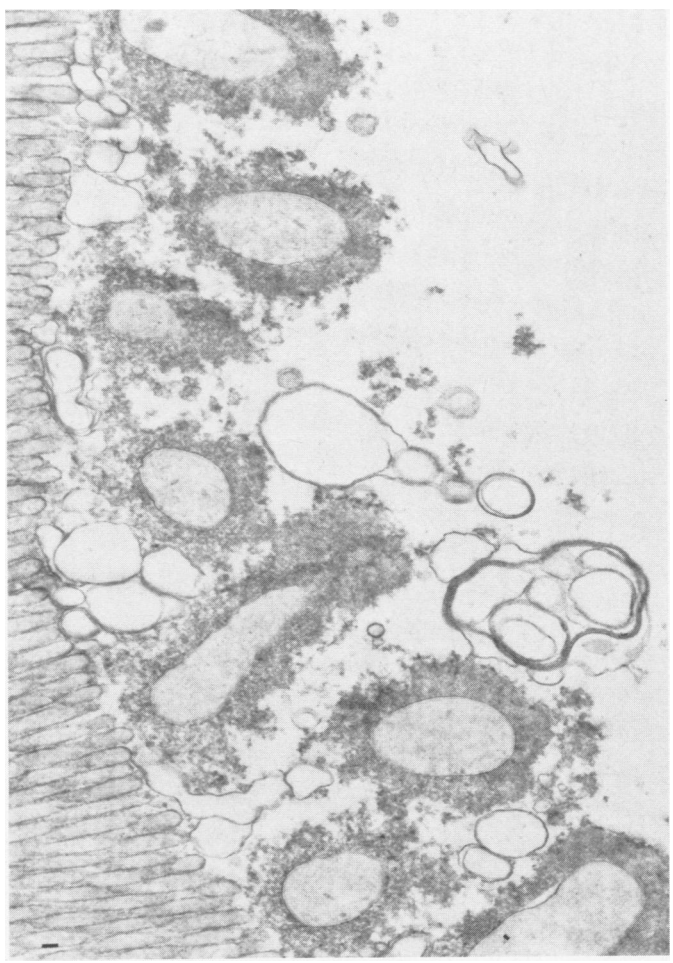

Fig. 6. As Figure 5. Note the heavily consolidated stabilization of the more accessible outer bacterial glycocalyces and the less consolidated stabilization of the less accessible inner glycocalyces.

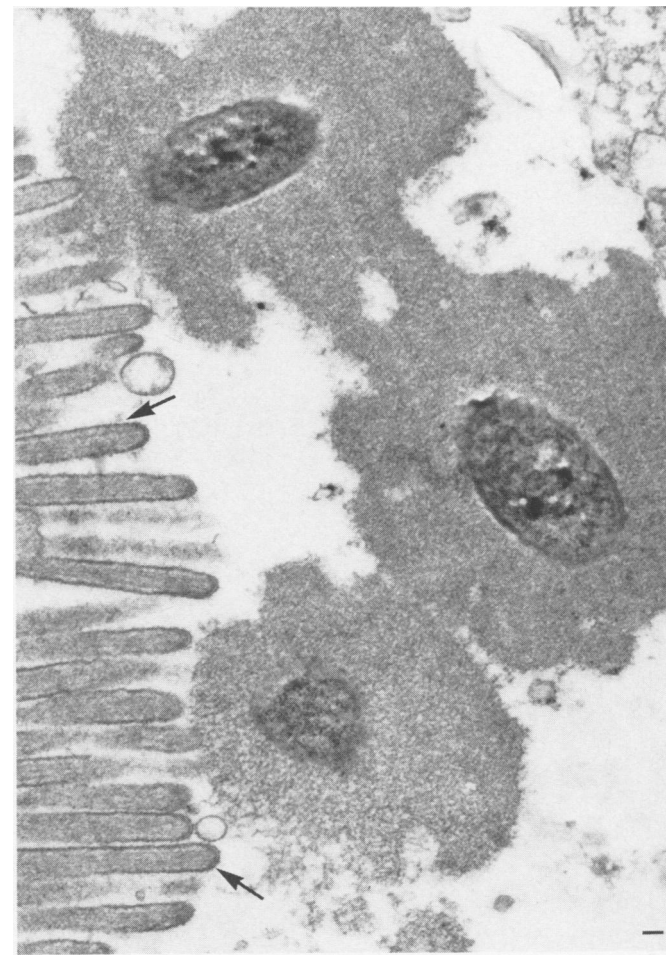

Fig. 7. As Figure 5. Note the consolidated stabilization of the bacterial glycocalyx, whose component fibers are clearly seen at this magnification and the lack of stabilization of the tissue cell glycocalyx (arrows) seen at the microvillar border.

medium for the detection of K99 antigen in calf enterotoxigenic strains of Escherichia coli. Infection & Immunity 15:676-678. 1977.
11. **ISAACSON, R.E.** K99 surface antigen of Escherichia coli: purification and partial characterization. Infection & Immunity 15:272-279. 1977.
12. **ISAACSON, R.E.** Pili of enterotoxigenic Escherichia coli. In S.D. Acres, A.J. Forman, H. Fast, Eds. Proceedings Third International Symposium on Neonatal Diarrhea, Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada. pp. 213-236. 1981.
13. **ISAACSON, R.E., H.W. MOON** and **R.A. SCHNEIDER.** Distribution and virulence of Escherichia coli in the small intestines of calves with and without diarrhea. Am. J. vet. Res. 39:1750-1755. 1978.
14. **LAM, J., R. CHAN, K. LAM** and **J.W. COSTERTON.** Production of mucoid microcolonies by *Pseudomonas aeruginosa* within infected lungs in cystic fibrosis. Infection & Immunity 28:546-556. 1980.
15. **LUFT, J.H.** Ruthenium red and ruthenium violet. I. Chemistry, purification, methods of use for electron microscopy, and mechanism of action. Anat. Rec. 171:347-368. 1971.
16. **MACKIE, E.B., K.N. BROWN, J. LAM** and **J.W. COSTERTON.** Morphological stabilization of capsules of group B streptococci, types Ia, Ib, II and III with specific antibody. J. Bact. 138:609-617. 1979.
17. **MOON, H.W., S.C. WHIPP** and **S.M. SKARTVEDT.** Etiologic diagnosis of diarrheal diseases of calves: frequency and methods for detecting enterotoxin and K99 antigen production by Escherichia coli. Am. J. vet. Res. 37:1025-1029. 1976.
18. **MORRIS, J.A., C. THORNS, A.C. SCOTT, W.J. SOJKA** and **G.A. WELLS.** Adhesion *in vitro* and *in vivo* associated with an adhesive antigen (F41) produced by a K99- mutant of the reference strain Escherichia coli B41. Infection & Immunity 36:1146-1153. 1982.
19. **MORRIS, J.A., C.J. THORNS** and **W.J. SOJKA.** Evidence for two adhesive antigens on the K99 reference strain Escherichia coli B41. J. Gen. Microbiol. 118:107-113. 1980.
20. **ROSEMAN, S.** Complex carbohydrates and intercellular adhesion. *In* Biology and Chemistry of Eukaryotic Cells Surfaces. Vol. 7. p. 317. E.Y.C. Lee and E.E. Smith, Eds. London: Academic Press. 1974.
21. **SMITH, H.W.** and **M.B. HUGGINS.** The influence of plasmid-determined and other characteristics of enteropathogenic Escherichia coli on their ability to proliferate in the alimentary tract of piglets, calves and lambs. J. Med. Microbiol. 11:471-492. 1978.
22. **SUTHERLAND, I.W.** Bacterial exopolysaccharides — their nature and production. *In* Surface Carbohydrates of the Prokaryotic Cell. Sutherland, I.W., Ed., London: Academic Press. 1977.