RECOVERY OF INTESTINAL MEMBRANE BINDING SITES FOR K88 *E. coli* FROM PIG MUCOSAL ORGAN CULTURE

By

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I thank my son, Michael, for his loving tolerance; my husband, Ted, for his loving insistence that I complete the program; Duane Garner and Jim Blankemeyer for their acceptance of my nontraditional status; and God for making all things possible.

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ABSTRACT

Incorporation of ^{14}C-glucosamine by newborn to day old and 3 week to 6 week old pig jejunal and ileal mucosa, in organ culture, occurred throughout the 24-hour culture period. Uptake in both age groups and both areas of the intestine was similar with a somewhat greater incorporation by the older age groups.

Secretion of ^{14}C-glucosamine labeled components into the culture medium was demonstrated by gel filtration. Large molecular weight components eluted in the void volume in excess of 2 x 10^6 daltons. A second peak of activity was spread from approximately 690K to 25K daltons. All eluted fractions demonstrated binding to K88+ E. coli.

Immunoprecipitation of the solubilized media with K88+ pili and pilus antisera, following electrophoresis under SDS and reduced conditions demonstrated a subunit of approximately 35K daltons.

Putative receptors for K88+ E. coli from pig intestinal epithelium were released into the culture medium and were demonstrated by direct binding with K88+ E. coli or by immunoprecipitation.

Key Words: Intestine, organ culture, secretion, ^{14}C-glucosamine, immunoprecipitation, K88+ E. coli, receptor.

INTRODUCTION

Application of organ culture techniques (1) to culture of human small intestinal mucosal biopsies has shown that mucosal morphology, epithelial proliferation, and fat absorption are maintained for up to 24 hours (2). Primary intestinal epithelial cell cultures have been cultured in monolayers; however, they appear to lack immunological determinants specific for differentiated villus cells (3) and as such do not have the functional capabilities of a mature mucosal epithelium. Due to the limitation of the primary intestinal epithelial culture, organ culture of intestinal mucosal tissue has become an established method for the study of various aspects of intestinal metabolism.

Organ cultures have been carried out with intestinal mucosa for a number of species: human (4, 5, 6), rabbit (7), guinea pig (8), mouse (9), calf (10), and pig (11). The major information derived has been in relation to normal turn-over of brush border proteins and secretory activity, as well as examination of abnormal intestinal mucosa.

The present study was developed to approach the problem of defining bacteria-intestinal epithelial cell interrelationships that permit colonization of the intestine by enterotoxigenic *E. coli*.

This study demonstrates that *E. coli* binding proteins from pig intestinal epithelial mucosa maintained in organ cultures are released into the culture media.

METHODS

Materials: Chemicals, chromatography gels, polyacrylamide gels, detergent, isotopes, chromatography standards and electrophoresis standards were from sources as previously described (16). Fetal bovine serum was purchased from KC Biological Inc. (Lenexa, Kansas). Dulbecco's modified Eagles medium and NCTC 135 medium was acquired from Grand Island Biological Co. (Grand Island, New York).

Animals: Clinically healthy pigs, newborn, day-old, 3 weeks, and 6 weeks of age, from mixed breeds (Duroc and Yorkshire - Crosses) were used in this study.

Culture Procedures: The procedure was essentially as described by Staley et al. (10). Intestinal mucosal explants from the jejunum or ileum of 24 pigs were maintained by organ culture conditions for periods up to and including 24 hours. The media consisted of Dulbecco's medium plus NCTC 135 medium (9:1), 10 percent bovine fetal serum, and gentamicin (2 μg/ml). Radiolabeled ^{14}C-glucosamine (specific activity 7.6 mCi/mM) was added to a final concentration of 2 μCi/ml. The mucosa was maintained with non-radiolabeled medium prior to incubation at 4° C. Preliminary organ cultures demonstrated that 0.1 g of mucosa per 5 ml of medium was the most efficient tissue to medium ratio. Cultures in triplicate were established for each timed culture interval, for a total of 12 cultures/animal. Six animals were examined for each culture period. The culture medium containing mucosal explants were

oxygenated with 95 percent oxygen/5 percent carbon dioxide, and incubated at 37° C. Cycloheximide (1.0 mg/ml) was added to paired mucosal cultures as a check on viability. Cultures with cycloheximide were preincubated for one hour before the addition of radiolabeled glucosamine.

Preparation of organ culture explants for radioactive assay: Explants were removed from the culture environment at 3, 6, 12, or 24 hours of culture, and placed into 154 mM NaCl (4° C). The explants were washed twice to remove free ^{14}C-glucosamine and homogenized in 5 mM EDTA. The homogenized protein preparations were precipitated with 10 percent trichloroacetic acid, centrifuged, and the pellet lipid extracted (12). Following air drying, the pellet was dissolved in 0.4 N NaOH. Aliquots of the solubilized precipitate were transferred to scintillation vials, 15 ml of Bray's solution added and the radioactivity determined by liquid scintillation counting. Protein was determined by the dye binding method of Bradford (13).

Preparation of organ culture medium for gel filtration and radioactive assay: In order to measure the secreted radiolabeled glycoproteins, the incubation medium was collected from each organ culture preparation and pooled. The pooled samples were centrifuged at 27,000 x g for one hour to remove cellular debris. The medium was then dialyzed and concentrated with an Amicon filtration cell using a YM-10 filter with an exclusion limit of 10K daltons. Following removal of the free radiolabeled glucosamine the total radioactivity of the medium was determined. The medium was then solubilized with 1 percent Zwittergent-detergent (Calbiochem.-Behring, La Jolla, CA) (14). The solubilized membranes were again centrifuged at 27,000 x g for one

hour. An aliquot of the supernatant (approximately 20-30,000 cpm) was subjected to gel filtration on Sepharose CL-4B and eluted with phosphate-buffered-saline (PBS) pH 7.2 with 0.1 percent Zwittergent to provide 0.5 ml fractions. The Sepharose column was previously calibrated with Blue dextran 2M, thyroglobulin 690K, IgG 160K, and chymotryps in 25K daltons. The eluted fractions were split into two portions, one for determination of radioactivity by liquid scintillation counting and the other was used to determine binding activity with K88+ E. coli.

K88+ Strain 263 E. coli culture and characterization: E. coli 08:K87aK88ab:H19, was grown on minca agar (15) for 16-18 hours at 37° C, for expression of the K88 surface antigen. The bacteria were washed from the agar with sterile PBS (4° C), centrifuged and resuspended to a concentration of 10^8 organisms/ml. The presence of K88+ antigen was determined morphologically by electron microscopy with negative staining, and by hemagglutination utilizing guinea pig erythrocytes (16). Bacterial suspensions were serial diluted and added to 1.0 percent (vol/vol) guinea pig red cells, incubated at 4° C and the presence of hemagglutination recorded after 2 hours.

Demonstration of binding of K88+ E. coli with solubulized organ culture medium: Of the fractions eluted from Sepharose, 250 µl was placed into 0.5 ml conical polypropylene microcentrifuge tubes. E. coli which were proven positive for the presence of pili by hemagglutination were added to the tubes to a final concentration of 10^4 organisms/ml. The tubes were mixed, and incubated overnight at 4° C. The following day the tubes were centrifuged to produce a bacterial pellet. The pellet was washed, resuspended in PBS and centrifuged

twice to remove unbound radiolabeled membrane. The pellet was then resuspended in 0.25 ml PBS and transferred to scintillation vials containing 15 ml of Bray's solution for scintillation counting. Appropriate controls using the Sepharose eluted fractions in the absence of E. coli were placed in polypropylene tubes and handled in the same manner to demonstrate that the radiolabeled solubilized membranes did not precipitate or adhere to the centrifuge tubes.

Isolation of K88 surface antigen (pili) of E. coli and production of pilus antisera: K88+ pili were isolated and antisera produced in rabbits as previously described (16).

Immunoprecipitation: The immunoprecipitation procedure was an adaptation of the technique described by Vitetta et al. (17). Pilus antigen (200 µg) was incubated with radiolabeled, solubilized, culture media, such as was prepared for gel filtration (approximately 500 µg protein), for 30 minutes at 37° C, followed by the addition of pilus antisera to a final dilution of 1:2. The preparation was then incubated at 37° C for 30 minutes, followed by overnight at 4° C. Precipitates were pelleted in a microcentrifuge at maximum speed for 2 minutes and washed 4-5 times with 0.01 M Tris-MCl buffer (pH 7.2). The pellets were then solubilized by the addition of 2 percent sodium dodecyl sulfate (SDS), 5 percent mercaptoethanol and immersion in boiling water for 10 minutes. Radioactivity was then determined in 100 µl aliquots by liquid scintillation counting.

Polyacrylamide gel electrophoresis of immunoprecipitates: The method of Laemmli (18) was used in all electrophoretic studies. In general, 200 µg (approximately 20,000 cpm) of immunoprecipitated radiolabeled membrane from organ culture media was adjusted to 0.25 M

Tris-HCl, containing 10 percent glycerol, 2 percent SDS, and 5 percent mercaptoethanol, and 1 percent Bromphenol blue. The separation gel consisted to 12.5 percent acrylamide: bis-acrylamide in a ratio of 37.5:1. Tube gels were subjected to electrophoresis at 3 mA for approximately 18 hours and then at 15 mA for 5-6 hours. Gels were either fixed and stained in 0.1 percent Coomassie blue in 7 percent acetic acid and scanned with a Beckman DU-8 spectrophotometer with gel scanner attachment (Beckman Instruments Inc., Irving, CA) at A590, or sectioned into 2 mm segments, extracted overnight with 0.1 percent SDS, and the radioactivity determined by scintillation counting. The approximate molecular weights (m.w.) of the major peaks of radioactivity were calculated by the procedure of Weber and Osborn (19).

RESULTS

Glucosamine incorporation by acid precipitable components of jejunal ileal mucosal cultures: Each point on the graphs represents 12 separate cultures of 6 different animals. The incorporation of ^{14}C-glucosamine into jejunal mucosal cultures of newborn-day old (NB-DO) animals versus 2 week-6 week (3W-6W) is shown in Figure (1). The uptake of radiolabeled glucosamine by 3W-6W jejunal cultures was greater in acid precipitable protein than NB-DO jejunal cultures for all of the culture periods. An approximate 8-fold increase in incorporation by the 3W-6W culture occurred by the 24-hour culture period. Incorporation by ileal cultures of NB-DO versus 3W-6W animals is presented in Figure (2). Uptake was similar in both groups through the 12-hour culture period. An apparent difference in uptake also occurred in 3W-6W ileal cultures by the 24-hour culture period.

Secretion of radiolabeled glycoproteins: The elution profiled of solubilized (NB-DO) jejunal and ileal culture media from Sepharose CL-4B are shown in Figures (3) and (4) respectively. The jejunal medium elution profile contained a large m.w. component which eluted in the void volume, a size in excess of blue dextran. The second peak of activity was spread from approximately 690K to less than 25K daltons. Binding studies with intact K88+ *E. coli* demonstrated parallel binding with the peaks of radioactivity. The NB-DO ileal medium following gel filtration produced a pattern similar to the jejunal elution

profile; however, several large m.w. components were much more evident in the void volume. Binding with intact K88+ E. coli was again parallel with the peaks of radioactivity, with very prominent binding in association with the large m.w. components.

The elution profiles of solubilized 3W-6W jejunal and ileal culture media following gel filtration on Sepharose are shown in Figures (5) and(6) respectively. The 3W-6W jejunal medium was similar to the NB-DO jejunal culture medium, with the exception that the large m.w. component was more broad in its m.w. range, with only a portion in the void volume. The second peak of radioactivity was spread from 690K to less than 25K. Binding with E. coli was generally very poor, but was evident in all fractions. The elution profile of 3W-6W ileal culture medium upon gel filtration showed only a slight amount of radiolabeled large m.w. component in the void volume; however, it was followed by two large peaks of radioactivity, one from 690K to 160K and one from 160K to 25K daltons. Binding activity with K88+ E. coli occurred in concert with the large void volume component, then at 690K and a third peak of binding activity between 160K and 25K daltons.

Binding of K12 E. coli with the pooled concentrated meium was negligible, 0.08 percent as compared to K88+ E. coli.

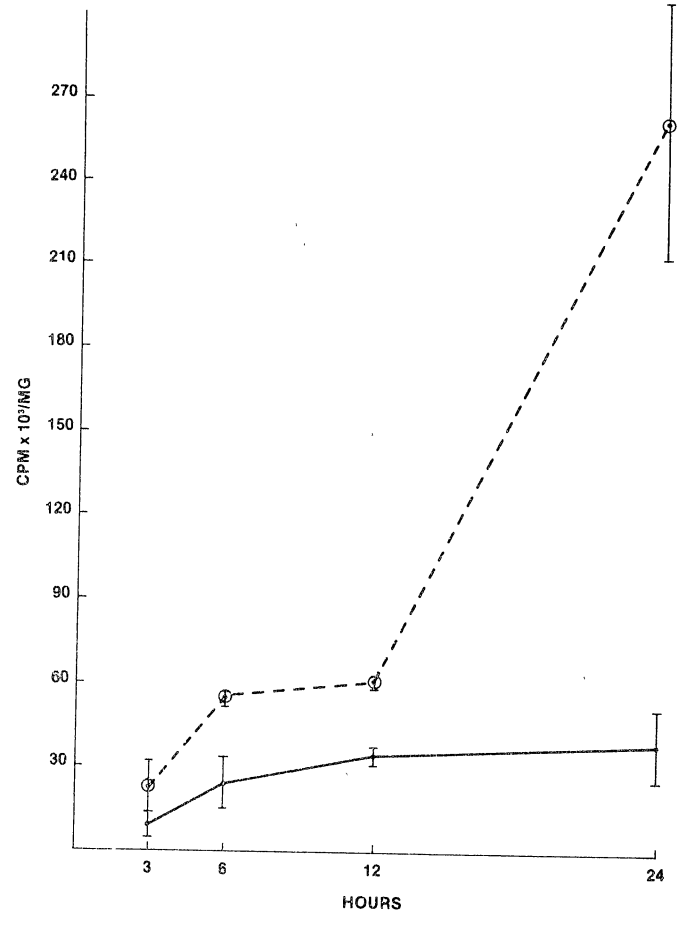
Double diffusion reactivity between brush border antibodies and organ culture medium or cellular debris recovered from culture medium: Brush border antibodies produced prominent precipitation bands with the original brush borders which were used for the immunogen as well as with the solubilized cellular debris from organ culture medium and jejunal or ileal organ culture medium precipitation bands with the original brush borders as shown in Figure (7).

Polyacrylamide gel electrophoresis of immunoprecipitates: Comparisons of NB-DO jejunal solubilized culture medium to ileal culture medium which were immunoprecipitated with K88+ pili and K88+ antisera are shown in Figures (8A) and (8B). The major bands of radioactivity in the jejunal immunoprecipitates were found at 35K and 33K daltons. The ileal immunoprecipitates showed one major band of radioactivity at 35K daltons. Comparison of 3W-6W jejunal solubilized culture medium immunoprecipitates to ileal culture medium immunoprecipitates are shown in Figures (9A) and (9B). The major band of radioactivity in both jejunal and ileal immunoprecipitates occurred at 35K daltons.

Gel scans for dye binding protein of all immunoprecipitates failed to demonstrate any detectable protein bands that correlated with the areas of radioactivity. Prominent protein bands were found in association with the K88+ pili at approximately 23K and with the heavy and light chains of the pilus antibody (data no included).

Figure 1. Incorporation of ^{14}C-glucosamine (CPM mg protein) into acid precipitable protein from NB-DO (·) and 3W-6W (Θ) pig jejunal mucosa in organ culture for a 24-hour period.

Figure 2. Incorporation of ^{14}C-glucosamine (CPM mg protein) into acid precipitable protein from NB-DO (·) and 3W-6W (Θ) pig ileal mucosa in organ culture for a 24-hour period.


270
240
210
180
150
120
90
60
30
CPM x 10³/MG
3
6
12
24
HOURS

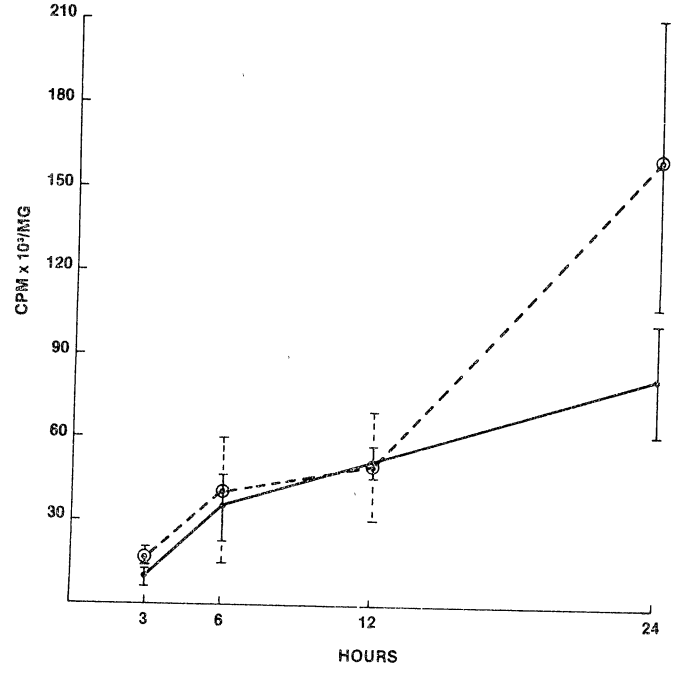
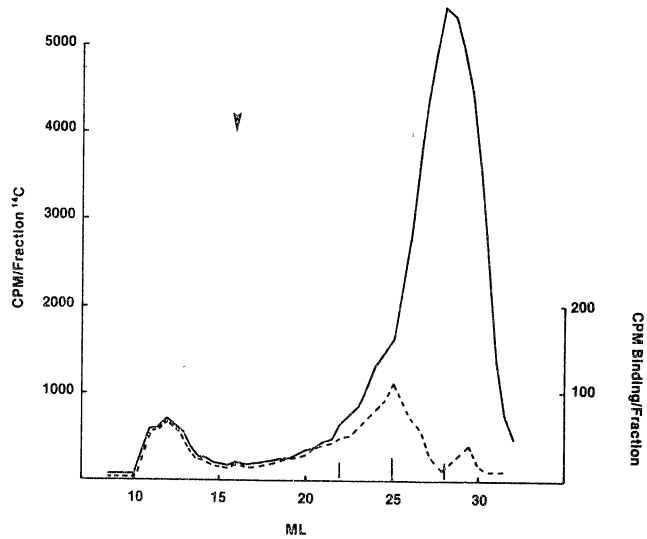

210
180
150
120
90
60
30
CPM x 10³/MG
3
6
12
24
HOURS

Figure 3. Elution pattern from pooled, solubilized, dialyzed, concentrated, medium from NB-DO pig jejunal cultures subjected to gel filtration on Sepharose CL-4B. Data plotted as radioactivity (CPM fraction C^{14}, solid line) per ml recovered from the column. The 3 calibration markers are standard proteins at fraction 22 (thyroglobulin), fraction 25 (lgC), and fraction 28 (chymotrypsin). Arrow indicates void volume. The binding of the labeled fractions to K88+ E. coli presented on a lesser scale (CPM binding fraction, dotted line).

Figure 4. Elution pattern from pooled, solubilized, dialyzed, concentrated, medium from NB-DO pig ileal cultures subjected to gel filtration on Sepharose CL-4B. Data plotted and calibration markers same as Figure 3.


5000
4000
3000
2000
1000
CPM/Fraction ¹⁴C
200
100
CPM Binding/Fraction
10
15
20
25
30
ML

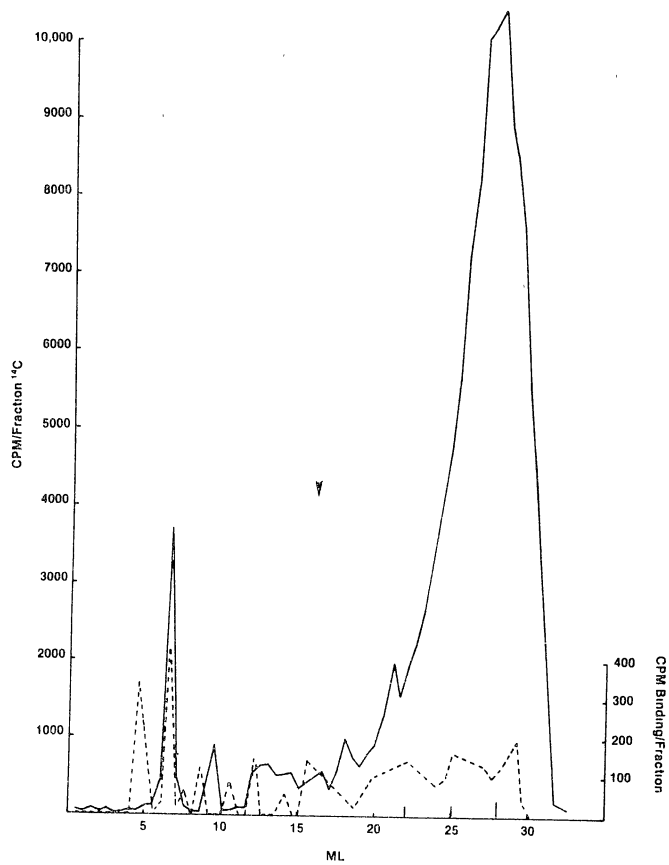
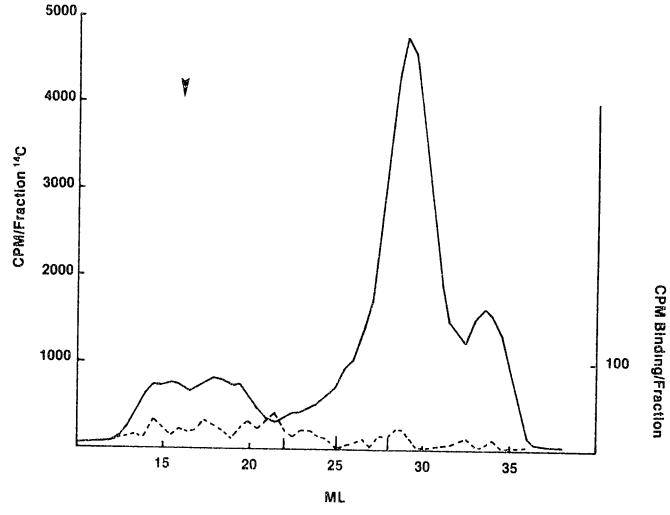

10,000
9000
8000
7000
6000
5000
4000
3000
2000
1000
CPM/Fraction ¹⁴C
400
300
200
100
CPM Binding/Fraction
5
10
15
20
25
30
ML

Figure 5. Elution pattern from pooled, solubilized, dialyzed, concentrated, medium from 3W-6W pig jejunal cultures subjected to gel filtration on Sepharose CL-4B. Data plotted and calibration markers same as Figure 3.

Figure 6. Elution pattern from pooled, solubilized, dialyzed, concentrated, medium from 3W-6W pig ileal cultures subjected to gel filtration on Sepharose CL-4B. Data plotted and calibration markers same as Figure 3.


5000
4000
3000
2000
1000
CPM/Fraction ^{14}C
100
CPM Binding/Fraction
15
20
25
30
35
ML

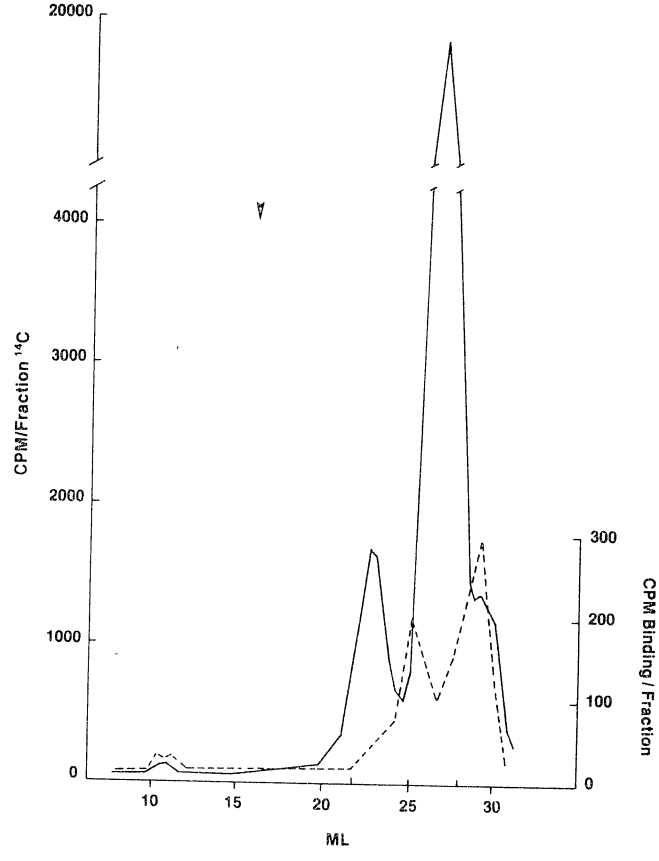
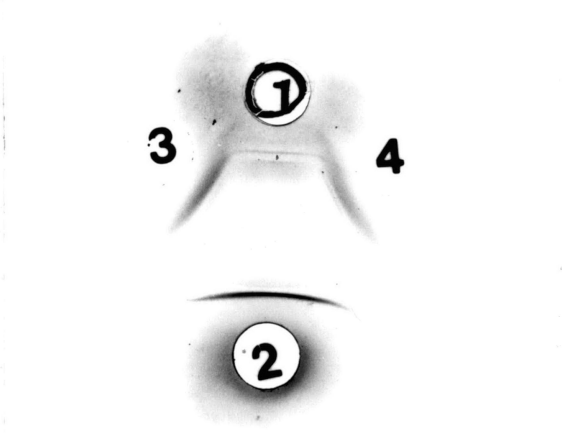

20000
4000
3000
2000
1000
0
CPM/Fraction ^{14}C
300
200
100
0
CPM Binding / Fraction
10
15
20
25
30
ML

Figure 7. Double diffusion Ouchterlony plate. The center well contained antibodies raised against intestinal cell membranes of susceptible pigs. The surrounding wells with visible precipitation lines were: 1) concentrated media from jejunal organ culture, 2) membrane pellet from media of jejunal organ culture, 3) and 4) brush border membranes from two different pigs. The remaining wells with no precipitation lines contained PBS. Media from ileal organ cultures produced similar results.


1
3
4
2

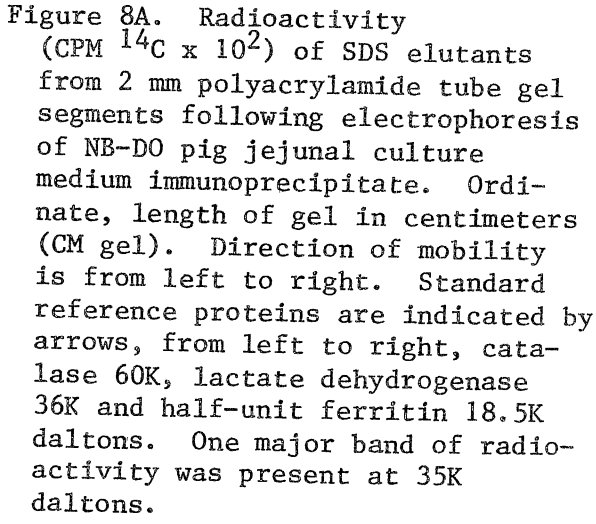


Figure 8A. Radioactivity (CPM $^{14}C \times 10^2$) of SDS elutants from 2 mm polyacrylamide tube gel segments following electrophoresis of NB-DO pig jejunal culture medium immunoprecipitate. Ordinate, length of gel in centimeters (CM gel). Direction of mobility is from left to right. Standard reference proteins are indicated by arrows, from left to right, catalase 60K, lactate dehydrogenase 36K and half-unit ferritin 18.5K daltons. One major band of radioactivity was present at 35K daltons.

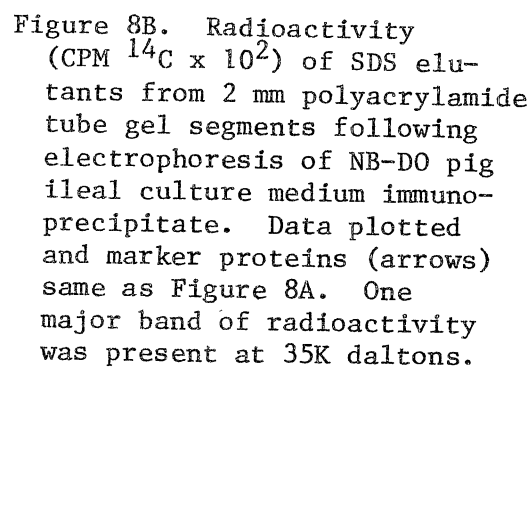


Figure 8B. Radioactivity (CPM $^{14}C \times 10^2$) of SDS elutants from 2 mm polyacrylamide tube gel segments following electrophoresis of NB-DO pig ileal culture medium immunoprecipitate. Data plotted and marker proteins (arrows) same as Figure 8A. One major band of radioactivity was present at 35K daltons.

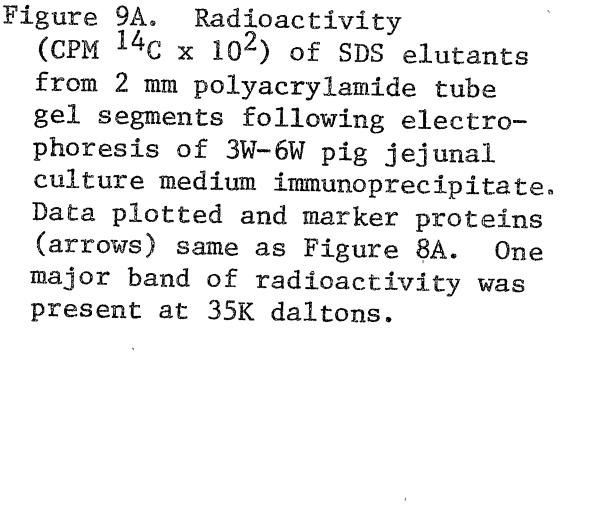


Figure 9A. Radioactivity (CPM $^{14}C \times 10^2$) of SDS elutants from 2 mm polyacrylamide tube gel segments following electrophoresis of 3W-6W pig jejunal culture medium immunoprecipitate. Data plotted and marker proteins (arrows) same as Figure 8A. One major band of radioactivity was present at 35K daltons.

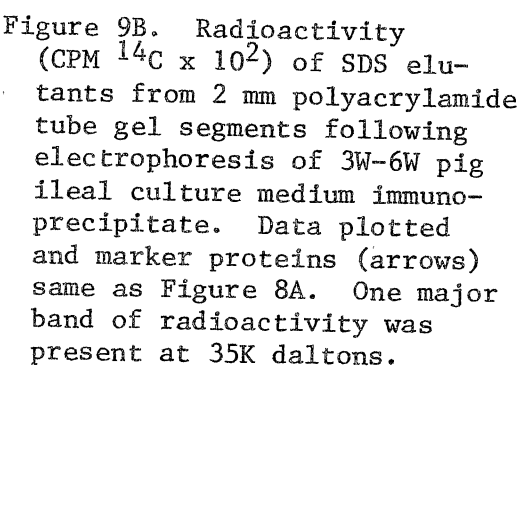
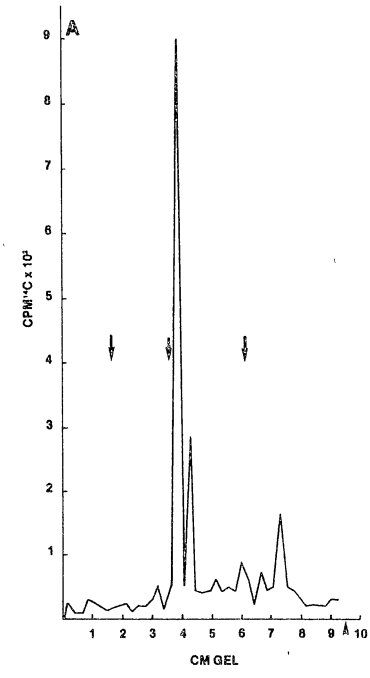
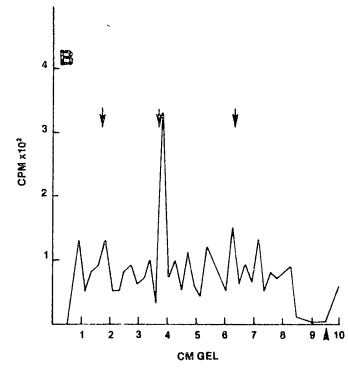
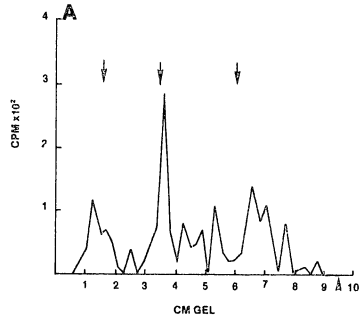


Figure 9B. Radioactivity (CPM $^{14}C \times 10^2$) of SDS elutants from 2 mm polyacrylamide tube gel segments following electrophoresis of 3W-6W pig ileal culture medium immunoprecipitate. Data plotted and marker proteins (arrows) same as Figure 8A. One major band of radioactivity was present at 35K daltons.


A
CPM ^{14}C x 10^3
CM GEL


B
CPM x10^3
CM GEL


A
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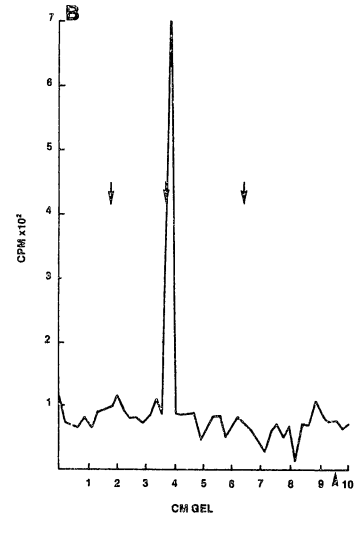
CM GEL


B
CPM x10^3
CM GEL

DISCUSSION

The susceptibility of neonatal animals to E. coli related diarrheas appears to be dependent on the presence of appropriate intestinal epithelial glycoprotein receptors. These receptors have been recovered from solubilized pig (16) intestinal brush borders by bioaffinity chromatography and immunoprecipitation. Relatively little is known about the receptor in terms of its chemical structure, function and spectrum in binding microorganisms, biogenesis and turn-over rate, availability in relationship to the general metabolic status of the animal, or its affinity for other intestinal microbes. Since the intestinal organ culture procedure has been used to study intestinal secretion of antibodies (7) and glycoproteins (10, 12), it was plausible that the release of E. coli binding glycoproteins could be demonstrated in organ culture.

In both age categories of pigs studied and in both jejunal and ileal mucosal organ cultures, the incorporation of ^{14}C-glucosamine occurred throughout the culture period up to and including 24 hours. A similar uptake has been demonstrated in calf intestinal organ cultures (10). Danielsen et al. (20) has also shown incorporation of ^{35}S-methionine by pig intestine in organ culture. The total incorporation was similar for both age categories of pigs and both areas of the intestine up to and including 12 hours of culture. After 12 hours the intestinal organ cultures of older animals in both areas of the

intestine displayed a greater degree of incorporation of radiolabeled glucosamine. The incorporation by human mucosa of radiolabeled leucine (4) or glucosamine (5) has been shown to be essentially linear during the first 24-hour culture period; this was also true for the calf (10). The reason for this increase in response of the 3W-6W pig intestinal mucosa after 12 hours of culture as compared to the NB-DO intestinal mucosa was not readily apparent. It may suggest that the 3W-6W mucosa required a longer period of equilibration in culture before the inhibitory influence of exogenous hormones was exhausted. A number of hormones have been shown to influence incorporation of membrane precursors, i.e., cortisol and vaso-active-intestinal peptide (10). In addition, the presence of thyroxine in embryonic check duodenal organ cultures enhanced the release of enzymes and the incorporation of ^{3}H-glucosamine (21). The release of radiolabeled glycoproteins (10, 12) and enzymes (4, 5, 11) from intestinal organ cultures has been reported and apparently reflected vesiculation of the microvillous membranes (21) and may reflect the means whereby cell membrane is replaced. The presence of cycloheximide in organ culture media inhibited incorporation of glucosamine by approximately 80-85 percent as compared to controls and was used as an indicator of cell viability.

This study was not designed to compare the relative secretion and/or vesiculation of radiolabeled cell membrane components and as such simply showed that both age categories and both areas of the intestine in organ culture eliminated a wide range of m.w. radiolabeled components into the culture media which bound to E. coli. From every culture, radiolabeled components eluted in the void volume from Sepharose CL-4B. This would place their molecular weights in excess

2×10^7 daltons for protein and 5×10^6 for polysaccharides. Goblet cell mucin was estimated by Forstner, et al. (22) at approximately 2×10^6 daltons. It would therefore appear that the void volume radioactive peak activity represented goblet cell mucin. Secretion of goblet cell mucin by human small intestinal mucosa has been previously reported (2). The remaining radiolabeled material represented multiple m.w. components with a range of 690K to less than 25K daltons. Previous reports have shown that papain treatment of intestinal brush borders yielded complex glycoproteins which, when subjected to gel filtration on Sepharose 4B, eluted in a pattern very similar to the results reported here. Further, intestinal enzyme activities were found in association with the peak radiolabeled activity (23). The m.w. range of Sepharose-eluted components, in this study, was in excess of most known intestinal enzymes (24); however, peak radioactivity occurred at less than 25 daltons. Binding to E. coli was demonstrated by the entire range of m.w. components. The 3W-6W ileal culture media displayed greater binding activity than other culture media; however, the total radioactivity of the chromatographed media was also greater than other cultures, and may not reflect a comparatively greater binding activity.

Immunoprecipitation procedures have been utilized to demonstrate aminopeptidase N and sucrase-isomaltase enzymes (21) as well as receptors for IgE (25). This study utilized a similar technique for the demonstration of the E. coli binding proteins present in solubilized jejunal and ileal organ culture media. Metabolically radiolabeled secreted glycoproteins were solubilized and exposed to purified K88+ pili, following 37° C incubation for 30 minutes a visible precipitate

was evident. Addition of pilus antisera produced a prominent flocculent precipitation. Upon polyacrylamide gel electrophoresis in the presence of SDS and mercaptoethanol a major peak of radiolabeled activity was detected in the gels from the media of organ cultures of all age categories of pigs. Attempts at immunoprecipitation with pilus antisera and organ culture medium failed to produce any precipitate, and therefore this procedure appears to be a specific demonstration of the subunit structure of the E. coli binding proteins which are released into the organ culture media. These results are essentially similar to those obtained with E. coli binding glycoproteins recovered from iodinated ileal brush borders by bioaffinity chromatography, with the exception that the smaller component previously demonstrated was not present. The repeatability between culture media of different areas of the intestine as well as different age categories is a reasonable indication that this is a reliable estimate of the subunit structure of the E. coli receptor proteins. Those immunoprecipitates which demonstrated peaks of radioactivity of 100 cpm over background may be the results of overloading the polyacrylamide gels. As mentioned in "Results," none of the radiolabeled peaks bound Coomassie blue, and therefore they may represent polysaccharides; however, attempts at staining with periodic acid Schiff reagent were inconclusive.

The binding with E. coli that was demonstrated utilizing the fractions eluted from Sepharose as compared to the immunoprecipitates requires some comparative comments: (1) the subunits recovered by electrophoresis on reduced gels, from immunoprecipitates, were too small to be subunits of mucin (22); (2) mucin did bind to E. coli, as evident by the binding of void volume elutants from Sepharose gel

filtration; (3) the subunits recovered at 35K daltons on reduced gels represent components that were too small to correlate with any known enzymes (24); (4) these subunits of the intestinal epithelial cell represented specific binding proteins or glycoproteins which in their native form have a wide range of molecular weights and represent multimeric forms of the same receptor.

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