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Interaction of *Escherichia coli* K88 Antigen with Porcine Intestinal Brush Border Membranes

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The fimbria-associated *Escherichia coli* antigen, K88, was purified to homogeneity as determined by polyacrylamide gel electrophoresis and immunodiffusion. This polymeric antigen consists of noncovalently linked subunits, containing little or no carbohydrate, and has a monomeric molecular weight of 23,000. When a binding assay employing differential filtration was used, K88 formed complexes with isolated porcine intestinal brush border membranes. The formation of complexes was inhibited by glycoproteins with terminal *N*-acetylglucosamine and *N*-acetylgalactosamine residues and to a lesser extent by free *N*-acetylhexosamines. These amino sugars may play a role in the interaction of this pathogenic strain of *E. coli* with the intestinal epithelia of pigs.

Noninvasive strains of *Escherichia coli* have two prerequisites for production of diarrheal disease: (i) they must produce an enterotoxin which can interact with the intestinal cells, and (ii) they must be able to proliferate in the intestine to reach significant numbers at their site of action. Both a heat-labile and a heat-stable form of enterotoxin are produced by *E. coli* and have been characterized previously (5-7). The means by which the bacteria overcome the normal flow through the intestine and proliferate in a particular locale are not so well defined. In the case of some *E. coli* strains which have been isolated from piglets with diarrhea, however, an adhesive mechanism which apparently allows the bacteria to colonize the small intestine has been shown to exist (9, 20). These strains produce surface fimbriae, approximately 0.2 to 1.3 μm in length, designated K88 antigen, which allow the bacteria to adhere to the mucosa of the small intestine. Many studies have shown an excellent correlation of the presence of the antigen on the bacteria, adhesion of the bacteria to the small intestine, and production of diarrhea in young pigs (1, 9, 15).

We have studied the interaction of K88 antigen with the small intestine of pigs. Such studies have been carried out previously with an indirect assay, agglutination of guinea pig erythrocytes (8). We have developed a direct and quantitative assay by using purified K88 antigen and hog intestinal brush border membranes and have used it indirectly to investigate structures in the membrane which may affect this interaction.

MATERIALS AND METHODS

Bacteria. *E. coli* D520 [O8:K27(A)−:K88(L):H−] was obtained from Ida Ørskov, Statens Seruminstitut, Copenhagen, Denmark. Bacteria were maintained on ox heart broth agar plates (2.5% ox heart infusion [Difco Laboratories, Detroit, Mich.], 1% agar, 0.2% Na_2HPO_4).

Purification and iodination of K88 antigen. K88 antigen was purified by the method of Stirm et al. (18), except that the final high-speed centrifugation was omitted. It was stored at pH 5.3 as a precipitate at 4°C. K88 antigen was labeled with ^{125}I, using lactoperoxidase. To 0.5 ml of a 5-mg/ml solution of K88 antigen in 0.1 M sodium phosphate buffer (pH 7.0) were added 50 μl of 1-mCi/ml $Na^{125}I$ (carrier-free; ICN Pharmaceuticals, Inc., Irvine, Calif.), 100 μl of lactoperoxidase (Sigma Chemical Co., St. Louis, Mo.) bound to Sepharose 4B (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), and 5 μl of 0.03% H_2O_2. The mixture was incubated at 4°C, and after 30 min an additional 5 μl of 0.03% H_2O_2 was added. After 60 min, the mixture was filtered through glass wool to remove the lactoperoxidase and then dialyzed at 4°C against 0.1 M sodium phosphate buffer, pH 7.0, to remove the residual ^{125}I. The protein was stored at −20°C until use. Before use in a filter assay, the protein was filtered through a 1.2-μm membrane filter (Millipore Corp., Bedford, Mass.) and dialyzed against 2 mM tris(hydroxymethyl)aminomethane (Tris)-50 mM mannitol, pH 7.1. The protein concentration was determined by the method of Lowry et al. (12).

Preparation of brush border membranes from hog intestine. Small intestines from hogs were obtained from a local slaughterhouse. They were washed gently with 0.9% NaCl and frozen at −20°C before use. Brush border membranes used were the "P_2" fraction as obtained by the method of Schmitz et al. (14). Purification of the membranes was assessed by comparing the specific activity of sucrase by the method of Dahlqvist (3) in the homogenate and in the P_2 fraction. The material used in these studies had been purified 7- to 10-fold.

Binding assay. Hog brush border membranes (14 μg of protein) and ^{125}I-labeled K88 antigen were combined and diluted to a final volume of 125 μl with 50

mM mannitol–2 mM Tris, pH 7.1. After incubation at room temperature for 60 min, a 100-μl sample was withdrawn and quickly filtered by vacuum through a Millipore filter with a pore size of 1.2 μM. The filter was washed by vacuum with 1 ml of Tris-mannitol buffer and was counted for retained ^{125}I with a Beckman gamma counter. Specific activities of labeled K88 ranged from 2×10^6 to 5×10^6 cpm/mg of K88.

Background adsorption of K88 antigen to filters was determined by filtration of the same mixture as in the assay, but without the brush border membrane. These values were subtracted from the total counts bound to determine the net radioactivity adsorbed to membranes. All determinations were in duplicate, with a minimum of four experiments for each data point.

Inhibition studies. In these experiments, buffer, the solution to be tested for inhibition, and the brush border membrane were combined before the addition of ^{125}I-labeled antigen.

Glycoproteins. Ovine submaxillary mucin (OSM) was purified by the method of Tettamanti and Pigman (19). A+ and A− porcine submaxillary mucins (PSM) were prepared by the method of de Salequi and Plonska (4). A+ and A− refer to the ability of those mucins to inhibit agglutination of human blood group A cells by anti-blood group antiserum. α_1-Acid glycoprotein was isolated by the procedure of Schmid (13). Glycoproteins were desialylated by heating in 0.1 N H_2SO_4 for 1 h at 80°C (2). Galactose was removed from α_1-acid glycoprotein by the method of Spiro (17). All of the test solutions containing glycoproteins were prepared in 50 mM mannitol–2 mM Tris, pH 7.1. A+ PSM required sonication to obtain a homogeneous solution.

Other methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (10). Carbohydrate analysis of K88 antigen was carried out with gas chromatography by the method of Laine et al. (11).

RESULTS

Properties of purified K88 antigen. Examination of purified K88 antigen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed a single protein band with a molecular weight of 23,000 (Fig. 1). The same pattern was obtained with or without the addition of mercaptoethanol. The purified antigen also gave a single immunoprecipitin line against serum from two rabbits immunized with K88 antigen (Fig. 2). Only traces (less than 0.5 residue per μmol of K88 monomer) of carbohydrate (mannose, galactose, and glucose) were detected by gas chromatographic analysis. Amino acid analysis revealed no cysteine residues, suggesting, along with the data from sodium dodecyl sulfate-polyacrylamide gel electrophoresis, that K88 antigen is a macromolecular aggregate of monomeric units with a molecular weight of about 23,000 linked by noncovalent bonds.

Binding Assay. Binding measurements in which a centrifugation technique was used were not successful, therefore, another method, which employs filtration with a Millipore filter was developed. The results of a typical binding assay are shown in Fig. 3. The assay relies upon the fact that hog brush border membranes are retained by the 1.2-μm Millipore filter (94% of the sucrase activity was retained by the filter), whereas most (greater than 90%) of the free K88 antigen passes through the filter. Retention of

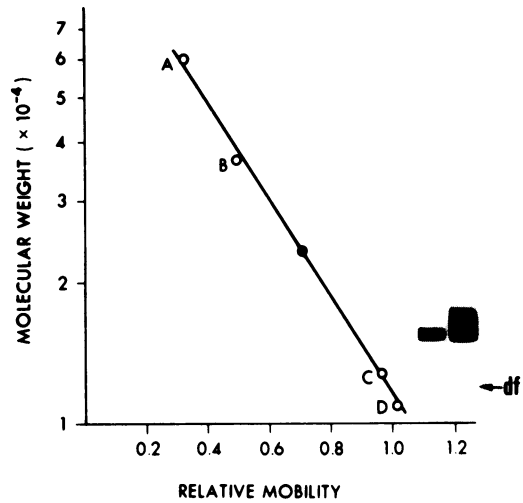

FIG. 1. *Molecular weight determination of K88 antigen by polyacrylamide gel electrophoresis in sodium dodecyl sulfate with mercaptoethanol. A, Catalase (M_r = 60,000); B, alcohol dehydrogenase (M_r = 37,000); C, chymotrypsin (M_r = 13,000); D, chymotrypsin (M_r = 11,000). ●, K88 antigen. The insert, which shows an independently run sodium dodecyl sulfate-polyacrylamide gel, demonstrates the purity of the K88 antigen used in these studies. Amounts of 20 and 100 μg of K88 were applied to the gels shown. df, Dye front.*

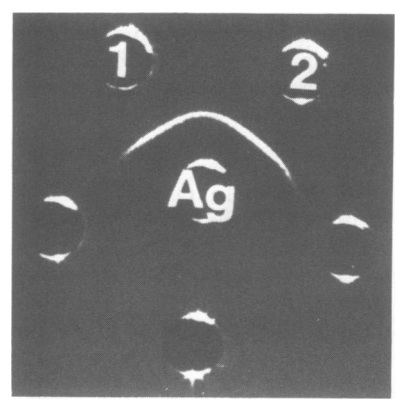

FIG. 2. *Immunodiffusion pattern of K88 antigen versus antiserum to K88 antigen prepared in two rabbits. Wells 1 and 2 contained the antisera, and the center well contained K88 antigen.*

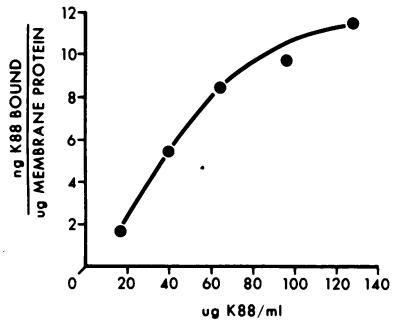

FIG. 3. *Binding of ^{125}I-labeled K88 antigen to purified porcine intestinal brush border membranes. Experimental procedures are described in the text.*

^{125}I on the filter is thus the result of binding of K88 antigen to the brush border membranes. Apparent saturation of binding occurred with about 11 ng of K88 antigen bound per μg of brush border membrane protein. Brush border membrane preparations from four hogs were tested separately in this assay, and all gave similar results.

To demonstrate that the binding is a specific interaction rather than random trapping of a percentage of the long, threadlike K88 antigen by the brush border preparation, we performed a competition experiment with unlabeled K88 antigen (Fig. 4). The concentration of ^{125}I-labeled K88 antigen was maintained at 100 μg/ml in all of the assays, whereas the concentration of unlabeled K88 was varied. As the concentration of the unlabeled protein was increased, the amount of ^{125}I-labeled K88 antigen retained by the membrane decreased. However, even at very high concentrations of unlabeled K88 antigen, about 20% of the control value of ^{125}I-labeled K88 antigen bound was retained by the filter, suggesting that there was some nonspecific binding. Most (80%) of the binding of the antigen to the filter was subject to competition by the unlabeled inhibitor and therefore may be considered specific.

Using the assay described here, we investigated the effect of sugars and glycoproteins on the binding of K88 antigen to hog brush border membranes. The concentration of K88 was 100 μg/ml in all experiments. The results, expressed as a percentage of the control value of binding remaining, showed that glucose, mannose, galactose, fucose, lactose, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-mannoside, or β-methyl-δ-glucoside produced no significant inhibition at concentrations of up to 100 mM. *N*-Acetylgalactosamine, *N*-acetylmannosamine, and *N*-acetylglucosamine at 100 mM concentrations inhibited binding of K88 antigen by 40, 29, and 14%, respectively. As shown in Table 1, several of the glycoproteins inhibited binding, with the greatest effects shown by asialo OSM (45% inhibition), asialoagalactosyl α_1-acid glycoprotein (38% inhibition), and asialo A+ PSM (28% inhibition). The effect of A+ PSM could not be accurately assessed because the retention on the filter of K88 antigen in the absence of added brush border membrane increased dramatically in the presence of this glycoprotein. Further studies indicated that the retention of the ^{125}I-labeled K88 antigen by the filter in the presence of A+ PSM was due to aggregates formed between the K88 antigen and the mucin

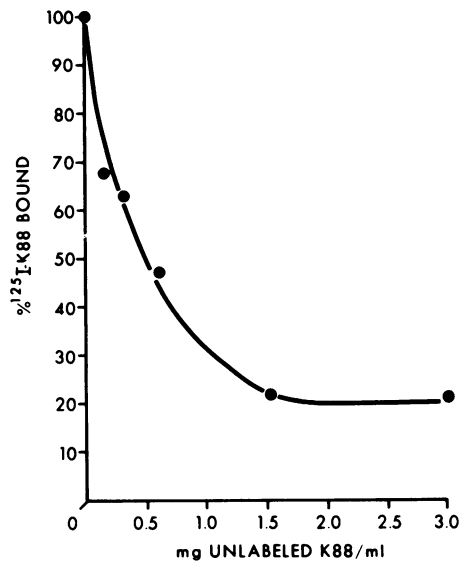

FIG. 4. *Inhibition of ^{125}I-labeled K88 antigen binding to brush border membranes by unlabeled K88 antigen. Experimental procedures are described in the text.*

TABLE 1. *Effect of glycoproteins on K88 binding to porcine intestinal brush border membranes*

Glycoprotein added[a]	Inhibition of K88 Binding (%)
OSM	5 ± 7[b]
OSM (without sialic acid)	45 ± 11
A− PSM	9 ± 13
A− PSM (without sialic acid)	16 ± 8
A+ PSM	—[c]
A+ PSM (without sialic acid)	28 ± 2
α_1-Acid glycoprotein (without sialic acid)	22 ± 13
α_1-Acid glycoprotein (without sialic acid and galactose)	38 ± 1

[a] Concentration of glycoprotein in binding assay, 0.4 mg/ml.

[b] Mean ± standard error of the mean; $n = 6$.

[c] Greatly increased background.

and that this interaction was dose related. Up to 78% of the labeled K88 could be retained by the filter in the absence of added membrane by 100 μg of an A+ PSM. Ten micrograms of A+ PSM was required to precipitate 50% of the labeled K88 antigen.

DISCUSSION

The results shown in Fig. 3 and 4 indicate that the assay described here reflects specific binding of K88 antigen to hog brush border membranes, since the binding is saturable and is subject to competition by the unlabeled antigen. The assay method was made possible by selecting a filter pore size which, while retaining nearly all of the hog brush border membrane preparation, allowed most of the free K88 antigen to pass through the filter.

The results of the experiments testing the inhibition of binding by sugars and glycoproteins suggest a role for *N*-acetylhexosamines in the binding process. *N*-Acetylglucosamine, *N*-acetylgalactosamine, and *N*-acetylmannosamine exerted the greatest influence on the binding, albeit substantially less than some of the glycoproteins tested. The inhibitory effects of the glycoproteins show a similar pattern with regard to their terminal sugars. OSM, which has terminal sialic acid residues, does not inhibit binding. However, after desialylation of OSM, revealing terminal *N*-acetylgalactosamine residues, this glycoprotein inhibits substantially the binding of K88 antigen to hog brush border membranes. A− PSM shows little inhibitory effect, even after removal of sialic acid residues. A+ PSM, which contains terminal *N*-acetylgalactosamine residues, interacted with K88. A+ PSM formed an aggregate with the K88 antigen in the absence of brush border membranes and prevented assessment of binding by the filter method. Up to 78% of the labeled K88 antigen was retained by the filter in the absence of membrane by adding 100 μg of A+ PSM. Asialo A+ PSM did not form a precipitate but inhibited binding by 28%. α_1-Acid glycoprotein showed some inhibitory effect in all of the forms tested, the largest effect seen with the derivative containing terminal *N*-acetylglucosamine residues (i.e., without sialic acid and galactose). A different specificity pattern was found by Gibbons et al. (8) with an indirect assay measuring the inhibition of agglutination of guinea pig erythrocytes by K88. They concluded that β-D-galactosyl residues are significant in the binding. Although some inhibition was seen with asialo α_1-acid glycoprotein, no inhibition was observed with up to 100 mM β-methyl-D-galactopyranoside or D-galactose. The difference in conclusion may result from the difference in the assay system, the guinea pig erythrocyte model versus the direct assay with brush border membrane preparations and purified K88 antigen.

It appears from the present study that *N*-acetylhexosamines play some role in the attachment of K88 antigen to hog brush border membranes. Since some strains of pigs are resistant to this pathogenic *E. coli*, these animals may lack the carbohydrate receptors on the mucosa for the K88 antigen. Conversely, the binding of the bacterium to the intestinal mucosa may be reduced by virtue of carbohydrate structures present in salivary or intestinal secretions of resistant pigs. It is also important to note the possible role of hydrophobic interactions in the binding of K88 antigen to hog brush border membranes, as reported by Smyth et al. (16). It is likely that a combination of elements will be found to be involved in bacterial adhesion to intestine mediated by fimbriae and colonization factors and that the varied interplay among these elements may determine the species specificity of the interaction.

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