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Interaction of a 60-Kilodalton D-Mannose-Containing Salivary Glycoprotein with Type 1 Fimbriae of *Escherichia coli*

JEGDISH P. BABU,[1]* SOMAN N. ABRAHAM,[2] MUSTAFA K. DABBOUS,[1,3] AND EDWIN H. BEACHEY[2,4,5]

Veterans Administration Medical Center[5] and Departments of Periodontics,[1] Medicine,[2] Biochemistry,[3] and Microbiology and Immunology,[4] The University of Tennessee, Memphis, Memphis, Tennessee 38163

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A 60-kilodalton glycoprotein previously isolated and purified from human saliva (J. P. Babu, E. H. Beachey, D. L. Hasty, and W. A. Simpson, Infect. Immun. 51:405–413, 1986) was found to interact with type 1 fimbriae and prevent adhesion of type 1 fimbriated *Escherichia coli* to animal cells in a D-mannose-sensitive manner. Purified salivary glycoprotein agglutinated type 1 fimbriated *E. coli* and, at subagglutinating concentrations, blocked the ability of type 1 fimbriated *E. coli* to attach to human buccal epithelial cells or agglutinate guinea pig erythrocytes. Both interactions were inhibited by α-methyl-D-mannoside but not by α-methyl-D-glucoside. Complexing of the glycoprotein to type 1 fimbriae was demonstrated by molecular sieve chromatography and modified Western blots. When mixed with type 1 fimbriae, the radiolabeled salivary glycoprotein coeluted with type 1 fimbriae from a column of Sepharose 4B. When blotted from a sodium dodecyl sulfate gel to nitrocellulose sheets, the glycoprotein interacted directly with type 1 fimbriae applied to the blots. Both of the latter interactions also were blocked by α-methyl-D-mannoside but not by α-methyl-D-glucoside. Chemical modification of the glycoprotein with sodium metaperiodate abolished its ability to interact with isolated type 1 fimbriae or type 1 fimbriated *E. coli*. These results suggest that the carbohydrate moiety of the 60-kilodalton glycoprotein serves as a receptor for type 1 fimbriae in the oral cavity, and we postulate that the interaction may cause agglutination and early removal of *E. coli*, thereby preventing colonization by these organisms of oropharyngeal mucosae and dental tissues.

The mucosal surfaces of the gastrointestinal, respiratory, and urinary tracts are constantly bathed in mucus and other secretions that are rich in glycoproteins and glycolipids that are analogs to mucosal cell receptors for various bacterial adhesins (2, 8). These soluble receptor analogs provide an alternate binding site for bacterial attachment, resulting in reduced bacterial association with mucosal epithelial cells. Binding of bacteria to soluble receptors in mucosal secretions often results in aggregation of bacteria and facilitates their early removal from mucosal surfaces.

Human saliva has been shown to contain a number of glycoproteins (5, 22), some of which are rich in dicarboxylic amino acids, proline, and glycine (5, 9, 22) and to contain bacterial agglutinating properties (11, 12, 14–16). These agglutinating compounds include high-molecular-weight mucinous glycoproteins (14, 21, 26), some of which are blood group reactive (25), lysozyme (24), and immunoglobulins. Recently, we isolated a 60-kilodalton (kDa) glycoprotein from human whole saliva, which demonstrated agglutinating activity with certain strains of *Streptococcus mutans* (6). We showed that the polypeptide rather than the oligosaccharide component of the glycoprotein molecule mediated this reaction. In this paper, we report that the glycoprotein interacted with type 1 fimbriated *Escherichia coli* and their isolated fimbriae in a D-mannose-sensitive manner. At subagglutinating concentrations, the salivary glycoprotein inhibited attachment of *E. coli* to human buccal epithelial cells and inhibited their ability to agglutinate guinea pig erythrocytes. Treatment of the glycoprotein with sodium metaperiodate or addition of D-mannose, but not D-glucose, abolished these inhibitory activities in these reaction mixtures. Taken together, our studies indicate that, in contrast to the interaction with *S. mutans* through its protein moiety (6), the glycoprotein interacted with type 1 fimbriae through its carbohydrate moiety. We postulate that the salivary glycoprotein, because of its interaction with both *S. mutants* and *E. coli*, plays a modulatory role in the bacterial ecology of the oropharyngeal cavity and dental tissues.

MATERIALS AND METHODS

Bacterial strain and culture conditions. *E. coli* CSH50 is a Cold Spring Harbor K-12-derived strain. The bacterial cells were cultured in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, MD. [Div. Becton Dickinson and Co., Paramus, N.J.]) under static conditions at 37°C for 48 h.

Purification of type 1 fimbriae. CSH50 fimbriae were purified by the method of Dodd and Eisenstein (10). Briefly, bacteria were washed with 0.5% NaCl and suspended in 5 mM Tris buffer, pH 7.8. Fimbriae were removed by mechanical agitation at high speed in an Osterizer Blender with five 2-min bursts. Defimbriated cells were sedimented at 27,000 × *g* for 30 min, and the supernatant was then subjected to ultracentrifugation at 227,000 × *g* for 2 h. The pellet of semipure fimbriae was suspended in a small volume of 5 M urea–5 mM Tris (pH 7.0) and incubated for several hours at 37°C. The urea-buffer mixture was subsequently diluted to 1 M by addition of an appropriate volume of Tris buffer. This suspension was layered on top of an equal volume of 1 M sucrose–5 mM Tris, and pure fimbriae were obtained by centrifuging for 16 h at 200,000 × *g*. The purity of the fimbrial preparation was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electron microscopy.

Immunization of rabbits. Two New Zealand White rabbits were each immunized with an injection of 300 μg of purified CSH50 fimbriae emulsified in complete Freund adjuvant, followed by a boost of 200 μg of fimbriae 2 weeks later in

* Corresponding author.

incomplete Freund adjuvant (4). Serum was obtained before immunization and at 2-week intervals thereafter.

Isolation and purification of salivary glycoproteins. The salivary glycoprotein was isolated and purified as described previously (6). Briefly, paraffin-stimulated whole saliva from several donors was pooled, clarified, and lyophilized. The lyophilized material was dissolved in 0.02 M phosphate–0.15 M NaCl, pH 7.4 (PBS), passed over a Sephadex G100 column, and monitored at 230 nm with a spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). The materials in the fractions of the second peak were further purified by ion exchange chromatography over a DEAE-Biogel column. The first peak material eluted from this column was further resolved into two peaks by reverse-phase high-pressure liquid chromatography. The fractions of the second peak were pooled and lyophilized and are hereafter designated as the salivary glycoprotein. Purity and relative molecular mass were assessed by SDS-PAGE. For some experiments, purified salivary glycoprotein was radiolabeled by reductive methylation with [^{3}H]formaldehyde (specific activity; 100 mCi/mmol; New England Nuclear Corp., Boston, Mass.) as described by Grinnel (13) and as previously described elsewhere (7). The specific activity of the radiolabeled glycoprotein was 2,600 cpm/μg.

$NaIO_4$ oxidation. The purified glycoprotein (2 mg) was dissolved in 1.0 ml of 0.05 M sodium acetate buffer (pH 4.5). An equal volume (0.5 ml) of 0.2 M $NaIO_4$ dissolved in the same buffer was mixed with the agglutinin solution and incubated for 24 h at ambient temperature in the dark. Excess $NaIO_4$ was neutralized with 4 M sodium borohydride in acetate buffer. In control experiments, the salivary glycoprotein was treated as described above except that $NaIO_4$ was deleted from the reaction mixture. All samples were extensively dialyzed against distilled water and then lyophilized.

Production of monoclonal antibodies. Preparation of the hybridoma clone from which the D-mannose-specific anti-glycoprotein monoclonal antibody, A07, used in the present study was derived as described in a previous report (6). The unrelated murine monoclonal antibody used in this study was raised against purified type V collagen.

***E. coli*-induced guinea pig erythrocyte agglutination.** The number of bacterial cells to be added to the test system was initially determined by titrating the bacteria for erythrocyte-agglutinating activity. The penultimate lowest concentration of bacteria that produced a strong agglutination reaction was used for the inhibition test. Twofold dilutions (50 μl) of the salivary glycoprotein (2 mg/ml) or $NaIO_4$-treated salivary glycoprotein in PBS was combined with an equal volume of 0.5% guinea pig erythrocytes and bacteria in a microtiter plate. The mixtures of bacteria, salivary glycoprotein, and guinea pig erythrocytes were incubated at 37°C for 60 min before they were examined for agglutination inhibition.

Bacterial adherence to human buccal epithelial cells. Adhesion assays were performed as previously described (3, 4). Test mixtures consisting of 10^5 epithelial cells in 0.1 ml of PBS were mixed with an equal volume of 10^8 *E. coli* CSH50 suspended in various concentrations of salivary glycoprotein (25 to 100 μg) or 100 μg of $NaIO_4$-treated salivary glycoprotein. The mixture was rotated at ambient temperature for 30 min, and unattached bacteria were removed by differential centrifugation. The number of adherent bacteria was determined microscopically on stained smears of epithelial cells. The concentrations of glycoprotein used in this assay did not induce agglutination of *E. coli*.

Bacterial adherence to immobilized glycoprotein. A 96-well flat-bottomed enzyme immunoassay plate (Costar, Cambridge, Mass.) was coated with purified salivary glycoprotein (10 μg/ml) in carbonate buffer (0.1 M, pH 9.6). The glycoprotein-coated wells were then treated with 0.1 ml of 3% bovine serum albumin (BSA) in PBS for 1 h at 37°C. The wells were washed four times with 0.9% NaCl–0.05% Tween 20 (solution A). A sample of type 1 fimbriated *E. coli* (50 μl containing 10^8 cells) was then added to the wells and mixed with an equal volume of a 1/50 dilution of mouse ascites fluid containing anti-glycoprotein monoclonal antibody. As a control, the bacterial cells were similarly mixed with monoclonal antibody raised against an unrelated antigen (type V collagen). Additionally, the bacterial cells were also mixed with an equal volume of a 2% solution of α-methyl-D-mannoside or α-methyl-D-glucoside. The glycoprotein-coated wells were incubated at 37°C for 3 h and washed six times with solution A. Adherent bacteria were fixed by incubation for 30 min at 60°C. Rabbit anti-fimbrial serum diluted 1/500 in 3% BSA solution was added to appropriate wells and incubated for 2 h at 37°C, followed by standard enzyme-linked immunosorbent assay procedures.

Agglutination of *E. coli* by salivary glycoprotein. A 20-μl drop of bacterial suspension was mixed with the same amount of salivary glycoprotein (10 mg/ml) on a glass slide. Agglutination was assessed visually over a 1-min period.

Direct binding of isolated type 1 fimbriae to salivary glycoprotein. Samples of purified salivary glycoprotein were electrophoresed on a 7.5% SDS-PAGE gel (18) under reducing conditions. After SDS-PAGE, the bands of salivary glycoprotein were electrophoretically transferred onto nitrocellulose paper by the method of Towbin et al. (27). After transfer, the nitrocellulose paper strips were either placed in 1% BSA in PBS for 1 h or placed in 2% α-methyl-D-mannoside suspended in BSA-PBS for 2 h at 37°C and then incubated with 50 μg of isolated type 1 fimbriae of *E. coli* per ml in BSA-PBS. The strips were washed extensively with 0.9% NaCl–0.05% Tween 20 and then incubated for 1 h at 37°C with a 1:750 dilution of polyclonal anti-type 1 fimbria serum in BSA-PBS. After extensive washing, the strips were treated with a 1:1,500 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.), washed thoroughly, and treated with horseradish peroxidase color development reagent (Bio-Rad Laboratories, Richmond, Calif.) in 100 ml of a 25 mM Tris–0.5 M NaCl solution supplemented with 20 ml of cold methanol and 100 μl of H_2O_2. The interaction of isolated type 1 fimbriae with the salivary glycoprotein was visualized as darkly staining bands in the region corresponding to the immobilized salivary glycoprotein on the nitrocellulose strips.

For direct visualization, the nitrocellulose strip with transferred salivary glycoprotein was stained with 1% amido black for 30 s and washed extensively with water.

Interaction of isolated type 1 fimbriae with ^{3}H-labeled salivary glycoprotein. Isolated *E. coli* fimbriae (100 μg) were subjected to gel filtration on a Sepharose 4B column (9 mm by 18 cm) equilibrated with PBS. The column was eluted with PBS at 16 ml/h, elution was monitored at 230 nm with a Beckman spectrophotometer, and 0.5-ml fractions were collected. ^{3}H-labeled salivary glycoprotein (100 μg; specific activity 2,600 cpm/μg) was similarly chromatographed on the same column after thorough washing with 50 ml of PBS. A sample (0.2 ml) of each fraction was suspended in 5 ml of Scintiverse (Fischer Scientific Co., Pittsburgh, Pa.) and assayed for radioactivity in a scintillation counter (Tri-Carb liquid scintillation spectrometer; Packard Instrument Co.,

Inc., Rockville, Md.). The column was washed again with 50 ml of PBS, and a mixture of fimbriae (100 μg) and ^{3}H-labeled salivary glycoprotein (100 μg) previously incubated for 2 h at ambient temperature was chromatographed under identical conditions. The radioactivity in the chromatographic fractions was monitored as described above. Elution patterns were obtained by plotting radioactivity, expressed as counts per minute, or absorbance as a function of elution volume.

RESULTS

The salivary glycoprotein was purified as previously described by molecular-sieve, ion-exchange, and reverse-phase chromatography (6). The glycoprotein was judged to be pure by SDS-PAGE and was used in the following studies.

Interaction of salivary glycoprotein with type 1 fimbriated *E. coli*. The purified glycoprotein at a final concentration of 5 mg/ml in PBS caused visible agglutination of a suspension of type 1 fimbriated *E. coli* in slide agglutination tests. However, glycoprotein concentrations below 2 mg/ml did not induce bacterial agglutination. Agglutination caused by glycoprotein (5 mg/ml) was reversed by adding a drop of 2% α-methyl-D-mannoside but not α-methyl-D-glucoside.

The agglutination of guinea pig erythrocytes induced by *E. coli* was completely blocked by both salivary glycoprotein (50 μl; 2 mg/ml) and α-methyl-D-mannoside (50 μl; 2% solution). Bacterial hemagglutination was not affected by α-methyl-D-glucoside (50 μl; 2% solution). The ability of the glycoprotein to block *E. coli* hemagglutination was abolished when the glycoprotein was treated with sodium metaperiodate.

Further, we also tested the effect of salivary glycoprotein on the adhesion of *E. coli* to human buccal epithelial cells. Adhesion of type 1 fimbriated *E. coli* to buccal epithelial cells was inhibited by α-methyl-D-mannoside and by the glycoprotein (Table 1). The degree of inhibition by the glycoprotein was dose dependent. Again, the glycoprotein treated with sodium metaperiodate and α-methyl-D-glucoside had no inhibitory effect on *E. coli* adhesion to epithelial cells. Collectively, these results suggested that the salivary glycoprotein interacted with type 1 fimbriated *E. coli* in a D-mannose-sensitive manner, probably through its carbohydrate moiety.

Interaction of salivary glycoprotein with isolated type 1 fimbriae. To determine whether the salivary glycoprotein reacted directly with type 1 fimbriae, we mixed ^{3}H-glycoprotein with isolated and purified fimbriae, and then analyzed the mixture by gel filtration chromatography on Sepharose 4B column. Approximately 70% of the radiolabeled glycoprotein coeluted with the fimbriae in the void volume from the column (Fig. 1C). Addition of α-methyl-D-mannoside, but not α-methyl-D-glucoside, prevented coelution of the labeled material with the fimbrial peak; all of the radiolabel was eluted with the glycoprotein peak in the internal volume of the column resin (Fig. 1D).

TABLE 1. Effect of salivary glycoprotein on type 1 fimbriated *E. coli* adherence to human buccal epithelial cells

Inhibitor	No. (±SE) of adherent bacteria/epithelial cell[a]
PBS	59 ± 12
α-Methyl-D-mannoside (2%)	11 ± 5
α-Methyl-D-glucoside (2%)	56 ± 9
Salivary glycoprotein (100 μg)	12 ± 4
Salivary glycoprotein (50 μg)	21 ± 7
Salivary glycoprotein (25 μg)	48 ± 5
$NaIO_4$-treated salivary glycoprotein (100 μg)	57 ± 6

[a] Epithelial cells (10^5) were mixed with 10^8 *E. coli* and incubated for 30 min, and the bacteria that adhered to the epithelial cells were counted.

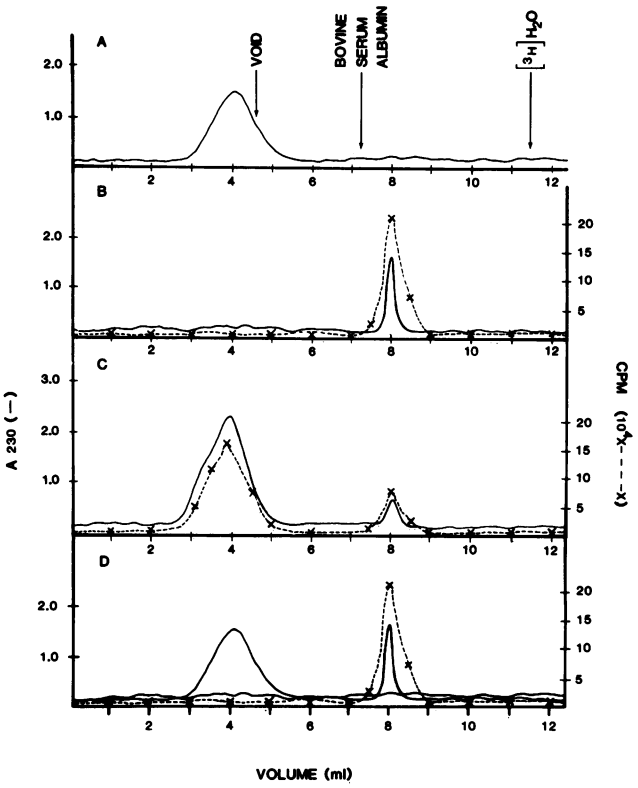

FIG. 1. Gel filtration chromatography on a column of Sepharose 4B of (A) type 1 fimbriae, (B) ^{3}H-labeled salivary glycoprotein, (C) a mixture of type 1 fimbriae and ^{3}H-labeled salivary glycoprotein, and (D) C in the presence of D-mannose.

To confirm the interaction of the glycoprotein with isolated fimbriae further, we subjected the glycoprotein to SDS-PAGE and electrophoretically transferred it to nitrocellulose paper. The nitrocellulose blot of glycoprotein was then exposed to isolated type 1 fimbriae, followed by anti-type 1 fimbrial antiserum. The type 1 fimbriae reacted strongly with the glycoprotein band (Fig. 2, lane B). The interaction was completely blocked by α-methyl-D-mannoside (Fig. 2, lane C) but not by α-methyl-D-glucoside. Taken together, these results indicated that the salivary glycoprotein interacted through its carbohydrate moiety with D-mannose binding sites on type 1 fimbriae. In contrast to these results, fimbriae dissociated into their subunits failed to interact with glycoprotein in immunoblots performed as described above (data not shown). No reactive bands were seen when the glycoprotein on the nitrocellulose sheet was treated with anti-type 1 fimbrial antiserum, thus ruling out the possibility of any cross-reactivity between the glycoprotein and the antibodies against type 1 fimbriae used in the assay.

To examine whether the 60-kDa salivary glycoprotein is the only component of saliva which reacts with type 1 fimbriae, we subjected human whole saliva to SDS-PAGE and reacted it with isolated fimbriae on Western blots as described above. Examination of the Western blots revealed

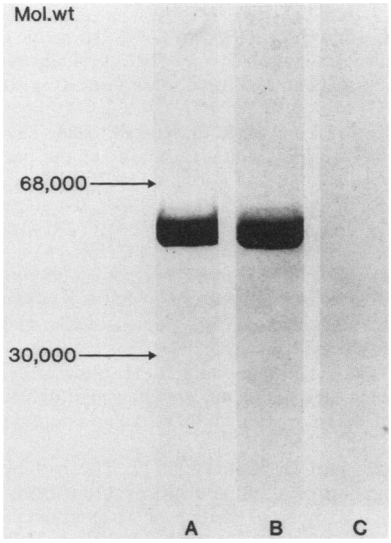

FIG. 2. Western blots of the salivary glycoprotein on nitrocellulose. Lanes were (A) stained with amido black, (B) treated with isolated type 1 fimbriae and reacted with antiserum against type 1 fimbriae, or (C) treated with isolated type 1 fimbriae in the presence of α-methyl-D-mannoside and reacted with antiserum against type 1 fimbriae.

the 60-kDa glycoprotein to be the predominant reactive band. In addition, two faint immunoreactive bands with approximate molecular masses of 110 and 180 kDa were also seen (data not shown).

Inhibition of interaction of glycoprotein with type 1 fimbriated *E. coli* by a D-mannose-specific monoclonal antibody. To confirm the mannose specificity of the interaction between the glycoprotein and type 1 fimbriated *E. coli*, we examined the effect of a D-mannose-specific monoclonal antibody, A07, which was previously raised against the 60-kDa salivary glycoprotein (6), on the glycoprotein-fimbrial interactions. In this assay, type 1 fimbriated *E. coli* was allowed to attach to the immobilized glycoprotein in the presence of A07 monoclonal antibody. As a control, we used a monoclonal antibody raised against an unrelated antigen (type V collagen). Additionally, the effects of α-methyl-D-mannoside and α-methyl-D-glucoside were also tested. The D-mannose-specific antibody, A07, and α-methyl-D-mannoside blocked attachment of type 1 fimbriated *E. coli* to the glycoprotein (Fig. 3). The presence of α-methyl-D-glucoside or the unrelated monoclonal antibody (anti-type V collagen) had no inhibitory effect (Fig. 3). These results once again confirmed that the glycoprotein interacted with type 1 fimbriated *E. coli* in a mannose-sensitive manner.

DISCUSSION

In this paper we report a specific interaction between a 60-kDa salivary glycoprotein and type 1 fimbriae of *E. coli*. This interaction was demonstrated by (i) formation of a complex detectable by column chromatography and (ii) direct binding of isolated type 1 fimbriae to the salivary glycoprotein immobilized on nitrocellulose. Furthermore, interaction of salivary glycoprotein with type 1 fimbriae inhibited the ability of type 1 fimbriated *E. coli* to agglutinate guinea pig erythrocytes and to adhere to buccal epithelial cells.

Type 1 fimbriae of *E. coli* are hairlike appendages which mediate attachment of the organisms to various eucaryotic cells. This interaction is inhibitable by D-mannose and its derivatives, suggesting that this sugar is part of the receptor on eucaryotic cells for the binding of type 1 fimbriated *E. coli*. Previous chemical analysis of the salivary glycoprotein revealed the presence of approximately 17% (wt/wt) D-mannose (6). Furthermore, we have previously described a mannose-specific monoclonal antibody, A07, raised against this glycoprotein, which not only inhibited adhesion of type 1 fimbriated *E. coli* to buccal epithelial cells but also protected against urinary tract infection in mice challenged with type 1 fimbriated *E. coli* (1). Salivary glycoprotein treated with $NaIO_4$ failed to interact with type 1 fimbriae. The $NaIO_4$-treated glycoprotein failed to block *E. coli* adhesion to epithelial cells and to hemagglutinate guinea pig erythrocytes. Additionally, the interactions of salivary glycoproteins with isolated fimbriae or fimbriated *E. coli* was found to be mannose sensitive. The data, taken together, strongly suggest that the type 1 fimbriae interact with mannose or mannoselike residues of the salivary glycoprotein.

These results contrast with our previous studies of the interaction of the same glycoprotein with cells of *S. mutans* (6). Rather than interacting through the carbohydrate moiety, *S. mutans* cells interacted through the protein moiety of the glycoprotein. Thus, the glycoprotein has the capacity to agglutinate different species of bacteria by different mechanisms.

We showed that the salivary glycoprotein can interact with isolated fimbriae and fimbriated *E. coli*. However, efforts to demonstrate binding of salivary glycoprotein to dissociated fimbriae immobilized on nitrocellulose were unsuccessful. This is probably due to the lack of D-mannose binding sites on the fimbrial subunits themselves. Binding requires either a quaternary fimbrial structure or perhaps an as yet undefined adhesive subunit (19, 20).

The biological significance of the interaction of the salivary glycoprotein with type 1 fimbriae of *E. coli* may be comparable to that suggested for Tamm-Horsfall protein and type 1 fimbriated *E. coli* in the urinary tract (17, 23). Tamm-Horsfall protein is a D-mannose-rich glycoprotein present in urinary mucin, and Orskov et al. (23) reported that type 1 fimbriated *E. coli* bound specifically to and was

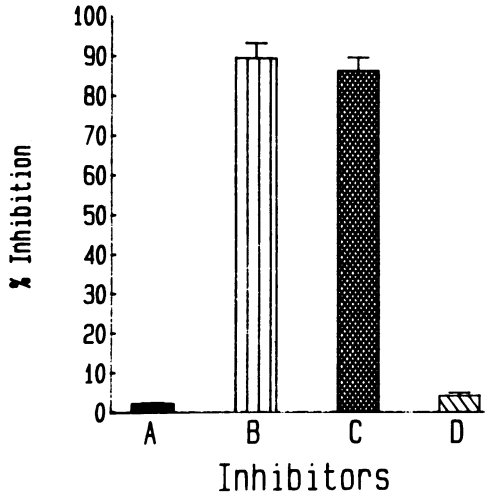

FIG. 3. Enzyme-linked immunosorbent inhibition assays of attachment of type 1 fimbriated *E. coli* to immobilized glycoprotein, using monoclonal antibody against D-mannose, A07 (B), monoclonal antibody against unrelated antigen (A), α-methyl-D-mannoside (C), or α-methyl-D-glucoside (D) as the inhibitor.

agglutinated by this glycoprotein. They proposed that, by this mechanism, fimbriated *E. coli* becomes entrapped in urinary mucin and is rapidly eliminated by urine flow (23). Nonfimbriated-phase variants which escape entrapment are also eliminated from the mucosal surface because they lack adhesive capability (2). Recently, it was suggested that salivary fibronectin, by virtue of its interaction with both gram-positive and gram-negative bacteria in the oral cavity, may modulate the ecology of the buccal cavity (3, 7). In a similar manner, we speculate that the salivary glycoprotein reported here may modulate the ecology of the buccal cavity by virtue of its interaction both with cells of *S. mutans* and with cells of fimbriated *E. coli*. The infrequent presence of *E. coli* in the oropharyngeal cavity may be a reflection of the powerful agglutinating properties of the 60-kDa glycoprotein and perhaps other D-mannose-containing glycoproteins in human saliva.

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