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Binding of *Escherichia coli* and *Salmonella* Strains to Members of the Carcinoembryonic Antigen Family: Differential Binding Inhibition by Aromatic α-Glycosides of Mannose

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Various *Escherichia coli* and *Salmonella* strains bound to glycoproteins of the family of carcinoembryonic antigens (CEA). As judged from plateau regions of the binding curves, CEA, nonspecific cross-reacting antigen of M_r 55,000 (NCA-55), and biliary glycoprotein of M_r 85,000 (BGP-85) showed similar binding activities. The binding to ovalbumin was significantly lower and the binding to fetuin was insignificant under identical experimental conditions. The binding of *E. coli* and *S. typhi* to the different glycoproteins was similar as judged from the binding curves. In comparison with α-methyl-D-mannopyranoside, aromatic α-glycosides of mannose were more potent binding inhibitors of *E. coli* but not of salmonellae to CEA and NCA-55. These results are similar to those previously obtained with intestinal epithelial cells and yeast cells (N. Firon, S. Ashkenazi, D. Mirelman, I. Ofek, and N. Sharon, Infect. Immun. 55:472–476, 1987). The binding of *E. coli* to CEA was inhibited by purified type 1 fimbriae. On the basis of the distribution of CEA-like glycoproteins in tissues and body fluids, the results indicate that glycoproteins of the CEA family may be involved in the recognition of bacteria and the regulation of bacterial colonization.

The commensal and symbiotic microorganisms, i.e., members of the normal microbial flora, that live in association with body surfaces of humans and animals are often highly specialized bacteria with different mechanisms for attachment, especially to surface epithelium cells, suggesting an evolutionary adaptation to a specific host. Microorganisms, in health and disease, show tissue tropism, i.e., they colonize certain sites dependent on the existence of receptors on the respective tissues that permit bacterial binding (8). It is well known that different bacteria as members of the resident flora of humans as well as pathogenic bacteria possess lectinlike surface adhesins, mostly localized on fimbrial structures, with a specificity for carbohydrates on tissue glycoprotein receptors (4, 31). Glycoproteins that bind to bacterial lectins may be secreted or shed by epithelia as mucus or slime (24). Secretion or shedding of glycoproteins with specificity for bacterial lectins such as the Tamm-Horsfall glycoprotein or the low-M_r glycoconjugates recently described in urine (25) may be understood as an unspecific host defense mechanism which prevents the binding of pathogenic bacteria with appropriate sugar specificity.

In recent years, significant evidence has accumulated that lectins of type 1-fimbriated *Escherichia coli* bind to D-mannosyl residues of glycoproteins present on buccal, intestinal, and bladder epithelia as well as on leukocytes (1, 32, 38). In addition to *E. coli*, binding to D-mannosyl residues of glycoproteins has been described for *Salmonella*, *Klebsiella*, *Citrobacter*, *Morganella*, and *Aeromonas* species (8). Our knowledge of the nature of the glycoproteins involved in the binding of bacteria via D-mannosyl residues is limited. By affinity chromatography on a column of immobilized type 1 fimbriae of a lysate of polymorphonuclear leukocytes, three bands were resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (28). Using a similar approach, a receptor for type 1 fimbriae of *E. coli* has been isolated from guinea pig erythrocytes (14). No information is available on receptor glycoproteins of human epithelia.

Recently, we showed that *E. coli* binds to carcinoembryonic antigen (CEA) and to the nonspecific cross-reacting antigen of M_r 55,000 (NCA-55) (20). CEA is normally produced by human colonic epithelial cells (10, 13, 19), and NCA-55 has been isolated by perchloric acid extraction from human granulocytes as well as from normal spleen and lung tissues (3, 6, 22). In the present study, the investigations were extended to additional bacteria and glycoproteins. Inhibition experiments indicated that the binding characteristics are similar to those described for receptors on intestinal epithelium and *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Strains and culture methods. *E. coli* strains were isolated from clinical materials. Enteropathogenic *E. coli* strains (EPEC) and enterohemorrhagic *E. coli* strains (EHEC) were isolated from feces of newborn children suffering from diarrhea. Strains of *Yersinia enterocolitica*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Shigella boydii*, *Salmonella typhi*, *Salmonella java*, and *Salmonella paratyphi* were isolated from feces of adults with enteric infections and were kind gifts from J. Bockemühl and S. Aleksic, Hygienisches Institut, Hamburg, Federal Republic of Germany, and K. Pieschulla, Hygienisch-Bakteriologisches Institut, Bielefeld, Federal Republic of Germany. All strains were passaged on DST agar (Oxoid, Wesel, Federal Republic of Germany) and, for the expression of fimbrial adhesins, in Luria broth.

Glycoproteins. Purified ovalbumin (crystallized) and fetuin

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(>99% pure) were from Sigma, Deisenhofen, Federal Republic of Germany. CEA was purified as described previously (27). NCA-55 was purified from human spleen by perchloric acid extraction and immunosorbent purification over the monoclonal antibody (MAb) T84.1 (22, 37). MAb T84.1 does not bind NCA-95, a product of a CEA family gene distinct from the genes encoding CEA, NCA, and biliary glycoprotein (BGP) (3, 5, 6). The sequence of the 24 N-terminal amino acids matches the sequence published for NCA (26). BGP was purified from human bile by perchloric acid extraction and immunosorbent purification over MAb T84.1 as described previously (22). To exclude contamination by NCA, the preparation was passed over an immunosorbent to which an anti-NCA MAb was bound (MAb 33, described in reference 7 and kindly provided by P. Burtin, Villejuif, France). The M_r of our BGP preparation was 85,000. Internal sequences of BGP purified in our laboratory were used to identify a cDNA specific for BGP (16). The meconium antigen of M_r 165,000 (NCA2) was purified as previously described (33). The sequence of the 30 N-terminal amino acids of this glycoprotein has been published (33). The concentration of each glycoprotein was determined by amino acid analysis. The amounts and concentrations of glycoproteins refer to their protein moieties.

Antisera. Bound bacteria were detected by the following antisera (numbers in parentheses refer to the dilutions used for binding and inhibition assays): *E. coli* 084, homologous antiserum induced against strain 084 after passage on DST agar (1:1,500); other *E. coli* strains, antiserum obtained from Dako, Hamburg, Federal Republic of Germany (1:100); all salmonellae, polyvalent antiserum from Behring, Marburg, Federal Republic of Germany (1:100). All antisera were produced in rabbits. The binding of *Shigella* and *Yersinia* strains to CEA was analyzed by an acridine orange stain.

Inhibitors. Methyl-α-D-mannopyranoside and 4-methylumbelliferyl-α-D-mannopyranoside were from Sigma. *p*-Ethoxyphenyl-α-D-mannopyranoside and *p*-methoxyphenyl-α-D-mannopyranoside were kindly provided by F. G. Loontiens, University of Ghent, Ghent, Belgium. Purified type 1 fimbriae were a kind gift from J. E. Shively, Beckman Research Institute of the City of Hope, Duarte, Calif.

Hemagglutination assay. A hemagglutination test routinely used for the determination of mannose-sensitive or mannose-resistant hemagglutination was done with guinea pig erythrocytes. Agglutination of erythrocytes in the absence but not in the presence of 25 mM methyl-α-D-mannopyranoside was defined as mannose-sensitive hemagglutination (17).

Binding and inhibition assays. Microtiter plates (type Maxisorp; diameter per well, 7 mm; Nunc, Wiesbaden, Federal Republic of Germany) were coated with 50 μl of glycoprotein solutions at amounts indicated on the abscissae of figures or in the figure legends. Glycoprotein solutions in 0.2 M carbonate buffer (pH 8.3) were used to coat the wells of microtiter plates overnight at room temperature. Unspecific binding sites were blocked by a solution of 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (2 h, room temperature). The wells were washed three times with PBS containing 0.05% Tween 20 (vol/vol). This solution was also used for subsequent washing steps. Bacteria were suspended in PBS and adjusted to a turbidity corresponding to 6×10^8 to 2×10^9 bacteria per ml. A 100-μl portion of the suspension was added to the wells and incubated overnight at 4°C. After washing three times with PBS, 100 μl of solutions of first antibodies in PBS were incubated in each well with the attached bacteria for 1 h at 37°C. After washing, goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad, Munich, Federal Republic of Germany) was added (100 μl per well; 1 h, 37°C). After washing, the color reaction was developed as described previously (36). Controls included (i) wells coated with BSA only and (ii) binding of antisera to glycoproteins in the absence of bacteria. Background optical density values never exceeded 0.3 optical density unit. Background values obtained in the absence of bacteria were subtracted from the values obtained in the presence of bacteria. Determinations were performed as duplicates. Binding and inhibition curves shown in a given figure are based on data obtained in a single experiment.

The fractions of glycoproteins immobilized on the wells of microtiter plates were determined for CEA, ovalbumin, and fetuin. For CEA, the amount remaining in the coating solution was determined by a CEA-specific immunoassay (Hoffmann-La Roche, Basel, Switzerland). For ovalbumin and fetuin, the bound fractions were determined by glycoprotein preparations trace labeled with ^{125}I-labeled ovalbumin or ^{125}I-labeled fetuin, respectively. The bound fractions amounted to 32% for CEA, 42% for ovalbumin, and 20% for fetuin of the amount added over the concentration range used for the bacterial binding studies. The bound fraction remained constant over the experimental concentration range. Because of the high structural homology between the glycoproteins of the CEA family, 32% binding was assumed also for NCA, BGP, and NCA2.

For screening experiments, bacteria bound to microtiter plates were stained with 50 μl of a solution of acridine orange (Api Bio Merieux) per well, washed, and examined for bacteria in a UV inverted microscope.

For inhibition experiments with α-D-mannosides, suspensions containing 6×10^8 to 2×10^9 bacteria per ml were spun down, the supernatant was discarded, and the bacteria were resuspended in PBS containing the concentrations of α-D-mannosides indicated on the abscissae of figures. For inhibition experiments with type 1 fimbriae, fimbriae were dissolved in PBS and added to CEA-coated wells (140 μg of protein per well; 50 μl) for 2 h at 37°C. After preincubation with fimbriae, the bacterial suspension was added to the well and incubated overnight in the presence of fimbriae.

Pretreatment of bacteria. Heat sensitivity of the adhesion mechanism was tested by heating the bacteria to 80°C. Type 1 fimbriae were destroyed by UV irradiation for 40 min as described previously (34).

Detection of type 1 fimbriae by Western immunoblot. Fimbriae were isolated from *E. coli* 084 by the method of Salit and Gotschlich (30). The fimbriae were submitted to SDS electrophoresis under nonreducing conditions and transferred to nitrocellulose. Type 1 fimbriae were detected by an antiserum induced against type 1 fimbriae from *E. coli* kindly provided by K. Jann, Max-Planck-Institut für Immunbiologie, Freiburg, Federal Republic of Germany.

RESULTS

Binding of enterobacteriaceae to CEA. Different isolates and laboratory strains of *E. coli*, salmonellae, shigellae, and yersiniae were tested with respect to adhesion of 250 ng of CEA added to the wells of microtiter plates. All strains were tested after culture in Luria broth. As shown in Table 1, the majority of *E. coli* isolates from urine and feces and two EPEC strains tested bound to CEA. The strains binding to CEA showed a mannose-sensitive hemagglutination, in contrast to the strains that did not bind to CEA. Interestingly,

TABLE 1. Binding of enterobacteriaceae to CEA

Bacterial strain	No. bound/ no. tested
Escherichia coli isolates	20/23
EPEC strains	2/2
EHEC strains	0/3
Salmonella typhi	4/5
Salmonella paratyphi A and B	5/6
Salmonella java	1/1
Salmonella (others)	4/8
Shigella spp.	0/4
Yersinia enterocolitica	0/5

three EHEC strains did not bind. Nearly all the strains of *Salmonella typhi* and *Salmonella paratyphi* showed binding, compared with one-half of the remaining strains. Among these remaining strains, those binding to CEA exhibited mannose-sensitive hemagglutination, whereas strains not binding to CEA showed either mannose-insensitive or no hemagglutination. *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, and *Shigella boydii*, as well as different serotypes of *Y. enterocolitica* did not bind.

Binding of *E. coli* to fetuin, ovalbumin, and members of the CEA family. Increasing amounts of different glycoproteins immobilized on the wells of microtiter plates were incubated with constant numbers of *E. coli* 084. Fetuin was chosen as a glycoprotein lacking high-mannose-type carbohydrate chains (23). In contrast, ovalbumin contains carbohydrate side chains of the high-mannose type (35). In Fig. 1a, the binding of *E. coli* to fetuin and ovalbumin is compared with the binding to CEA. In comparison with ovalbumin, the CEA binding curve is significantly steeper and a binding plateau is approached at 10 ng bound to the wells, whereas at 25 ng of ovalbumin, a plateau is not reached. Binding of *E. coli* to fetuin is insignificant. When the amounts of glycoproteins added to the wells were increased up to 4,000 ng, binding of *E. coli* to ovalbumin approached a plateau at the level obtained with CEA, whereas binding to fetuin remained low (data not shown). In Fig. 1b, the binding of *E. coli* to immobilized CEA is compared with the binding to two distinct products of the CEA gene family, NCA and BGP. The amounts of glycoproteins at which binding plateaus are reached are not significantly different from the amount of CEA at which a plateau is reached.

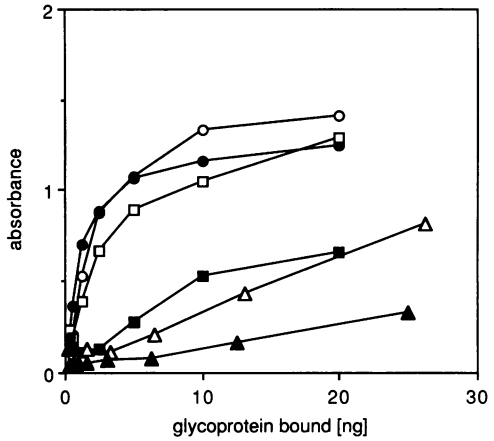

FIG. 2. Binding of *Salmonella typhi* to CEA (○), NCA (●), BGP (□), meconium antigen of M_r 165,000 (NCA2) (■), ovalbumin (△), and fetuin (▲).

Binding of *Salmonella typhi* to fetuin, ovalbumin, and members of the CEA family. The binding patterns of *Salmonella typhi* to ovalbumin, fetuin, CEA, NCA, and BGP were not different from the binding patterns of *E. coli* (Fig. 2). As an additional CEA-related antigen, the meconium antigen of M_r 165,000 (NCA2) was tested for binding. The NCA2 binding curve was not as steep as the binding curves of CEA, NCA, and BGP. A binding plateau was not reached in the concentration range tested.

Inhibition of binding of *E. coli* and salmonellae to CEA by aromatic α-glycosides of mannose. Three different aromatic α-glycosides of mannose, *p*-ethoxyphenyl-α-mannoside, *p*-methoxyphenyl-α-mannoside, and 4-methylumbelliferyl-α-mannoside, were investigated with respect to their inhibition of the binding of *E. coli* to CEA. The inhibitory activity of 4-methylumbelliferyl-α-mannoside was highest, followed by the two remaining aromatic mannosides (Fig. 3). To reach 50% inhibition, more than 700-fold the amount of methyl-α-mannoside is needed in comparison with 4-methylumbelliferyl-α-mannoside (Table 2). The binding of two *Salmo-*

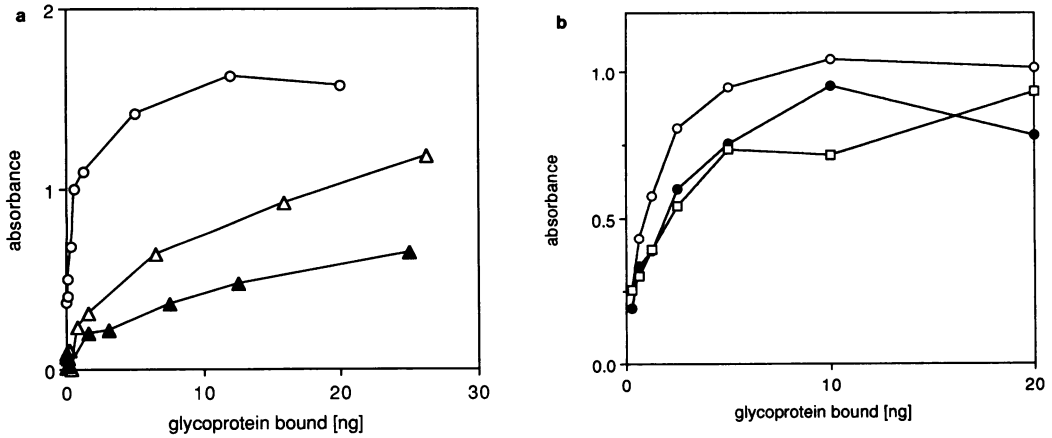

FIG. 1. (a) Binding of *E. coli* (strain 084) to CEA (○), ovalbumin (△), and fetuin (▲). (b) Binding of *E. coli* (strain 084) to CEA (○), NCA-55 (●), and BGP-85 (□).

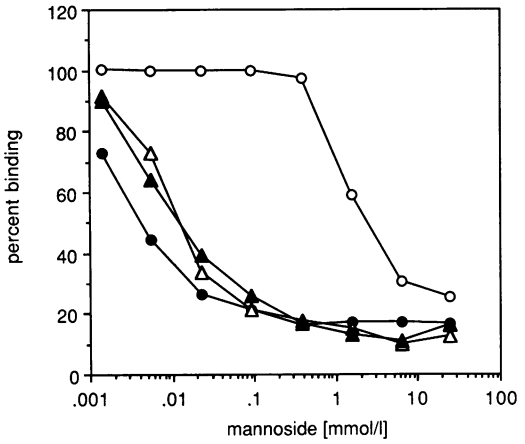

FIG. 3. Inhibition of binding of *E. coli* (strain 084) to CEA by different α-glycosides of D-mannose. ○, methyl-α-D-mannoside; ●, 4-methylumbelliferyl-α-D-mannoside; △, *p*-ethoxyphenyl-α-D-mannoside; ▲, *p*-methoxyphenyl-α-D-mannoside (250 ng of CEA added per well).

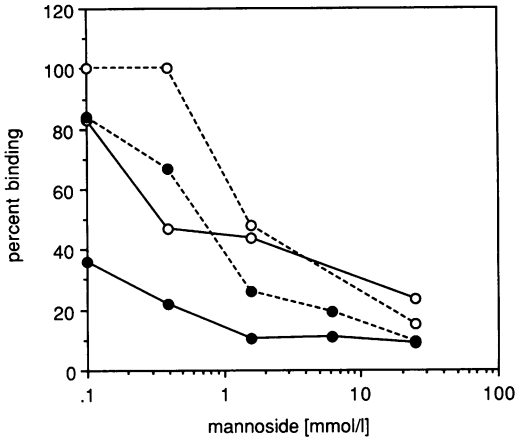

FIG. 4. Inhibition of binding of *Salmonella typhi* (○) and *Salmonella derby* (●) to CEA by methyl-α-D-mannoside (——) and 4-methylumbelliferyl-α-D-mannoside (– – –) (250 ng of CEA added per well).

nella strains, *Salmonella typhi* and *Salmonella derby*, to CEA was investigated in the presence of 4-methylumbelliferyl-α-mannoside and methyl-α-mannoside. In contrast to *E. coli*, higher amounts of 4-methylumbelliferyl-α-mannoside compared with methyl-α-mannoside were needed to reach 50% binding inhibition (Fig. 4).

Inhibition of binding of *E. coli* and salmonellae to NCA by 4-methylumbelliferyl-α-mannoside and methyl-α-mannoside. The results of inhibition experiments using 4-methylumbelliferyl-α-mannoside and methyl-α-mannoside as inhibitors and NCA as the immobilized glycoprotein are demonstrated in Fig. 5 and Table 2. The results are comparable to those obtained with CEA. For *E. coli*, less than 1/1,000th the amount of 4-methylumbelliferyl-α-mannoside in comparison with methyl-α-mannoside was needed to reach 50% inhibition. In contrast, with *Salmonella virchow* and *Salmonella bovis*, higher amounts of the aromatic mannosides were needed to reach binding inhibitions comparable to those reached by methyl-α-mannoside.

Inhibition of binding of *E. coli* to CEA by purified type 1 fimbriae and physical treatment. After preincubation of CEA-coated wells with purified type 1 fimbriae, *E. coli* (strain 084) was added and incubated as usual. In the presence of type 1 fimbriae, binding was 10.9% compared with binding without fimbriae added (100%). UV irradiation of bacteria and heating to 80°C resulted in a complete loss of binding.

Detection of type 1 fimbriae. Fimbriae were isolated from *E. coli* 084 and submitted to Western immunoblots. Using an antiserum against purified type 1 fimbriae from *E. coli*, bands corresponding to M_r 17,000 and two-, three-, and fourfold this M_r were detected.

TABLE 2. Relative inhibition by *p*-methoxyphenyl-α-mannoside, *p*-ethoxyphenyl-α-mannoside, and 4-methylumbelliferyl-α-mannoside of *E. coli* (strain 084) binding to CEA and NCA

Inhibitor	Relative inhibitory activity[a]		
	CEA	NCA	Guinea pig ileal epithelial cells[b]
Methyl-α-mannoside	1	1	1
p-Methoxyphenyl-α-mannoside	208	ND[c]	ND
p-Ethoxyphenyl-α-mannoside	192	ND	240
4-Methylumbelliferyl-α-mannoside	735	1,036	1,015

[a] Determined at 50% inhibition.
[b] According to reference 11.
[c] ND, Not determined.

DISCUSSION

In the present study, the binding of different enterobacteriaceae (*E. coli* and *Salmonella*, *Shigella*, and *Yersinia* strains) to glycoproteins of the CEA family was investigated. Among the bacteria tested, only *E. coli* and salmonellae bound to CEA, NCA, BGP, and, to a lesser extent, to a meconium antigen of M_r 165,000. (NCA2). The binding of *E. coli* and *Salmonella typhi* to CEA and related antigens was compared with their binding to two unrelated glycoproteins. Ovalbumin was selected as a glycoprotein with carbohydrate side chains of the high-mannose type (35), and fetuin was selected as a glycoprotein of the biantennary type lacking high-manose-type oligosaccharides (23). The results of the binding studies can be summarized as follows. (i) As judged from the plateau regions of the binding curves, CEA, NCA, and BGP bind *E. coli* and *Salmonella typhi* with similar avidity. The binding of *Salmonella typhi* to the meconium antigen is less expressed. (ii) The bacterial binding to fetuin is insignificant. Binding to ovalbumin, a high-mannose-type glycoprotein, approaches a plateau only at about 100-fold the amount of CEA.

As shown previously, the binding of *E. coli* is significantly reduced after deglycosylation of CEA (20). Since methyl-α-D-mannoside inhibits the binding of *E. coli* to CEA and NCA, D-mannosyl residues in CEA are probably involved in binding of *E. coli* and, as shown below, of *Salmonella* species to CEA, NCA, and probably BGP. It has been suggested that the structure α-D-Man*p*-(1→3)-β-D-Man*p*(1→4)-D-GlcNAc in oligomannoside-type structures is most potent in inhibiting the binding of type 1-fimbriated *E. coli* to yeast cells (12) or guinea pig erythrocytes (21). According to Neeser et al. (21), the crucial requirement for maximal binding lies in the occurrence of a nonsubstituted α(1→3)-linked terminal D-mannosyl residue initiating the first an-

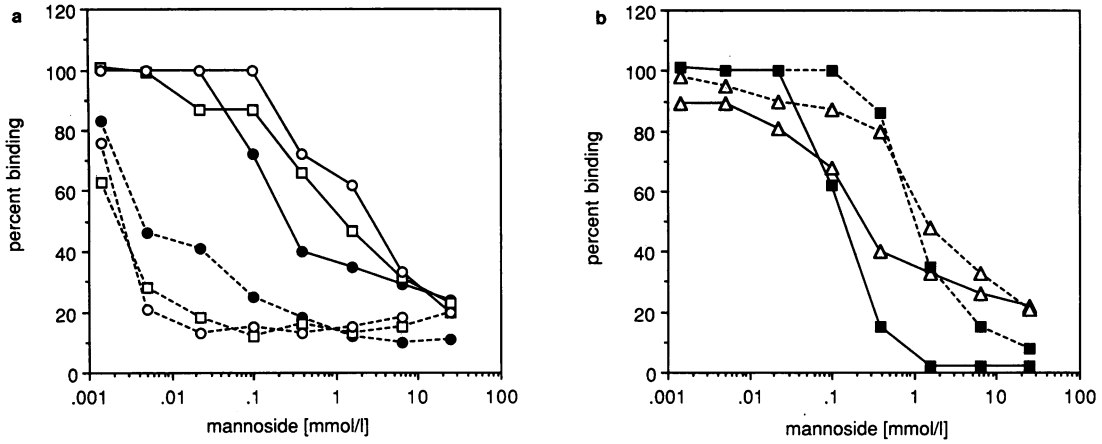

FIG. 5. (a) Inhibition of binding of *E. coli* 084 (○), 126 (●), and 128 (□) to NCA-55 by methyl-α-D-mannoside (——) and 4-methylumbelliferyl-α-D-mannoside (– – –) (125 ng of NCA added per well). (b) Inhibition of binding of *Salmonella virchow* (△) and *Salmonella bovis* (■) to NCA-55 by methyl-α-D-mannoside (——) and 4-methylumbelliferyl-α-D-mannoside (– – –) (125 ng of NCA added per well).

tenna. Such structures have been described in ovalbumin (35) and CEA (40). Additional oligomannoside structures with moderate binding activities for type 1-fimbriated *E. coli* have been described previously (21). Obviously, the carbohydrate structure of CEA, BGP, and NCA meets the requirements for high-affinity binding of *E. coli* better than the carbohydrates of ovalbumin. The binding pattern of *Salmonella typhi* is similar to that of *E. coli*. It is interesting that the meconium antigen of M_r 165,000 (NCA2) binds *Salmonella typhi* to a lesser extent than CEA, NCA, and BGP. This finding is consistent with the absence of high-mannose-type side chains in NCA2 (39). In meconium of newborns, CEA is absent or a minor component (22). In colon washings of adults, high amounts of CEA have been found (10). This could indicate that the high-mannose-type carbohydrate appears after birth at a time when the large bowel is colonized by bacteria.

It is well established that aromatic derivatives of mannose are powerful inhibitors of the adherence of type 1-fimbriated *E. coli* to intestinal epithelial cells (11, 31). This finding has been attributed to a hydrophobic binding region adjacent to the binding site of type 1 fimbriae which may interact with a hydrophobic region of the epithelial receptor. In the present study, α-methyl-mannoside and aromatic α-mannosides were used to inhibit the binding of *E. coli* and different *Salmonella* strains to CEA and NCA. The relative inhibitory activity of the different mannosides was well comparable with the data obtained with guinea pig ileal epithelial cells (11) (Table 2). In contrast to *E. coli*, aromatic mannosides are less efficient inhibitors than α-methyl-mannoside of the binding of mannose-specific *Salmonella typhimurium* to intestinal epithelial cells (11). Similarly, aromatic mannosides are less efficient than α-methyl-mannoside in inhibiting the binding of different *Salmonella* strains to CEA (Fig. 4) and NCA (Fig. 5b).

Our previous data (20) and the data presented here strongly suggest that CEA, NCA, and probably BGP bind to type 1 fimbriae of *E. coli*. This suggestion is further corroborated by the findings that binding was sensitive to UV irradiation (34) and that purified type 1 fimbriae inhibited the binding of *E. coli* to CEA by nearly 90%. Furthermore, bands the M_rs of which correspond to monomers, dimers, trimers, and tetramers of *E. coli* type 1 fimbriae (30) were shown by Western immunoblots using an antiserum induced against purified type 1 fimbriae.

Our findings indicate that CEA, NCA, and BGP bind *E. coli* and salmonellae with high avidity and, in comparison with unrelated glycoproteins, relatively high specificity. With respect to the biological meaning of these findings, the occurrence of the glycoproteins in different tissues has to be considered. CEA, which is bound to the plasma membrane via a phosphatidylinositol anchor (15), is localized at the apical border of epithelial cells lining the large intestine. Recent data on CEA gene expression and synthesis by normal colonic epithelium in organ culture indicate that the expression of the CEA gene is comparable to that of colonic cancer cells and that CEA is secreted into the culture medium (19). In the membranes of normal granulocytes, products of two different NCA genes and of the BGP gene are present (2, 3, 5, 9). Since both colonic epithelial cells and granulocytes bind type 1 fimbriated bacteria via D-mannosyl residues (28, 31, 38), products of the CEA gene family are probably recognition molecules for type 1-fimbriated bacteria.

One would predict a relatively low evolutionary conservation of receptors involved in bacterial binding in the context of colonization and host defense mechanisms. This is indeed the case for members of the CEA gene family (18, 29). Since members of the CEA gene family belong to the immunoglobulin supergene family (26), the products of these genes may represent a group of immunoglobulinlike molecules involved in the regulation of colonization and/or in the defense against type 1-fimbriated bacteria.

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