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Binding of Nonspecific Cross-Reacting Antigen, a Granulocyte Membrane Glycoprotein, to *Escherichia coli* Expressing Type 1 Fimbriae

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Nonspecific cross-reacting antigen (NCA) is a well-characterized membrane glycoprotein on granulocytes, macrophages, and lung epithelium. Structural studies at the protein and genomic levels have revealed that NCA is a member of the immunoglobulin supergene family, and hybridization studies showed that the transcript level of NCA is induced by treatment with gamma interferon. These studies, as well as the expression of NCA on granulocytes, suggest a role for NCA in immune response. For a first step in studying this possible role, we have examined the binding of two glycoforms of NCA designated NCA-50 (M_r, 50,000) and TEX-75 (M_r, 75,000). Here we report the results from binding assays which demonstrate carbohydrate-mediated binding of *Escherichia coli* expressing type 1 fimbriae and of isolated type 1 fimbriae to NCA-50. TEX-75 did not bind to the purified fimbriae but bound slightly to the bacterial strain. Inhibition studies showed that the binding to NCA-50 involved interaction of mannose moieties on NCA-50 and lectins on the fimbriae. The binding of NCA-50 to bacterial fimbriae was confirmed by electron microscopy studies, using immunolabeling techniques. In addition, we show that the surface expression of NCA-50 (and presumably of other NCA species) on isolated polymorphonuclear leukocytes is increased following activation with the bacterial peptide formylmethionyl-leucyl-phenylalanine, consistent with a role for NCA in immune response.

Nonspecific cross-reacting antigen (NCA) is a highly glycosylated membrane protein found on granulocytes, macrophages, and lung epithelium and in colonic adenocarcinoma. The first NCA form (50 kDa) (NCA-50) was identified by von Kleist et al. (49) and Mach and Pusztaszeri (31) in 1972 and named for its immunological cross-reactivity with carcinoembryonic antigen (CEA), a well-characterized tumor-associated antigen (43). Since then, several other NCA forms with apparent molecular masses of 50, 75 (designated TEX-75, for tumor-extracted antigen), 90, 95, and 160 kDa (3, 9, 27, 31, 49) have been identified. Protein and nucleotide sequencing have resolved much of the confusion regarding the structures and relatedness of these antigens (2, 18, 21, 32, 35, 38, 46, 48). NCA-50 and TEX-75 were found to have identical protein sequences and numbers of glycosylation sites but to be distinct glycoproteins differing significantly in carbohydrate content (21). These same studies revealed the likelihood that TEX-75 (isolated in this laboratory) (27) is equivalent to the NCA-90 identified by Audette et al. (3), at least with regard to the protein sequence. NCA-95 was found by others to be a unique antigen and to share >85% sequence homology with NCA-50 and TEX-75 (9). Tissue distribution studies have revealed that NCA-95 is found on granulocytes only, while NCA-50 and TEX-75 are found on granulocytic and epithelial cells (9). Although not confirmed, it is generally believed that the 160-kDa form of NCA is biliary glycoprotein, a structurally related (4, 22, 23), but distinct, glycoprotein. To date, only two genes are known for NCA, one encoding NCA-95 (2) and the other encoding NCA-50 or TEX-75 (32, 46), indicating that much of the size diversity observed for the NCA family members is due to differences in the glycosylation patterns of the respective antigens.

Although much is known concerning the structure and tissue distribution of these antigens, only recently have researchers reported possible biological roles for NCA and CEA. Oikawa et al. (34) proposed that NCA functions as a homotypic adhesion molecule, a function similar to that suggested for CEA (6). Oikawa's conclusions were based on the observation of a slight increase (~10%) in cell-cell aggregation of Chinese hamster ovary cells following transfection of NCA cDNA. The relevance of this finding as it relates to NCA antigens on granulocytes, however, remains to be determined. In other studies designed to examine alternative roles, Leusch and coworkers (30) recently described the bacterial binding properties of CEA and provided evidence that NCA had similar properties.

The role of cell surface glycoproteins in mediating bacterial and viral binding is well recognized (40, 51). Many bacteria express long proteinaceous appendages, called fimbriae or pili, that contain lectins which enable the microorganism to adhere to surface glycoproteins in a carbohydrate-specific manner. Oligosaccharide sequences identified as receptors for fimbriae include Manα1-3Man for type 1 fimbriae, NeuAcα2-3Gal for S fimbriae, and Galα1-4Gal for P fimbriae (5, 41). The most common and thoroughly characterized of these structures are mannose-specific type 1 fimbriae that are found in about 70% of *Escherichia coli* strains (37). Numerous studies have demonstrated the involvement of type 1 fimbriae in bacterial binding to epithelial and phagocytic cells, and there is a positive correlation between the degree of expression of type 1 fimbriae and the susceptibility to phagocytosis by granulocytes in serum-free medium (33, 42). On the basis of the above information and the finding that NCA-50 contains a high amount of high-

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mannose sugars (see Results), we chose the mannose-specific type 1 fimbriae for our studies as a possible ligand for NCA-50. Here we extend the initial observations of Leusch et al. (30) and provide direct evidence that NCA-50 binds specifically to type 1 fimbriae. We further examine the type 1 fimbriae binding properties of TEX-75, the expression of NCA on granulocytes, and the carbohydrate compositions of NCA-50 and TEX-75.

MATERIALS AND METHODS

Bacteria. *E. coli* 38 is a clinical isolate from a patient with a urinary tract infection and was obtained from the Department of Infectious Diseases, City of Hope Medical Center. *E. coli* HB101, which does not express fimbriae (Fim$^-$) (8, 28), was purchased from the American Type Culture Collection. Both strains were grown at 37°C under static conditions for 48 h in Luria-Bertani (LB) broth and serially diluted three times before use. These conditions favor the growth of cells with the Fim$^+$ phenotype over cells with the Fim$^-$ phenotype (36). Bacteria were tested in a yeast agglutination assay for their ability to mediate mannose-specific binding. Bacteria were considered mannose sensitive when visible agglutination could be prevented by the addition of 20 mM α-methylmannoside (MMan) but not with α-methylgalactoside (MGal) or α-methylglucoside (MGlu). Confirmation that *E. coli* 38 expressed type 1 fimbriae was accomplished by using anti-type 1 polyclonal antibodies which were generously provided by S. N. Abraham, Washington University, St. Louis, Mo. (data not shown).

Isolation of type 1 fimbriae. Type 1 fimbriae isolated from *E. coli* CSH50 (39) were a generous gift from M. B. Goetz, Department of Infectious Diseases, VA Medical Center, University of California, Los Angeles. The purification procedure followed the method of Eshdat et al. (12), and the purity of the fimbriae was examined by electron microscopy, amino acid analysis, and immunoreactivity with anti-type 1 polyclonal antibodies.

Isolation of NCA-50 and TEX-75. Both glycoproteins were isolated from a liver metastasis of a colon adenocarcinoma as previously described (21). The purity of the glycoproteins was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis with the anti-NCA-reactive monoclonal antibody T84.1E3 (50). This antibody was raised against CEA and cross-reacts with other CEA family members, including NCA-50 and TEX-75. Concentrations of glycoproteins and type 1 fimbriae were determined by amino acid analysis, and the mole amounts given refer to the protein moiety only.

Carbohydrate analysis and endo H digestion of NCA-50 and TEX-75. Approximately 50 pmol of NCA-50 and 120 pmol of TEX-75 were used for carbohydrate analysis performed by established procedures (20). For determination of neutral sugars, the protein samples were placed in glass hydrolysis tubes, lyophilized to dryness, and resuspended in 15 μl of 2 M aqueous trifluoroacetic acid. The tubes were sparged of oxygen with helium and sealed, and the samples were hydrolyzed for 4 h at 100°C. A similar technique was used for the detection of amino sugars and sialic acid, except that the samples were hydrolyzed with 6 M HCl for 4 h at 100°C or with 0.1 M trifluoroacetic acid for 8 h at 50°C, respectively. Following hydrolysis, the samples were dried, resuspended in deionized water, and analyzed with a Bio-LC system (Dionex Corp.) equipped with an amperometric detector. For digestion with endoglycosidase H (endo H) (Boehringer Mannheim), the samples (~150 pmol each) were suspended in 10 μl of 0.12% SDS (wt/vol), boiled for 3 min, diluted with 15 μl of 50 mM NaH_2PO_4 buffer at pH 6.0, and digested with 5 μl of endo H (2 U/ml) for 48 h at 37°C. After digestion, the samples were loaded onto a 10% SDS-polyacrylamide gel, electrophoresed, and blotted onto nitrocellulose with a PolyBlot electrotransfer system (American Bionetics). The blots were sequentially incubated with the anti-NCA-reactive monoclonal antibody T84.1E3 (mouse ascitic fluid diluted 1:500 in phosphate-buffered saline [PBS]) and horseradish peroxidase-labeled goat anti-mouse antibody (1:500 dilution), and the specific protein bands were visualized by addition of the chromogenic substrate 4-chloro-1-naphthol.

Bacterial agglutination assay. *E. coli* 38 was washed in saline (0.15 M NaCl) and 5×10^8 bacteria were incubated with 2 μM NCA-50 in 20 μl of saline for 20 min at room temperature. A 10-μl aliquot was heat fixed on a glass slide, stained with crystal violet (1%) for 1 min, washed with deionized water, and examined under a light microscope. The glycoproteins TEX-75, ovalbumin, thyroglobulin, ribonuclease B, and fetuin and Fim$^-$ *E. coli* HB101 cells served as controls.

Biotinylation of bacteria and type 1 fimbriae. Bacteria were washed twice in PBS and 50 mM bicarbonate buffer (pH 8.5), respectively. The cells were resuspended in the bicarbonate buffer containing 10 μg of NHS-LC-biotin (Pierce) per ml to a density of 5×10^9/ml and incubated at 37°C for 100 min. Before use in the binding and inhibition assays, the freshly biotinylated bacteria were washed extensively with saline. Isolated type 1 fimbriae (5 mg/ml) were incubated in bicarbonate buffer with NHS-LC-biotin (200 μg/ml) at 4°C for 2 h, washed with saline by using a Centricon microconcentrator, and stored at −40°C until use.

Binding assay. Microtiter plates (Dynatech) were individually coated with a dilution row of each glycoprotein ranging from 2 to 500 nmol/liter. NCA-50, ribonuclease B, fibronectin, TEX-75, ovalbumin, or fetuin were resuspended in 70 μl of coating buffer (35 mM $NaHCO_3$, 15 mM Na_2CO_3, 0.02% NaN_3 [wt/vol], pH 9.6) and wells were coated by incubation at 37°C for 2 h. Nonspecific binding sites were blocked with 200 μl of 1% bovine serum albumin (BSA) (wt/vol) in saline at 37°C for 1 h. Biotinylated bacteria (5×10^9/ml) or biotinylated fimbriae (20 μg/ml) were resuspended in saline and incubated at 37°C for 1 h in a volume of 50 μl per well. The plate was incubated with 100 μl per well of streptavidin-alkaline phosphatase conjugate diluted 1:4,000 in TBS at 37°C for 1 h, and enzyme activity was visualized after the addition of 100 μl of *p*-nitrophenylphosphate (1 mg/ml containing 10% [vol/vol] ethanolamine, pH 9.8) per well. Color development was recorded after 1 h at 37°C by monitoring the A_{405} values on a Titertek Multiscan ELISA reader. Between each incubation step, the plate was washed three times with 0.05% Tween 20 in PBS (vol/vol). Various negative controls, prepared by coating the wells with coating buffer only, adding unbiotinylated bacteria, not using bacteria, or using *E. coli* HB101 instead of *E. coli* 38, were used.

Inhibition assay. Inhibition studies were carried out as described above for the binding assay using different sugars (MMan, MGlu, and MGal) on NCA-50-coated microtiter plates (4 μg/ml). The biotinylated bacteria or type 1 fimbriae were preincubated with various sugar concentrations (0.005 to 100 mM) for 30 min at room temperature before being added to the wells. Controls were prepared by incubating *E. coli* 38 in the absence of sugars, using *E. coli* HB101, and using wells coated with coating buffer only.

Electron microscopy. Samples of bacteria and type 1 fimbriae were applied to Formvar-coated and glow-dis-

charged copper grids, negatively stained with 1% phosphotungstic acid for 30 s and examined with a Philips transmission electron microscope model CM 10 operating at 80 kV. Binding of NCA-50 to *E. coli* 38 was carried out in a volume of 20 μl (0.5 mg of NCA-50 per ml) for 4 h. Unbound NCA-50 was removed by centrifugation, and the bacteria were washed with saline, applied to a grid, and stained as described above. Incubation with ovalbumin or incubation of *E. coli* HB101 (Fim$^-$) with NCA-50 or ovalbumin served as negative controls. For immunolabeling, the procedure described above was modified to allow the incubation of NCA-50 directly on grids coated with bacteria. For these studies, 10 μl of a bacterial suspension (10^9/ml) was adsorbed to a grid for 2 min at room temperature, the excess liquid was removed, and the grid was placed face down on a drop of 3% BSA in saline (wt/vol) for 30 min to reduce nonspecific binding. All of the following incubation steps were performed in saline with 3% BSA. The BSA-blocked grids were placed on a drop of NCA-50 (0.5 mg/ml) for 4 h at room temperature. After three washes with saline, the grids were incubated with T84.1E3 (1 μg/ml) for 30 min, washed again, incubated with a 1:20 dilution of goat anti-mouse antibody conjugated with 5-nm-diameter gold particles (Janssen Life Sciences Products) for 30 min, washed, and stained as described above. Controls were performed under the conditions as described above but without the incubation with either T84.1E3 or NCA-50.

Fluorescence-activated cell sorting (FACS) analysis of fMLP-activated PMNs. Polymorphonuclear leukocytes (PMNs) were isolated from whole blood by centrifugation through Percoll (19) and washed repeatedly with PBS. The purified PMNs were resuspended in PBS with 3% BSA (wt/vol) to a concentration of 2×10^6 cells per ml and stimulated with formylmethionyl-leucyl-phenylalanine (fMLP) at a concentration of 10^{-5} M. Following incubation periods of 0, 5, 10, 20, 40, 60 and 80 min, the stimulated cells were washed twice in PBS and resuspended in 50 μl of labeling buffer (PBS containing 3% BSA [wt/vol], 0.03% NaN_3 [wt/vol], 5 μg of cytochalasin B [wt/vol]) at 4°C. The PMNs were then incubated with $F(ab')_2$ fragments of the NCA-reactive monoclonal antibody T84.1E3 (2 μg/50 μl) for 1 h at 4°C. After being washed, the cells were resuspended in 100 μl of the labeling buffer and again incubated for 1 h at 4°C with a goat anti-mouse fluorescein isothiocyanate (FITC)-labeled $F(ab')_2$ fragment (Jackson Immunoresearch) diluted 1:100 with labeling buffer. The labeled cells were then washed several times with PBS and analyzed with a Becton Dickinson FACS IV flow cytometer. Negative controls included elimination of the primary T84.1E3, $F(ab')_2$ fragment or substitution of the secondary antibody with a FITC-labeled mouse anti-human $F(ab')_2$ fragment (Jackson Immunoresearch).

RESULTS

Carbohydrate differences in NCA glycoforms. Two purified glycoforms of NCA, NCA-50 (50 kDa) and TEX-75 (75 kDa), were digested with endo H in order to compare their respective complement of high-mannose oligosaccharides. This enzyme treatment was chosen by considering the carbohydrate specificity of type 1 fimbriae. Treatment with endo H decreased the apparent molecular weight of NCA-50 to approximately 40,000 but had little effect on the molecular weight of TEX-75 (Fig. 1). Taking into account that the molecular masses of fully deglycosylated NCA-50 and TEX-75 are both 35 kDa (21), the decrease in molecular size

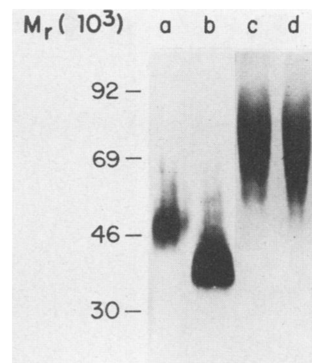

FIG. 1. Western blot analysis of NCA-50 (lanes a and b) and TEX-75 (lanes c and d) before (lanes a and c) and after (lanes b and d) treatment with endo H. The samples were electrophoresed (10% acrylamide gel), blotted onto a nitrocellulose filter, and detected as described in the text. The M_r values of the standard proteins used (Amersham rainbow markers) are indicated to the left of the gel.

after endo H treatment suggests that >50% of the glycosylated side chains of NCA-50 are high in mannose, whereas TEX-75 contains none or few high-mannose side chains. The carbohydrate compositions of NCA-50 and TEX-75 are presented in Table 1. NCA-50 contains considerably more mannose (a saccharide found in all N-linked oligosaccharides) and less galactose (indicative of complex oligosaccharides) than TEX-75, results consistent with the presence of more high-mannose structures in NCA-50. The values for NCA-50 are in close agreement with those values obtained for granulocyte NCA-50 isolated from normal spleens (11).

Bacterial binding to NCA-50 and TEX-75 in solution. NCA-50 exhibited strong binding properties to a strain of *E. coli* (designated strain 38) isolated from a patient with a urinary tract infection. This strain was found to express type 1 fimbriae by a yeast agglutination assay and by detection with anti-type 1 polyclonal antibodies (data not shown). Incubation of *E. coli* 38 with 2 μM NCA-50 resulted in visible agglutination of the bacterial suspension, suggesting the presence of multiple binding sites on NCA-50 (Fig. 2A). In comparison, incubation of *E. coli* 38 with TEX-75 failed to result in any detectable agglutination (Fig. 2B). For controls, we also incubated *E. coli* 38 with other glycoproteins such as ovalbumin, thyroglobulin, or ribonuclease B that contain high-mannose oligosaccharide chains, and fetuin which has complex chains. None of these controls agglutinated this

TABLE 1. Carbohydrate composition of NCA-50 and TEX-75

Sugar	Composition[a] (mol%) (range)	
	NCA	TEX
Sialic acid	3.1 (2.8–3.5)	2.5 (0.7–4.3)
Fucose	11.3 (10.6–11.4)	11.9 (11.5–12.2)
Mannose	46.0 (45.3–46.8)	23.5 (23.5–23.6)
Galactose	14.0 (14.0–14.2)	21.7 (20.7–22.6)
N-Acetylglucosamine	34.7 (33.7–35.7)	38.5 (37.8–39.1)
N-Acetylgalactosamine	1.0 (0.9–1.0)	1.8 (1.6–2.0)

[a] Mean value for four determinations. Hydrolysis and analysis conditions are provided in the text. For comparison, the mole percentage values previously reported (11) for granulocyte NCA from normal spleens are as follows: 2.5 to 5.8 for sialic acid, 8.3 to 12.2 for fucose, 37.8 to 50.6 for mannose, 12.1 to 12.8 for galactose, 22.1 to 31.4 for *N*-acetylglucosamine, and 0 for *N*-acetylgalactosamine.

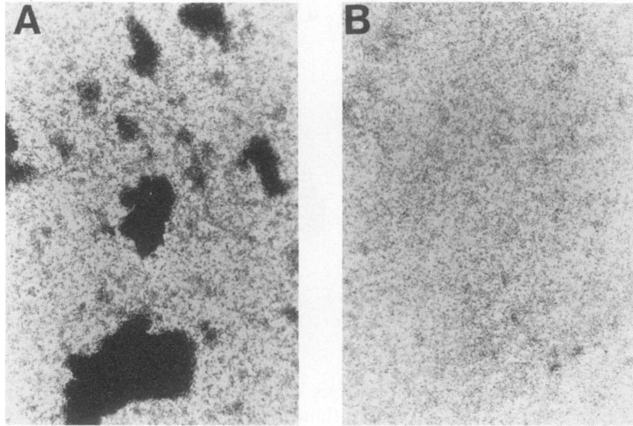

FIG. 2. Photomicrographs of stained and heat-fixed *E. coli* 38 after incubation with glycoproteins for 20 min at room temperature in saline and staining with crystal violet. Bacteria incubated with 2 μM NCA-50 (A) or with 2 μM TEX-75 (B) (incubation with 2 μM fetuin, thyroglobulin, ribonuclease B, or ovalbumin gave identical results). Magnification, ca. ×180.

strain of *E. coli*. In addition, we did not detect any agglutination of NCA-50, TEX-75, or the controls with Fim^- *E. coli* HB101 cells.

Specific binding of purified type 1 fimbriae and *E. coli* 38 to immobilized NCA-50 and TEX-75. To demonstrate specific binding of the fimbriae on *E. coli* 38 to NCA-50, a solid-phase binding assay was developed. In this assay, *E. coli* 38 and isolated type 1 fimbriae were biotinylated and added to microtiter plates coated individually with NCA-50, TEX-75, fibronectin, or glycoprotein controls containing high-mannose oligosaccharide chains (ovalbumin and ribonuclease B) or complex chains (fetuin).

Specific binding of *E. coli* 38 or purified type 1 fimbriae to immobilized NCA-50 is shown in Fig. 3. The results demonstrate a dose-dependent and saturable binding. *E. coli* 38 bound most strongly to isolated NCA-50, bound to a much lesser extent to TEX-75 and ovalbumin, and did not bind to fetuin (Fig. 3A). In contrast to *E. coli* 38, the isolated type 1 fimbriae bound specifically to NCA-50 and did not bind to TEX-75, fetuin, or ovalbumin (Fig. 3B). The interaction between type 1 fimbriae and deglycosylated NCA-50, ribonuclease B, and fibronectin was examined as well, but no binding could be detected (data not shown). Negative controls were performed to rule out nonspecific binding. These controls were prepared by adding biotinylated HB101 or unbiotinylated *E. coli* 38 to the glycoprotein-coated plates or by eliminating the bacteria or the glycoprotein-coating step. None of these controls showed detectable color development above the background level (0.15 absorbance units).

Inhibition of binding to type 1 fimbriae. In order to determine the specificity of the interaction between the carbohydrate moieties on NCA-50 and the bacterial fimbriae, inhibition studies were performed in the presence of three different α-methyl-D-pyranosides using the solid-phase assay described above. The presence of 1 mM MMan inhibited binding of *E. coli* 38 to the NCA-50-coated plates by 50%, and nearly complete inhibition was obtained with ~20 mM MMan (Fig. 4A). The other saccharides tested either failed to inhibit binding or slightly inhibited binding at much higher sugar concentrations (Fig. 4A). Adhesion of *E. coli* 38 to NCA-50 is at least 2 orders of magnitude more sensitive to

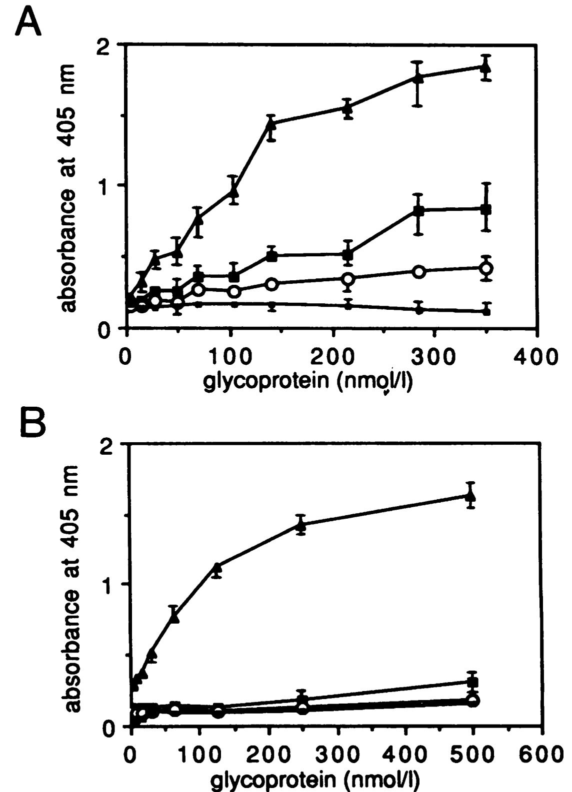

FIG. 3. Binding of biotinylated *E. coli* 38 (A) and biotinylated type 1 fimbriae (B) to various glycoproteins. (A) Biotinylated bacteria (2.5×10^8 in 50 μl of saline) were allowed to bind to microtiter wells, after the wells were coated with glycoproteins (3 to 350 nmol/liter) and nonspecific binding sites were blocked with 3% BSA in saline. (B) Biotinylated fimbriae (1 μg in 50 μl of saline) were added to wells that were coated with glycoproteins (2 to 500 nmol/liter) and blocked. After incubation of 1 h at 37°C, the wells were washed with PBS-Tween 20, and bound bacteria or fimbriae were detected with streptavidin-alkaline phosphatase conjugate. Binding activities for NCA-50 (▲), TEX-75 (■), ovalbumin (○), and fetuin (■) are shown. The data were collected in duplicate experiments with three replicates each. Each datum point represents the mean of the six determinations. Each bar represents the range of the absorbance readings for the six determinations.

MMan than to MGlu. The slight degree of binding of *E. coli* 38 to TEX-75 observed in the previous assays was not inhibited with any of the three sugars, suggesting that the binding was mediated by fimbriae with different sugar specificities or by other membrane components (data not shown).

In contrast to *E. coli* 38, the binding of purified type 1 fimbriae to NCA-50 was completely inhibited with 1 mM MMan, and 50% inhibition occurred with 0.08 mM MMan (Fig. 4B). Similar to the inhibition study with *E. coli* 38, inhibition by MGlu or MGal was detected only with much higher concentrations (>50 mM). The results of the inhibition assays show that the carbohydrate moiety of NCA-50 involved in binding to the bacterial fimbriae contains mannose and can be completely inhibited through competition with MMan.

Electron microscopic examination of NCA-50 binding to type 1 fimbriae. Electron microscopy studies were carried out to examine the morphology of the fimbriae on *E. coli* 38, to verify the purity of the isolated type 1 fimbriae, and to

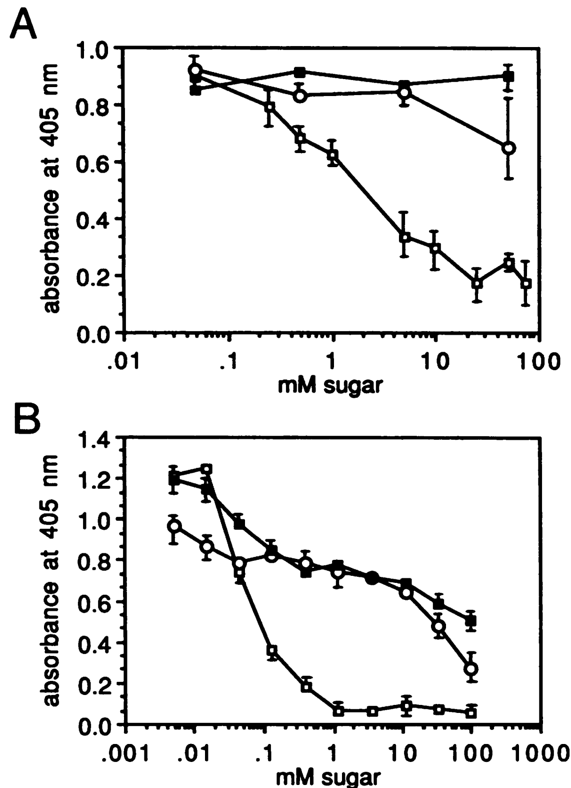

FIG. 4. Inhibition of binding of biotinylated *E. coli* 38 (A) and biotinylated type 1 fimbriae (B) to NCA-50 with MMan. (A) Biotinylated bacteria (2.5×10^8) were preincubated with various α-methylpyranosides (0.05 to 75 mM) in 50 μl of saline for 30 min at room temperature before adding to NCA-coated plates (4 μg of NCA-50 per ml). (B) Biotinylated fimbriae (1 μg) were preincubated in a similar fashion with different saccharide inhibitors (0.005 to 100 mM) and assayed for binding as described in the legend to Fig. 3. After incubation for 1 h at 37°C, the wells were washed with PBS-Tween 20, and bound bacteria or fimbriae were detected as described in Materials and Methods. Binding activities for MMan (□), MGlu (○), and MGal (■) are shown. The data were collected in duplicate experiments with three replicates each. Each datum point represents the mean of the six determinations. Each bar represents the range of the absorbance readings for the six determinations.

localize the binding of NCA-50 to the fimbriae. The fimbriae showed the characteristic diameter of 7 nm for type 1 fimbriae monomers and some fimbriae aggregates (Fig. 5A and B). Incubation of *E. coli* 38 with NCA-50 caused extensive agglutination of the bacterial fimbriae, consistent with the presence of multiple fimbriae binding sites (Fig. 5C). Immunolabeling experiments with anti-NCA-reactive monoclonal antibody T84.1E3 and a secondary, gold-labeled goat anti-mouse antibody demonstrated the localization of NCA-50 on the fimbriae (Fig. 5D). The gold label is especially concentrated in the agglutinated areas. In contrast, the negative controls (no prior incubation with NCA-50 or with T84.1E3) showed only a slight amount of nonspecific labeling (Fig. 5E).

Effects of fMLP stimulation on the expression of NCA. Others have shown that stimulation of human phagocytic cells enhances the fimbriae-mediated binding of bacteria, presumably by increasing the membrane expression of glycoprotein receptors (10, 26), and increases in expression of the receptors for complement (52). On the basis of the results of these studies, we evaluated the changes in membrane surface levels of NCA on human neutrophils by FACS following stimulation with fMLP. The levels of NCA expression on the cell surface after 10 and 40 min of stimulation are shown in Fig. 6 in comparison to unstimulated neutrophils. The level of expression of NCA increased progressively following stimulation with the maximal point (two- to threefold increase) at 40 min (Fig. 6). This increase in surface expression was detected almost immediately following activation, suggesting transportation of presynthesized NCA forms from intracellular granules to the cell surface. At longer times (60 and 80 min), the levels of NCA decreased to values between those observed for stimulations of 10 and 40 min (data not shown). The PMNs were stained with trypan blue and examined under the microscope after stimulation in order to assure that the decrease of surface expression after longer exposures to fMLP was not caused by a higher percentage of dead cells (data not shown).

DISCUSSION

In this study, we demonstrate the specific binding of *E. coli* expressing type 1 fimbriae to NCA-50, a well-characterized glycoprotein found on epithelial cells and granulocytes. The binding was demonstrated in a solid-phase assay using a clinical isolate of *E. coli* (designated strain 38) shown to express type 1 fimbriae and with fimbriae isolated from *E. coli* CSH50, a genetically characterized strain known to produce type 1 fimbriae only (Fig. 3). Here we show that purified type 1 fimbriae bound specifically to NCA-50 and not to TEX-75, ovalbumin, or fetuin (Fig. 3B). Likewise, no binding of type 1 fimbriae to ribonuclease B or fibronectin could be detected (data not shown). Electron microscopy studies demonstrated that NCA-50 bound along the lengths of the fimbriae (Fig. 5). These NCA-50 binding locations are consistent with the locations of the fimbriae mannose-specific lectin (FimH) (1, 29). In contrast to type 1 fimbriae that bound to NCA-50 only, *E. coli* 38 also bound to TEX-75 to some extent (Fig. 3). This slight degree of binding could be due to simultaneous expression of multiple types of fimbriae with different sugar specificities on the same bacteria or to nonspecific interactions with other bacterial outer membrane molecules, such as lipopolysaccharides and capsular antigens. Although we confirmed the presence of type 1 fimbriae by using a yeast agglutination assay and by using anti-type 1 polyclonal antibodies (data not shown), we cannot exclude the presence of other types of fimbriae, e.g., the morphologically indistinctive mannose-resistant P fimbriae. Further evidence that the slight degree of binding between *E. coli* 38 and TEX-75 is not mediated by type 1 fimbriae is the observation that bacterial binding to TEX-75 cannot be inhibited by MMan (data not shown). Additional studies are planned to examine the bacterial binding specificities of TEX-75.

Binding of the isolated type 1 fimbriae and *E. coli* 38 to NCA-50 was inhibited specifically with MMan in a dose-dependent manner (Fig. 4). This carbohydrate specificity is characteristic of type 1 fimbriae (41). The decrease in binding of type 1 fimbriae to NCA-50 with MGal or MGlu at molarities of >50 mM (Fig. 4) is in accordance with the results of Silverblatt et al. (44), who also observed a slight inhibition in binding of type 1 fimbriated *E. coli* to PMNs, using high sugar concentrations. It is likely that the observed inhibition of type 1 fimbriae-mediated binding at these higher sugar concentrations is due to nonspecific steric hindrance,

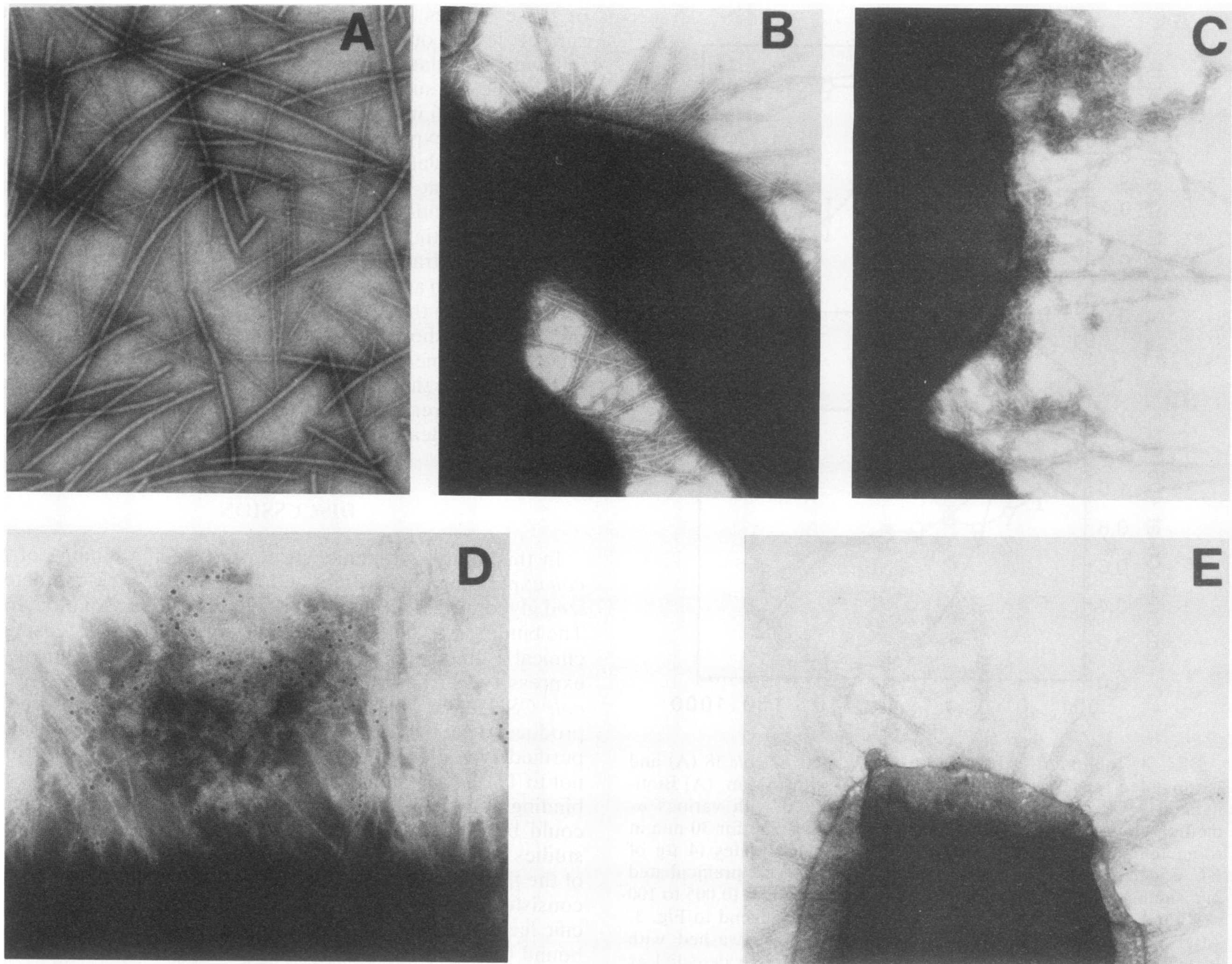

FIG. 5. Negatively stained electron micrographs of *E. coli* 38 and purified type 1 fimbriae. (A) Purified type 1 fimbriae. Magnification, ×52,000. (B) Untreated *E. coli* 38. Magnification, ×50,000. (C) *E. coli* 38 incubated with NCA-50 as described in the text. Note the cross-linking of fimbriae. Magnification, ×50,000. (D) *E. coli* 38 incubated with NCA-50 and bound NCA-50 detected with T84.1E3 and goat anti-mouse antibody conjugated with 5-nm-diameter gold particles. Magnification, ×100,000. (E) *E. coli* 38 incubated with primary and secondary antibodies, but without prior incubation with NCA-50. Magnification, ×73,000.

since the sugar specificity of type 1 fimbriae is well established (41).

Although the presence of mannose is necessary for mediating binding to type 1 fimbriae (41), the failure of ovalbumin and ribonuclease B (glycoproteins that contain high-mannose saccharides) to bind (Fig. 3) suggests that mannose alone is not sufficient. These results suggest a specific receptor/ligand-like interaction between type 1 fimbriae and the carbohydrate moieties on NCA-50. The precise structural elements recognized by the type 1 fimbriae lectin have not been elucidated; however, the current model predicts that the lectin recognizes a trisaccharide with the structure of α-D-Man(1-3)-β-D-Man(1-4)-D-GlcNAc and contains a hydrophobic region in close proximity to the saccharide binding site (41). It is thus possible that the lectin recognizes both a carbohydrate and protein sequence, presumably an oligomannose and a hydrophobic amino acid. Evaluation of the structural requirements for mediating binding to type 1 fimbriae will be examined in other studies.

The studies reported required large sample quantities which could be obtained efficiently only from a colonic carcinoma. NCA-50 from malignant tissue has been shown in numerous studies to be identical to that form found on normal lung epithelium and granulocytes (3, 9, 11, 17). In addition, our carbohydrate analyses (Table 1, Fig. 1) of tumor NCA-50 revealed the presence of a high percentage of high-mannose oligosaccharides, results consistent with the analyses performed on granulocyte NCA-50 isolated from normal spleens (11). These data indicate that NCA-50 isolated from malignant cells is identical to the NCA-50 from normal lung epithelium and phagocytic cells, leading to the conclusion that their bacterial binding properties would also be identical. With regard to TEX-75, immunological and molecular mass data suggest that the tumor form of TEX-75 is equivalent to the 75-kDa NCA form isolated from normal lung (17). In consideration of the fact that only two genes have been found for NCA, it is also likely that TEX-75 (isolated in this laboratory) (27) and the 90,000-molecular-

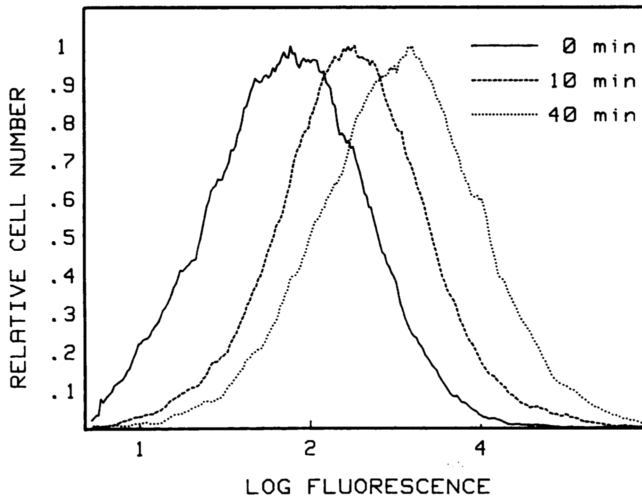

FIG. 6. FACS analysis of unstimulated PMN (——) and after stimulation with fMLP (10^{-5} M) for 10 and 40 min in 3% BSA in PBS. After activation, the PMN cells were suspended in PBS containing 3% BSA, 0.03% NaN_3, and 5 μg of cytochalasin B per ml and labeled with the $F(ab')_2$ fragments of the NCA-cross-reacting T84.1E3 antibody for 1 h at 4°C. Following washing, the cells were suspended in the same buffer and incubated for 1 h at 4°C with an FITC-labeled goat anti-mouse antibody.

weight form of NCA identified by Audette et al. on granulocytes (3) are identical with regard to the protein sequence. This likelihood is further supported by our recent study (21) in which multiple TEX glycoforms with masses from 65 to 85 kDa were isolated and found to share a protein core common with NCA-50. It thus follows that heterogeneity of glycosylation produces multiple glycoforms of NCA on normal and malignant cells.

Our data demonstrated that NCA-50 binds bacteria expressing type 1 fimbriae. Additional studies are required to understand the biological importance of these findings as they relate to the expression of NCA on granulocytes and epithelial cells. We are led to the conclusion that NCA has a role in immune or cellular recognition because of its tissue distribution, its domain structure (which is similar to the variable and constant regions of immunoglobulins [47]), and its expression (2.8-kb message) being induced by gamma interferon (25). The data presented here demonstrating an increase in cell surface expression of NCA on granulocytes following treatment with fMLP (Fig. 6) is consistent with a role for NCA in immune response to bacterial infections. Such a possibility is supported by the work of others who have demonstrated increased fimbriae-mediated bacterial binding to phagocytic cells following activation, presumably due to enhanced expression of the relevant glycoprotein membrane receptors (10, 26). A similar activation-dependent regulation of receptors for C3b and C3bi has been reported (52). While monitoring the increase in NCA expression after stimulation with fMLP (Fig. 6), we observed a decrease at 60 and 80 min (data not shown), which may reflect the release of NCA from the membrane by cleavage of the GPI anchor complex. A similar activation-dependent release of FcRIII, another GPI-anchored membrane receptor on neutrophils, was observed by Huizinga et al. after stimulation with fMLP (24). We are continuing to examine the bacterial binding properties of the various NCA antigens in order to elucidate the physicochemical requirements for binding and as a first step in understanding the significance of such binding.

These studies should complement the work of others who are examining the biological relevance of fimbriae-mediated binding of bacteria to phagocytes or studying the fimbriae glycoprotein receptors (for a review, see reference 33). The binding of *E. coli* expressing type 1 fimbriae in the absence of serum factors to human leukocytes has been shown to increase phagocytosis (44), chemiluminescence (7, 15, 16), protein iodination (39), degranulation (45), and activation of protein kinase C (13). Studies with latex particles coated with isolated type 1 fimbriae demonstrated that the fimbriae per se mediated the activation of the granulocytes (16). In the presence of serum factors, the expression of type 1 fimbriae was also shown to significantly increase the O_2 consumption and H_2O_2 production by activated granulocytes (16). As an initial attempt at identifying the type 1 fimbriae receptors, Rodriguez-Ortega and coworkers (40) have purified four prospective glycoprotein receptors from granulocytes with molecular weights of 45,000, 70,000 to 80,000, 100,000, and 150,000. No protein sequence analysis is available on these glycoproteins, precluding comparisons with the NCA forms. Using similar techniques, Giampapa et al. (14) have isolated a 65-kDa type 1 fimbriae receptor from guinea pig erythrocytes. Limited structural analysis of this receptor also prevents any comparison with the NCA we have isolated from human sources. Our future studies will include examination of possible relationships of these glycoprotein receptors with NCA and the biological significance of bacterial binding to NCA.

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