Recognition of Lewis x Derivatives Present on Mucins by Flagellar Components of Pseudomonas aeruginosa

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Pseudomonas aeruginosa binds to human respiratory mucins by mechanisms involving flagellar component-receptor interactions. The adhesion of P. aeruginosa strain PAK is mediated by the flagellar cap protein, FliD, without the involvement of flagellin. Two distinct types of FliD proteins have been identified in P. aeruginosa: A type, found in strain PAK, and B type, found in strain PAO1. In the present work, studies performed with the P. aeruginosa B-type strain PAO1 indicate that both the FliD protein and the flagellin of this strain are involved in the binding to respiratory mucins. Using polyacrylamide-based fluorescent glycoconjugates in a flow cytometry assay, it was previously demonstrated that P. aeruginosa recognizes Lex (or Lewis x) derivatives found at the periphery of human respiratory mucins. The aim of the present work was therefore to determine whether these carbohydrate epitopes (or glycotopes) are receptors for FliD proteins and flagellin. The results obtained by both flow cytometry and a microplate adhesion assay indicate that the FliD protein of strain PAO1 is involved in the binding of glycoconjugates bearing Le^x or sialyl-Le^x determinants, while the binding of flagellin is restricted to the glycoconjugate bearing Le^x glycotope. In contrast, the type A cap protein of P. aeruginosa strain PAK is not involved in the binding to glycoconjugates bearing Le^x, sialyl-Le^x, or sulfosialyl-Le^x glycotopes. This study demonstrates a clear association between a specific Pseudomonas adhesin and a specific mucin glycotope and demonstrates that fine specificities exist in mucin recognition by P. aeruginosa.

Pseudomonas aeruginosa is the major pathogen in the airways of patients suffering from cystic fibrosis (CF) and is currently responsible for most of the morbidity and mortality seen from this disease. This organism has been localized to the mucus of the airways of CF patients during colonization (3); thus, binding to mucin is of great importance in the pathogenesis of chronic airway colonization. Supporting the clinical observations, adhesion of this organism to human salivary and airway mucins has been demonstrated in vitro using liquid- and solid-phase adhesion assays (5, 7, 17, 19, 27, 28). Human airway mucins are a very broad family of polysizeable high-molecular-weight glycoproteins that are part of innate airway defenses. They are highly glycosylated and contain from one single to several hundred carbohydrate chains which form a combination of carbohydrate determinants of considerable diversity in CF and normal airway mucins (14, 20). Therefore the molecular interactions of P. aeruginosa and mucins is potentially a multiligand-adhesin phenomenon involving different carbohydrate epitopes, or glycotopes, of the mucin molecules as well as different peripheral structures on the organism.

By use of P. aeruginosa mutants, peripheral bacterial components, mostly flagellar proteins, have been identified as playing an important role in the binding of this bacterium to respiratory mucins (1, 2, 25). A role for pil which interact with the carbohydrate sequence GalNAcβ1-4Galβ (24), which is present in glycosphingolipids, such as asialo-GM1 and asialo-GM2 (11, 12), but not in human respiratory mucins, has also been excluded by use of nonpiliated mutants (18). However, the nature of the mucin determinants that are specifically recognized by the flagellar components is unknown.

Different approaches have been used in order to identify the mucin carbohydrate determinants responsible for the adhesion of P. aeruginosa to mucins. They are all based on the study of glycolipids or neoglycoconjugates bearing a single type of glycotope. Glycolipids or neoglycolipids have been used in solid-phase adhesion assays (16, 20). More recently, the synthesis of water-soluble polyacrylamide-based fluorescent glycoconjugates (4) has allowed the use of flow cytometry to analyze the interactions of glycotopes with various strains of P. aeruginosa (21). Under these conditions, a number of neutral and acidic Lewis blood group derivatives analogous to glycotopes found at the periphery of airway mucins are recognized by whole cells of P. aeruginosa (20–22). Some of these glycotopes such as the sialyl-Lewis x determinants are overexpressed in the airway mucins of patients chronically colonized with bacteria, especially in the muccins of patients suffering from CF (6).

The present study was therefore designed to determine if the P. aeruginosa flagellar protein FliD, which is a mucin-specific adhesin, recognizes any of the specific Lewis x determinants that bind to whole P. aeruginosa cells. A mutant of the flagellin gene of strain PAO1 was also used as a control. Flow cytometry and solid-phase binding assays were used to analyze the interactions of various mutants of P. aeruginosa defective in the expression of these flagellar proteins with polyacrylamide-based fluorescent neoglycoconjugates bearing neutral, sialylated, and/or sulfated Lewis x glycotopes.
MATERIALS AND METHODS

Neoglycoconjugates. The neoglycoconjugates (Gly-PAA) used in this study were made commercially and were obtained from Syntosome (Munich, Germany). In order to synthesize the neoglycoconjugates (Table 1), oligosaccharides (Gly) are linked via a 3-carbon spacer arm [-\((\text{CH}_2)_3\)-] to a polyacrylamide type matrix (PAA) (4). In these compounds, approximately every fifth amide group of the polymer chains is substituted by the carbohydrate on the spacer arm. Their molecular weights are about 40,000, and the carbohydrate content is about 20% (4). Neoglycoconjugates labeled with a fluorescent probe (Gly-PAA-Flu) were used for flow cytometry analysis.

Bacterial strains and culture conditions. The bacterial strains used in the study are shown in Table 2. They were grown in tryptic soy broth (TSB medium; Difco, Detroit, Mich.) for 18 h at 37°C. The following antibiotics were used to maintain plasmids and chromosomal insertions in P. aeruginosa strains PAO1 and PAK: gentamicin at 100 \(\mu\)g/ml and carbenicillin at 300 \(\mu\)g/ml for complementation experiments. After centrifugation of the cultures at 4,000 \(\times\) g for 30 min, the cell pellet was washed twice with phosphate-buffered saline (PBS) containing 1% BSA but omitting neoglycoconjugates from the incubation mixtures. They corresponded to the average number of bacteria of each well. Wells were considered valid. All experiments were performed at least four times controls. Only the experiments with little or no background binding to uncoated neoglycoconjugates were desorbed by adding a 0.5% solution of Triton X-100 for 1 min, the cell pellet was washed twice with PBS. The bacteria adhering to mucins or to neoglycoconjugates was quantified using a microtiter plate assay (27, 28) and the adhesion of strain PAO1 were calculated to be 39 ± 7 nM for Le\(\alpha\)-PAA-Flu, 31 ± 3 nM for sialyl-Le\(\alpha\)-PAA-Flu, and 59 ± 7 nM for sialofucosyl-Le\(\alpha\)-PAA-Flu (Table 3). The \(K_d\) values obtained for the binding of the fluorescent glycoconjugates to strain PAO1 were calculated to be 70 ± 8 nM for Le\(\alpha\)-PAA-Flu, 61 ± 6 nM for sialyl-Le\(\alpha\)-PAA-Flu, and 72 ± 9 nM for sialofucosyl-Le\(\alpha\)-PAA-Flu to strains PAO1 and PAO1-D were significant (\(P < 0.005\)), while no differences were found between the binding of sulfosialyl-Le\(\alpha\)-PAA-Flu to the parental strain and to the mutated strain.

The \(K_d\) values obtained for the binding of Le\(\alpha\)-, sialyl-Le\(\alpha\)-, and sulfosialyl-Le\(\alpha\)-glycophosphates to strain PAO1 and its fliD mutant. (i) Flow cytometry analysis. The \(K_d\) values obtained for the binding of the fluorescent glycoconjugates to strain PAO1 were calculated to be 39 ± 7 nM for Le\(\alpha\)-PAA-Flu, 31 ± 3 nM for sialyl-Le\(\alpha\)-PAA-Flu, and 59 ± 7 nM for sialofucosyl-Le\(\alpha\)-PAA-Flu (Table 3). The \(K_d\) values obtained for the binding to the fliD mutant PAO1-D were calculated to be 70 ± 8 nM for Le\(\alpha\)-PAA-Flu, 61 ± 6 nM for sialyl-Le\(\alpha\)-PAA-Flu, and 72 ± 9 nM for sialofucosyl-Le\(\alpha\)-PAA-Flu (Table 3). The differences between the binding of Le\(\alpha\)-PAA-Flu and sialyl-Le\(\alpha\)-PAA-Flu to strains PAO1 and PAO1-D were significant (\(P < 0.005\)), while no differences were found between the binding of sulfosialyl-Le\(\alpha\)-PAA-Flu to the parental strain and to the mutated strain.

RESULTS

Adherence of a fliD mutant of P. aeruginosa strain PAO1 to human respiratory mucins. The adhesion of strain PAO1 and a fliD mutant of this strain was examined. Using the microtiter plate adhesion assay, the number of bacteria adhering to mucins was (439 ± 86) × 10^2 CFU/well for the parental strain (PAO1) (Fig. 1). This number was significantly decreased (\(P < 0.05\)) with the fliD mutant (PAO1-D). Adhesion assays were also performed with strain PAO1-D complemented with the homologous fliD gene on the multicopy plasmid vector pPZ357 (strain PAO1-D(375Db)) or containing vector alone (strain PAO1-D(375)). Complementation restored adhesion to a level that was not significantly different from that of the wild-type parent strain [(349 ± 70) × 10^2 CFU/well], while it was significantly higher (\(P < 0.005\)) than that of the vector control. These results indicate that, as previously described for strains PAK and PAK-NP (2), the binding of P. aeruginosa strain PAO1 to respiratory mucins is partly mediated by the flagellar cap protein, Flid.

Binding of glycoconjugates bearing Le\(\alpha\), sialyl-Le\(\alpha\), and sulfosialyl-Le\(\alpha\) glycophosphates to strain PAO1 and its fliD mutant. Adhesion assay, of P. aeruginosa to human respiratory mucins and to neoglycoconjugates was quantified using a microtiter plate assay (27, 28). Respiratory mucins were prepared from sputum of a patient with chronic bronchitis by ultracentrifugation as described previously (6). Respiratory mucins were prepared from sputum of a patient with chronic bronchitis by ultracentrifugation as described previously (6). Respiratory mucins were prepared from sputum of a patient with chronic bronchitis by ultracentrifugation as described previously (6). Respiratory mucins were prepared from sputum of a patient with chronic bronchitis by ultracentrifugation as described previously (6). Respiratory mucins were prepared from sputum of a patient with chronic bronchitis by ultracentrifugation as described previously (6). 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(ii) Microtiter plate adherence assay. To confirm the role of FliD in the recognition of Le\textsuperscript{x} and sialyl-Le\textsuperscript{x}, which are oligosaccharides that are found in mucins, adhesion assays were performed using microtiter plates coated with the unlabeled glycoconjugates. The number of bacteria adhering to sialyl-Le\textsuperscript{x}-PAA was found to be significantly higher for strain PAO1 \((71 \pm 6) \times 10^2\) CFU/well than for strain PAO1-D \((11 \pm 2) \times 10^2\) CFU/well (Fig. 2). After complementation with the \(fliD\) gene, the adherence of PAO1-D to sialyl-Le\textsuperscript{x} was restored, but this was not the case after complementation with the vector alone. Similar results were obtained for the adherence to Lex-PAA (Fig. 3).

**TABLE 3. Binding of fluorescent glycoconjugates bearing Le\textsuperscript{x}, sialyl-Le\textsuperscript{x}, and 6-sulfosialyl-Le\textsuperscript{x} glycotopes to the \(fliC\) mutant of \(P. aeruginosa\) strain PAO1 and to its \(fliD\) mutants**

| Glycoconjugate | \(K_d\) (nM ± SD)
<table>
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<tr>
<td></td>
<td>PAO1</td>
</tr>
<tr>
<td>LPF</td>
<td>39 ± 7</td>
</tr>
<tr>
<td>Sialyl-LPF</td>
<td>31 ± 3</td>
</tr>
<tr>
<td>6-Sulfosialyl-LPF</td>
<td>59 ± 7</td>
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\(a\) The number of data points for Scatchard analysis ranged between 20 and 30.

\(b\) NS, not significant.

\(c\) LPF, Le\textsuperscript{x}-PAA-Flu.
respectively). These numbers are in the range of those seen with the PAO1 fliD mutant, supporting the conclusion that PAK FliD is not involved in binding to the Lewis glycotopes.

**DISCUSSION**

*P. aeruginosa* binding to human respiratory mucins is mediated by the interaction of a bacterial component(s) with carbohydrate moieties of mucins (5, 17, 27, 28). However, there are differences in binding from one strain to another for the same mucins. For instance, this binding is greater for strains 1244 and PAO1 than for strain PAK (17, 23). While it is possible that one or the other strain may possess multiple adhesins to mediate these differences, another possible explanation may be differences in receptor recognition mediated by the known adhesins. Concerning the mucin adhesins, only the flagellar system of *P. aeruginosa* strain PAK has been shown to date to play an important role in mucin binding (25), and the

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**FIG. 2.** Comparative binding of *P. aeruginosa* PAO1 and its fliD and fliC mutants to the polyacrylamide derivative bearing the sialyl-Le^a^ glycotope. PAO1-D, fliD mutant of PAO1; PAO1-D(375 Db), PAO1-D complemented with the complete fliD gene on a multicopy plasmid vector, pPZ375Db; PAO1-D(375), PAO1-D with the vector pPZ375; PAO1-C, fliC mutant of PAO1. Differences in binding between strain PAO1 and its mutants marked by one asterisk were not significant, whereas those marked by two asterisks were considered significant ($P < 0.05$) by Student’s t test. The binding of PAO1-D(375Db) and that of PAO1-D(375) were also significantly different.

**FIG. 3.** Comparative binding of *P. aeruginosa* PAO1 and its fliD and fliC mutants to the polyacrylate derivative bearing the Le^x^ glycotope. PAO1-D, fliD mutant of PAO1; PAO1-D(375 Db), PAO1-D complemented with the complete fliD gene on a multicopy plasmid vector, pPZ375Db; PAO1-C, fliC mutant of PAO1. Differences in binding between strain PAO1 and its mutants marked by one asterisk were not significant, whereas those marked by two asterisks were considered significant.
flagellar cap protein, FlID, was shown to be a mucin-specific adhesin for this strain (2). However, P. aeruginosa contains two distinct types of flagellar cap proteins, designated A and B types, which are inherited with their corresponding a- and b-type flagellins (1). The flagellar caps show only 43% identity at the amino acid level, even though they belong to the same species and no immunological cross-reactivity between them is detectable by use of polyclonal antibodies (1). This suggests that they do not share recognizable linear or conformational epitopes that may be involved in adhesion. The role of the B-type cap in adhesion had not been previously studied, since it was assumed that caps were structurally similar, but this has now been shown not to be the case (2). Strains carrying the B-type cap, however, make up only 20% of mucoid isolates from CF individuals (R. Ramphal, personal communication). Thus, the increased mucin binding of strains carrying B-type caps alluded to above may be aided by other adhesins. This investigation thus further demonstrates a role for the flagellar cap protein in mucin adhesion and points out important fine specificities in the recognition of carbohydrates by the FlID protein.

Human respiratory mucins, the other partner in the bacteria-mucin interaction, have significant numbers of potential receptors, since there are a large number of glycotopes at their periphery, especially neutral or acidic Lewis derivatives (13, 20). Using purified whole mucins, it has been difficult to define specific receptors. However, some progress has been made in defining mucin receptors, with the recognition of the involvement of certain sugars in binding of whole bacteria. One approach that has been successful at identifying potential glycotopes that are recognized by P. aeruginosa has been the synthesis of neoglycolipids containing one specific putative receptor (15, 19). Such an approach has now been applied to the study of mucin receptors with the ability to synthesize neoglycoproteins that carry glycotopes that can be obtained in significant quantities (4). In the context of CF, the Lewis derivatives such as Le\(^a\), sialyl-LE\(^a\), or 6-sulfosialyl-Le\(^a\) appear to be increased in airway mucins and have been shown to bind to whole P. aeruginosa cells (21, 22). Recognizing that the FlID protein was a mucin-specific adhesin provided an opportunity to test the interaction between a specific adhesin and single putative receptors.

Polyacrylamide-based glycoconjugates that have predetermined properties, such as molecular mass, solubility, matrix flexibility, distance between the glycotopes, and density of substitution by the different glycotopes, are a suitable tool for measuring interactions between lectins and their carbohydrate receptors (4, 9, 10). The adhesion of bacteria to these neoglycoconjugates can be studied by two different methods: an adhesion assay with unlabeled glycoconjugates applied to a microtiter plate or a flow cytometry assay with polyacrylamide-based glycoconjugates labeled with fluorescein. Fluorescent neoglycoconjugates bearing Le\(^a\) determinant and its sialylated or sulfated derivatives bound specifically to several strains of P. aeruginosa (21, 22). Scatchard analysis of the data obtained by flow cytometry showed that the affinity of the fluorescent glycoconjugate bearing the sialyl-Le\(^a\) glycopeptide was higher than that observed for the other glycoconjugates. The binding of fluorescent glycoconjugates bearing blood group A or sialy-N-acetyllactosamine to these strains was not saturable (no K\(_d\) could be calculated) and was therefore considered nonspecific (21). In the present work, the binding of polyacrylamide-based glycoconjugates bearing Le\(^a\), sialyl-Le\(^a\), and 6-sulfosialyl-Le\(^a\) to strains PAO1 and PAK and to their fliD or fliC mutants was compared. The results obtained for the binding of the neoglycoconjugates to P. aeruginosa strain PAO1 indicated that FlID protein was involved in the binding of this strain to glycoconjugates bearing Le\(^a\) and sialyl-Le\(^a\) glycotopes. This was confirmed by using a microtiter plate adhesion assay with unlabeled glycoconjugates bearing Le\(^a\) and sialyl-Le\(^a\) glycotopes. In contrast, mutation in the fliD gene of strain PAK did not change the binding of the fluorescent conjugates compared to that with the parental strain, indicating that the specific ligand of PAK FlID is not one of the Le\(^a\) derivatives that is recognized by the PAO1 FlID.

The studies performed with the strain PAO1 fliC mutant indicated that PAO1 flagellin may be involved in the specific binding of glycoconjugates bearing the Le\(^a\) glycopeptide, but not in the binding of the glycoconjugates bearing the sialyl-Le\(^a\) and 6-sulfosialyl-Le\(^a\) glycotopes, which recognize the FlID protein. This was confirmed for FlID by using a microtiter plate adhesion assay with unlabeled fluorescent glycoconjugates bearing Le\(^a\) glycotopes. This suggests a specificity of the interactions between protein and the glycotopes and not a nonspecific interaction with fluorescein, as could have occurred. Both strains bound to 6-sulfosialyl-Le\(^a\) glycotopes. However, their binding was not modified after mutations either in the fliD or in the fliC gene. This is assumed to be some form of nonspecific binding.

These data suggest that the interactions between P. aeruginosa and carbohydrates are more complex than has been thought and that different components of the adhesin-flagellar system, flagellin and FlID, which differ from one strain to another, do not necessarily recognize the same glycotopes of
human respiratory mucins. Since CF mucins are characterized by a high content of sialyl-Le^x glycotopes (6), the FlpD protein and flagellin may be involved in specific binding to these glycotopes in vivo. Recent results have also demonstrated that glycoconjugates bearing the 6-sulfosialyl-Le^x glycotope, also found in abundance in CF respiratory mucins (13), was recognized by whole P. aeruginosa (22). In the future, it will be important to find out the bacterial component involved in the binding to this ligand. In conclusion, the recognition of human respiratory mucins by the adhesin-flagellar system appears to be a multifactorial phenomenon, involving different flagellar components and different carbohydrate receptors. Further studies will still be necessary to link these in some quantitative way to airway colonization of CF patients. However, these studies define for the first time the association between a specific P. aeruginosa adhesin and a mucin glycotope.

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