Pseudomonas aeruginosa binds to neoglycoconjugates bearing mucin carbohydrate determinants and predominantly to sialyl-Lewis x conjugates

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Pseudomonas aeruginosa plays an important role in the colonization of the Airways of patients suffering from cystic fibrosis. It binds to the carbohydrate part of respiratory and salivary mucins and its binding to cystic fibrosis mucins is even higher, suggesting that qualitative or and quantitative modifications of the carbohydrate chains may be involved in this process. In order to find out the best carbohydrate receptors for Pseudomonas, a flow cytometry technique using a panel of polycrylicamide based glycoconjugates labeled with fluorescein was developed. The neoglycoconjugates contained neutral, sialylated or sulfated chains analogous to the outer membrane of Pseudomonas aeruginosa, responsible for the adhesion of another nonpiliated strain of P. aeruginosa, 1244-NP, was saturable except for the glycoconjugates containing blood group A or sialyl-N-acetyllactosamine epitopes. The measurement of Kd indicated that strain 1244-NP had a higher affinity for the glycoconjugate bearing the sialyl-Lea determinant than for all the other glycoconjugates studied. The role of sialic acid was confirmed by competition assay using mainly siaaly-Lex conjugates were observed. However, three strains out of four variations in the binding of these strains to the three glycoconjugates were observed. Individual blood group A determinants). We used also neoglycoconjugates containing Gal(c1-2)Galβ- and sialyl-N-acetyllactosamine determinants. The interaction of these glycoconjugates with the nonpiliated strain of Pseudomonas aeruginosa, 1244-NP, was saturable except for the glycoconjugates containing blood group A or sialyl-N-acetyllactosamine epitopes. The measurement of Kd indicated that strain 1244-NP had a higher affinity for the glycoconjugate bearing the sialyl-Lea determinant than for all the other glycoconjugates studied. The role of sialic acid was confirmed by competition assay using mainly sialylated mucin glycopeptides. In order to find out if this behavior was the same for pathological strains as for the 1244-NP mutant, four mucoid strains of Pseudomonas aeruginosa isolated from cystic fibrosis patients were analyzed with the Lea neoglycoconjugate, its sialylated and its sulfated derivatives. Individual variations in the binding of these strains to the three glycoconjugates were observed. However, three strains out of four had a higher affinity for the sialyl-Lea than for the 3′-sulfo-Lea derivative.

Key words: binding/mucin–carbohydrates/Pseudomonas aeruginosa/sialyl-Lewis x

Introduction

Adhesion of Pseudomonas aeruginosa, an opportunistic pathogen, to respiratory mucins and/or to epithelial glycoconjugates has attracted much attention because of its role as the major airway colonizer in immunocompromised hosts and patients with cystic fibrosis (Hoiby, 1982).

Cystic fibrosis (CF), a general exocrinopathy, is the most common severe genetic disease among Caucasians. In its most typical form, the severity of the disease is due to mucus hypersecretion and to chronic lung infection characterized by the predominance of three bacteria, Hemophilus influenzae and Staphylococcus aureus in early life, and thereafter Pseudomonas aeruginosa, often in a mucoid form, a pathogen which is responsible for most of the morbidity and mortality of the disease. CF is caused by mutations in the gene encoding cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel of low conductance activated by protein-kinase A (Riordan, 1993) which has probably other functions (Barasch et al., 1991; Stutts et al., 1995; Devidas and Guggino, 1997; Pasyk and Foskett, 1997). The relation between the CFTR defect and the specificity of airways infection by Pseudomonas aeruginosa is not yet elucidated.

The binding of Pseudomonas aeruginosa to the human airway mucosa may involve several host receptors and bacterial adhesins. Pili present on the surface of Pseudomonas aeruginosa recognize the sequence GalNAc(β1,4)Gal of asialo-GM1 and -GM2 receptors and may contribute to the binding of bacteria to epithelial cells (Lee et al., 1994), especially in a regenerating respiratory epithelium following damage (De Bentzman et al., 1996).

However, human airway mucins, a population of heavily glycosylated glycoproteins synthesized by goblet cells and bronchial glands of the human airway mucosa, are part of the mucus layer. The mucociliary escalator which normally removes inhaled particles or microorganisms. Their very diverse carbohydrate chains act as recognition sites for bacteria or viruses. Indeed, several reports have demonstrated that various strains of Pseudomonas aeruginosa adhere to mucins (Vishwanath and Ramphal, 1984; Ramphal et al., 1989; Sajjan et al., 1992; Carnoy et al., 1993; Devaraj et al., 1994) and that the adhesion process involves several adhesins localized on the outer membrane of Pseudomonas aeruginosa (Carnoy et al., 1994). More recently, the role of flagella in the binding of the bacteria to mucins (Ramphal et al., 1996; Arora et al., 1998) and to cell surface glycolipids (Feldman et al., 1998) has been emphasized and the flagellar cap protein, Fli D, has been shown to be responsible for the adherence of another nonpiliated strain of Pseudomonas aeruginosa, PAK-NP, to human bronchial mucins (Arora et al., 1998).

In the airways of CF patients suffering from cystic fibrosis colonized by Pseudomonas aeruginosa, most bacteria are embedded in the airway mucus and their interactions with respiratory mucins probably represent a major step in the development of the colonization process. Bronchial mucins secreted by patients suffering from cystic fibrosis (Roussel et al., 1975; Boat et al., 1976; Lamblin et al., 1977; Chace et al., 1985), by CF cells (Frates et al., 1983; Chen et al., 1989) or by xenograft models of CF cells (Zhang et al., 1995) are oversulfated, and it has been suggested that the AFS08 deletion, the most frequent mutation in CF, leads to a misfolding of CFTR which is retained in the...
A.Scharfman et al.

Fig. 1. Influence of the concentration of fluorescent glycoconjugate on the binding to *P. aeruginosa* 1244-NP. Bacterial suspension was incubated with increasing concentrations (from 6.25 to 250 nM) of Lex-PAA-flu (A), sialyl-Le^a^ (B) and sialyl-N-acetyllactosamine- (C) epitopes. After 30 min in the dark, a flow cytometry analysis was performed.

Table I. Synthetic neoglycoconjugates used in the study

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Neoglycoconjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Le^a^</td>
<td>Gal[β1–3][Fuc(α1–4)]GlcNAcβ-PAA-flu</td>
</tr>
<tr>
<td>Le^β^</td>
<td>Fuc(α1–2)Gal[β1–4][Fuc(α1–3)]GlcNAcβ-PAA-flu</td>
</tr>
<tr>
<td>Le^ε^</td>
<td>Gal[β1–4][Fuc(α1–3)]GlcNAcβ-PAA-flu</td>
</tr>
<tr>
<td>Blood group A determinant</td>
<td>GalNAc(α1–3)[Fuc(α1–2)]Galβ-PAA-flu</td>
</tr>
<tr>
<td>Gal(α1–2)Galβ</td>
<td>Gal(α1–2)Galβ-PAA-flu</td>
</tr>
<tr>
<td>Sialyl-Le^ε^</td>
<td>Neu5Ac(α2–3)Gal[β1–4][Fuc(α1–3)]GlcNAcβ-PAA-flu</td>
</tr>
<tr>
<td>3^′^-Sulfo-Le^ε^</td>
<td>HSO3-3Gal[β1–4][Fuc(α1–3)]GlcNAcβ-PAA-flu</td>
</tr>
</tbody>
</table>

PA-flu, Polyacrylamide carrier labeled with fluorescein.
Affinity of *P. aeruginosa* for sialyl-Lewis x epitopes

Fig. 2. Scatchard analysis of the binding of Lex (A), sialyl-Lex (B) and sialyl-N-acetyllactosamine (C) fluorescent derivatives to *P. aeruginosa* 1244-NP. After incubation of bacteria with increasing concentrations of the fluorescent glycoconjugates, flow cytometry analysis was performed.

Scatchard analysis of the data (Figure 2, Table I) showed that there was differences in the binding of the different fluorescent derivatives to *P. aeruginosa* 1244-NP. The binding of derivatives containing Le^a^, Le^b^, Le^x^, 3′-sulfo-Le^x^ or Gal(α1-2)Galβ epitopes was monophasic, suggesting that there was only one single class of receptors (Figure 2A); the dissociation constant (Kd) ranged from 87 nM for Gal(α1-2)Galβ to ∼50 nM for the other derivatives (Table I). The number of binding sites varied from 1569 ± 199 for the Le^b^-fluorescent derivative to 4500 ± 262 for the sulfo-Le^x^ derivative. In contrast, the binding of *P. aeruginosa* 1244-NP to sialyl-Le^x^-PAA-flu was biphasic (Figure 2B) suggesting that there were two classes of binding sites for this determinant, one with high affinity (Kd =12 nM ± 4.2, number of binding sites = 1106 ± 345) and another one with low affinity (data not shown). The data obtained for the binding of blood group A and sialyl-N-acetyllactosamine fluorescent derivatives demonstrated that the binding of these two derivatives was non specific as shown for sialyl-N-acetyllactosamine in Figure 2C.

Table I. Comparative binding of the different Gly-PAA-flu to *P. aeruginosa* 1244-NP

<table>
<thead>
<tr>
<th>Carbohydrate epitope</th>
<th>Number of binding sites</th>
<th>Dissociation constant (Kd nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sialyl-Le^a^</td>
<td>1106 ± 345</td>
<td>12 ± 4.2^d</td>
</tr>
<tr>
<td>Sulfo-Le^x^ a</td>
<td>4500 ± 262</td>
<td>49 ± 11</td>
</tr>
<tr>
<td>Le^b^ a</td>
<td>4264 ± 159</td>
<td>60 ± 5.3</td>
</tr>
<tr>
<td>Le^x^ a</td>
<td>2236 ± 273</td>
<td>51 ± 13</td>
</tr>
<tr>
<td>Le^x^ b</td>
<td>1569 ± 199</td>
<td>50 ± 12</td>
</tr>
<tr>
<td>Gal(α1-2)Galβ</td>
<td>3600 ± 332</td>
<td>87 ± 23</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD.

^aNumber of data point fitted for Scatchard analysis = 36.
^bNumber of data point fitted in Scatchard analysis = 18.
^cNumber of data point fitted in Scatchard analysis = 12.
^dSignificantly different when compared to Kd obtained with the other Gly-PAA-flu (p < 0.001).

Altogether the values obtained for Kd and number of binding sites showed that the affinity of fluorescent derivatives containing Le^a^, Le^b^, Le^x^, 3′-sulfo-Le^x^ or Gal(α1-2)Galβ epitopes for *P. aeruginosa* 1244-NP was in the same order of magnitude. The value of Kd obtained for the sialyl-Le^x^ derivative indicated that *P. aeruginosa* 1244-NP had an higher affinity for this neoglycoconjugate than for all the other glycoconjugates studied so far (p < 0.001).

Specificity of the binding of the Gly-PAA-flu to *P. aeruginosa* 1244-NP

To check the specificity of the interaction between fluorescent derivatives and *P. aeruginosa* 1244-NP, competition binding assays were performed for three of them, Le^a^, Gal(α1-2)Galβ, and sialyl-Le^x^PAA-flu.

Incubation of Le^x^-PAA-flu and bacteria in presence of 100-fold molar excess of the corresponding unlabeled Le^x^ derivative, resulted in a decrease of the logarithm of fluorescence intensity, when compared to control as shown in Figure 3 (4250 equivalent bound particles but 8250 in the absence of unlabeled Le^x^ polyacrylate derivative). Reduction of intensity of fluorescence was evidenced by the Kolmogorov-Smirnov two-sample test. On the contrary, the fluorescence intensity of the Le^x^-PAA-flu did not change when incubation was performed in presence of unlabeled Gal(α1-2)Galβ polyacrylate derivative. Moreover, Kd value obtained for the binding of Le^x^-PAA-flu was not modified (p > 0.05) when incubation was performed in presence of unlabeled Gal(α1-2)Galβ derivative.

Competitive binding between Gal(α1-2)Galβ-PAA-flu and the corresponding unlabeled polyacrylate derivative resulted in a decrease of the fluorescence intensity (5400 equivalent bound particles but 8150 in absence of unlabeled derivative). Reduction of intensity was evidenced by the Kolmogorov-Smirnov two-
sample test. Fluorescence intensity was not changed when incubation with Gal(α1–2)Galβ-PAA-flu was performed in presence of unlabeled Lex polyacrylate derivative.

Competition binding assays were also performed with sialyl-Lex-PAA-flu and unlabeled glycoconjugates bearing either sialyl-Le^x, Le^x or Gal(α1–2)Galβ epitopes. The presence of an excess of unlabeled sialyl-Le^x derivative in the incubation mixture led to an aggregation of the bacteria as visualized by an increase of the forward scatter (data not shown). The addition of Le^x and Gal(α1–2)Galβ-PAA to incubation mixtures did not modify the binding of sialyl-Le^x-PAA-flu to P. aeruginosa 1244-NP. In addition, Kd value obtained for the binding of sialyl-Le^x was not changed in presence of an excess of the unlabeled Gal(α1–2)Galβ derivative (p > 0.05), confirming the absence of cross-binding of these two derivatives to P. aeruginosa. Additional experiments also demonstrated that the Kd values of the binding of sialyl-Le^x-PAA-flu was not modified by addition of free stachyose in the incubation mixture. Altogether, these results demonstrated that there was no cross-reactivity between Le^x, Gal(α1–2)Galβ epitopes and sialyl-Le^x-PAA-flu to P. aeruginosa 1244-NP.

**Competitive binding of Gly-PAA-flu and airways mucins glycopeptides to P. aeruginosa 1244-NP**

Binding assays were also performed in presence of an excess of different airway mucin glycopeptides, neutral, mainly sialylated, or mainly sulfated glycopeptides (Ramphal et al., 1989). As demonstrated by the number of equivalent bound particles, the binding of Gly-PAA containing Le^x, sialyl-Le^x or 3′-sulfo-Le^x to P. aeruginosa was partly inhibited by mucin glycopeptides (Table III). The binding of Le^x-PAA-flu was equally inhibited by the three classes of glycopeptides, while the binding of 3′-sulfo-Le^x-PAA-flu was better inhibited by mainly sulfated glycopeptides and the binding of sialyl-Le^x-PAA-flu better inhibited by mainly sialylated glycopeptides (Table III).

**Table III. Inhibition of binding of Le^x, sialyl-Le^x, 3′-sulfo-Le^x-PAA-flu to P. aeruginosa 1244-NP after competition with airway mucin glycopeptides**

<table>
<thead>
<tr>
<th>Gly-PAA-flu</th>
<th>% of inhibition by glycopeptides^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutral</td>
<td>Manly sialylated</td>
</tr>
<tr>
<td>Le^x</td>
<td>17</td>
</tr>
<tr>
<td>Sialyl-Le^x</td>
<td>20</td>
</tr>
<tr>
<td>3′-Sulfo-Le^x</td>
<td>10</td>
</tr>
</tbody>
</table>

^aThe percentage of inhibition was calculated from the mean values of fluorescence intensity obtained after incubation with or without mucin glycopeptides.

**Binding of Le^x, sialyl-, and 3′-sulfo Le^x-PAA-flu derivatives to clinical strains of P. aeruginosa**

In order to determine whether pathological strains recognize also preferentially sialyl-Le^x determinants, the binding of Le^x, sialyl-Le^x, or 3′-sulfo-Le^x-PAA-flu to four mucoid strains isolated from CF patients was compared by using flow cytometry and Scatchard analysis. All these neoglycoconjugates bound to the four pathological strains. However, there were individual variations in the binding (Table IV). Strain 6118 bound equally to the three derivatives. In contrast, the three other strains (690, 6190, and 130308) bound better to the sialyl-Le^x derivative than to the 3′-sulfo-Le^x derivative. For two of these strains (690 and 6190), there was no significant differences between their binding to the Le^x and to the sialyl-Le^x derivatives.

**Table IV. Binding of Le^x, sialyl-Le^x and 3′-sulfo-Le^x-neoglycoconjugates to four strains of P. aeruginosa isolated from CF patients**

<table>
<thead>
<tr>
<th>Mucoid strains</th>
<th>Kd</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPF</td>
<td>Sialyl-LPF</td>
</tr>
<tr>
<td>690^a</td>
<td>80 ± 22</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>6118^b</td>
<td>70 ± 13</td>
<td>43 ± 34</td>
</tr>
<tr>
<td>6190^b</td>
<td>36 ± 14</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>130,308^b</td>
<td>100 ± 25</td>
<td>43 ± 6</td>
</tr>
</tbody>
</table>

LPF, Le^x-PAA-flu. N.S., not significantly.

^aNumber of data fitted in Scatchard analysis = 26.

^bNumber of data fitted in Scatchard analysis = 16.
Discussion

The aim of the present work was to set up a flow cytometry assay using polyacrylamide-based glycoconjugates labeled with fluorescein (Gly-PAA-flu) (Bovin et al., 1993), in order to compare the binding of *P. aeruginosa* to a panel of conjugates containing carbohydrate determinants analogous to the peripheral part of many carbohydrate chains present in human airway mucins (Roussel and Lamblin, 1996). Several proteins of the outer membrane or the flagella of *P. aeruginosa* have already been shown to be recognized by various human mucins (Carnoy et al., 1994; Ramphal et al., 1996; Arora et al., 1998), and several carbohydrate determinants have been identified as possible sites of attachment of this bacteria. In contrast to previous techniques used to identify carbohydrate receptors recognized by *P. aeruginosa*, such as chromatography of glyco- or neoglycolipids followed by bacteria overlay (Ramphal et al., 1991a; Rosenstein et al., 1992), flow cytometry allows the interactions between bacterial receptors to occur in a liquid phase. With this technique, using polyacrylamide based glycoconjugates labeled with fluorescein, carbohydrate receptors of *P. aeruginosa* surface lectins can be identified. The type of binding of the different glycoconjugates (Table I) to *P. aeruginosa* 1244-NP was not identical and was dependent on the carbohydrate epitope. The binding of blood group A and sialyl-N-acetyllactosamine fluorescein glycoconjugates was not saturable. It was saturable and monophasic for polyacrylate derivatives bearing Leα, Leβ, 3-sulfo-Leα and Gal(α1–2)Galβ epitopes while there was two binding sites, for the polyacrylate derivative containing the sialyl-Leα epitope, one of them with high affinity.

To assess the specificity of the binding, different competition experiments were performed using three different fluorescent conjugates. Competition experiments demonstrated that stachyose which does not exist in the mucin carbohydrate chains was not recognized by *P. aeruginosa* 1244-NP. The binding of Leα and Gal(α1–2)Galβ-PAA-flu to *P. aeruginosa* 1244-NP showed a decrease of 50% in the presence of an excess of the corresponding unlabeled polyacrylate derivative. However, it was not possible to obtain a total inhibition: this may be due to a nonspecific fixation of the fluorescein groups on the bacteria (Babiuk and Paul, 1970; Chatelier et al., 1995). Similarly, competing molecules, such as mucin glycopeptides, are not able to inhibit more than 50% of the binding of the fluorescent glycoconjugates. Incubation of sialyl-Leα with an excess of the unlabeled derivative led to an aggregation of the bacterial population; Komiyama and associates (Komiyama et al., 1987) have suggested a role of sialic acid in the aggregation of *P. aeruginosa* by saliva.

In the present study, Leα, Leβ, and Leδ determinants were identified as receptors for *P. aeruginosa* 1244-NP and the Kd values of the binding of these three neoglycoconjugates were not significantly different. On another hand, the binding of this bacteria to the blood group A and to the sialyl-N-acetyllactosamine neoglycoconjugates was found to be not saturable, attesting a nonspecific binding of these two neoglycoconjugates to *P. aeruginosa* 1244-NP which again may be due to the fluorescent groups. Since *P. aeruginosa* synthesizes an internal lectin, PA-IL, which binds to β-galactose in α or β anomeric configuration (Chen et al., 1998) and since its best ligands have a terminal Galβ, we also assayed the Gal(α1,2)Galβ-PAA-flu, although human mucins are not supposed to contain terminal Galβ, except in mucins with blood group B and mucins which contain a few Gal(α1,3)GalNAc chains (van Halbeek et al., 1994). The binding of *P. aeruginosa* to this determinant was in the same order of magnitude as the binding to Leα or Leδ determinants which have a terminal Galβ. Competition experiments revealed the absence of cross-binding between fluorescent polyacrylate derivatives carrying Leα and Gal(α1–2)Galβ derivatives, suggesting that these two epitopes are recognized by different lectins. Glick and Garber (1983) have reported that, even if most PA-IL is internal, small quantities of the lectin may exist on the outer membranes and therefore may be involved in the recognition of the Gal(α1–2)Galβ epitope. Since the pattern of the outer membrane proteins that act as bacterial adhesins for airway mucins varies from one strain of *P. aeruginosa* to the other (Carnoy et al., 1994), it will be necessary, in the future, to determine the different surface adhesins involved in the recognition of the different neutral carbohydrate chains.

Neoglycoconjugates containing acidic determinants, sialyl-Leα or 3′-sulfo-Leα, were also recognized by *P. aeruginosa* 1244-NP. The present study indicates that these neoglycoconjugates were much better receptors than the neoglycoconjugate containing sialyl-N-acetyllactosamine. As far as the binding of the sialyl-Leα derivative is concerned, strain 1244-NP had a better affinity for this derivative than for all the other glycoconjugates studied. The dissociation constant obtained for the binding of 1244-NP to 3′-sulfo-Leα containing glycoconjugates suggested that the preferential affinity of 1244-NP strain for the sialyl-Leα epitope was not due to the substitution of Leα by any acidic residue but indeed by the presence of sialic acid residue. The role of sialic acid was confirmed by competition binding assays showing that the binding of *P. aeruginosa* to the sialyl-Leα epitope was better inhibited by mainly sialylated mucin glycopeptides than by neutral or mainly sulfated glycopeptides, and also better by CF mucins than by non-CF mucins. CF mucins indeed contain more sialic acid than non-CF mucins (Davril et al., 1999). Competitive experiments demonstrated the absence of cross-binding between sialyl-Leα-PAA-flu and polyacrylate bearing neutral epitopes such as Leα and Gal(α1,2)Galβ, suggesting that the receptor for sialyl-Leα glycoconjugate had a restricted specificity. Therefore, for most strains analyzed in the present study, the sialyl-Leα polyacrylate derivative is a major receptor.

The role of sialic acid in the binding of *P. aeruginosa* has already been suggested by several reports (Baker et al., 1990; Ramphal et al., 1991a). Moreover, desialylation of human saliva mucin glycopeptides has also been shown to reduce their binding to the pilated strain 1244-NP (Carnoy et al., 1993).

Finally, the preferential inhibition of the binding of strain 1244-NP to the sialyl-Leα or the sulfated Leα-neoglycoconjugates by the corresponding sialylated or sulfated mucin glycopeptides is in favor of at least two adhesins having different affinities to these two neoglycoconjugates.

There are differences in the binding of *P. aeruginosa* PAK and 1244 strains, as well as their nonpiliated derivatives, to different glycoconjugates and in the pattern of outer membrane proteins recognized by airway mucins (Ramphal et al., 1991b; Carnoy et al., 1994). In order to find out whether the behavior of *P. aeruginosa* is the same for pathological strains as for the 1244-NP strain, four mucoid strains isolated from CF patients were also analyzed with the Leα neoglycoconjugate and its sulfated and sialylated derivatives. Three strains out of four bound better to the sialyl-Leα than to the 3′-sulfo-Leα derivatives. It should be noticed that the results obtained with these clinical isolates showed some variations in the binding to the three neoglycoconjugates from one experiment to another, as indicated by standard deviations. A similar problem has been encountered by Devraj et al. in studying the binding of *P. aeruginosa* to CF and non-CF...
glycopeptides (Devaraj et al., 1994). Moreover, Govan et al. have pointed out the instability of mucoid strains during in vitro cultures leading to reversion of the mucoid to nonmucoid forms (Govan et al., 1979).

These findings concerning sialylated or sulfated neoglycoconjugates are of special interest when considering the abnormalities of saliva and respiratory mucins secreted by CF patients. CF salivary mucins have been found to be more sulfated and more sialylated than mucins from controls (Carnoy et al., 1993) and, moreover, they are better recognized by various strains of P. aeruginosa. An overexpression of sialyl-Le\(^\alpha\) determinants has been observed in various inflammatory conditions, for instance among the acute phase glycoproteins synthesized in the liver under cytokine stimulation (Van Dijk et al., 1998). Recently, we have observed an increased sialylation and an increased expression of sialyl-Le\(^\alpha\) epitopes in the airway mucins from patients suffering from CF, in addition to increased sulfation (Davril et al., 1999). This increased sialylation was not specific for cystic fibrosis since it was also observed in the airway mucins secreted by severely infected patients suffering from chronic bronchitis. This observation may suggest that overexpression of sialyl-Le\(^\alpha\) determinants on glycoproteins synthesized by the airway mucosa may be viewed as the signature of local inflammation, in the same way as acute phase proteins synthesized by the liver which contain more sialyl-Le\(^\alpha\) epitopes during inflammation (Van Dijk et al., 1998). Several reports currently suggest that, in CF airways, the inflammatory response to aggression is abnormal (Birrer et al., 1998). Therefore, the attachment of P. aeruginosa to the sialyl-Le\(^\alpha\) epitope may be especially important in the development of lung colonization by P. aeruginosa (Heeckeren et al., 1997). Therefore, the attachment of P. aeruginosa to the sialyl-Le\(^\alpha\) epitope may be especially important in the development of lung infection in CF.

In conclusion, P. aeruginosa recognizes a whole set of neutral and acidic carbohydrate determinants, and especially the sialyl-Le\(^\alpha\) epitope. In the future, it will be important to find out (1) how many bacterial adhesins from P. aeruginosa may be involved in the airway colonization of CF patients, (2) which one is specific for the sialyl-Le\(^\alpha\) epitope, and (3) whether it is expressed in all strains colonizing CF patients.

Material and methods

**Bacteria strains and culture conditions**

Strain 1244-NP (provided by S. Lory from University of Washington) is an isogenic nonpiliated, nonmucoid strain (Ramphal et al., 1991b) which has already been shown to adhere to mucin carbohydrate chains (Carnoy et al., 1993) and to neoglycolipids (Ramphal et al., 1991a). Other strains were isolated from CF patients as mucoid isolates. All strains were grown in tryptic soy broth (TSB medium, Difco, Detroit) for 18 h at 37°C. After centrifugation of the cultures at 4000 x g for 30 min, the cell pellets were washed twice with a filtered physiologic saline containing 5% (v/v) TSB and then resuspended in the same solution. Optical density measurements were used to obtain a bacterial suspension of ~10^7 CFU/ml. The exact number of bacteria was determined by dilution and plating of the suspension.

**Preparation of human respiratory mucins and mucins glycopeptides**

High molecular weight mucins were prepared from sputum of patients (blood group O) suffering either from chronic bronchitis or from CF, by two steps of cesium bromide density-gradient ultracentrifugation as already described (Houdret et al., 1986). Mucins obtained after the first step of ultracentrifugation were treated by a cocktail of enzymes (Lo Guidice et al., 1994) to get rid of nucleic acids and proteoglycans, often associated with mucins of patients suffering from cystic fibrosis (Rahmoune et al., 1991), and then submitted to the second step of ultracentrifugation.

Glycopeptides were obtained by pronase digestion of mucins followed by fractionation by ion exchange chromatography allowing to obtain neutral, mainly sialylated and mainly sulfated glycopeptides (Ramphal et al., 1989).

**Binding assay**

Analysis of the binding of the different glycoconjugates to P. aeruginosa was performed by flow cytometry using fluorescent polymeric neoglycoconjugates obtained from Syntesone (Munich, Germany) (Bovin et al., 1993). In these compounds (Gly-PAAs-flu), a fluorescence-labeled polyacrylamide matrix is N-substituted every fifth amide group by a carbohydrate determinant on a spacer arm -(CH\(_2\))\(_3\)-. The carbohydrate/fluorescein molar ratio is 20:1. Unsubstituted sides group of the polymer were converted into -CONHCH\(_2\)CH\(_2\)OH. The different Gly-PAAs-flu used in the present study are listed in Table 1.

Bacteria cells were resuspended at a concentration of 2 x 10^6 CFU/ml in phosphate-buffered saline (PBS) containing 1% (w/v) bovine serum albumin (BSA), and 0.5 ml aliquots were incubated for 30 min in the dark with increasing amounts of the different Gly-PAAs-flu (6.25–250 nM). Controls were obtained by omitting glycoconjugates in the incubation mixture (Figure 3A). The mixtures were analyzed on a FACSscan cytofluorimeter (Becton-Dickinson) using FACSscan and LYSIS software for acquisition and analysis respectively. The light scatters channels were set on a linear gain scale, the flow rate was kept at approximately 200 events per second and 5000 events were collected. Acquisition was done on gated populations (by forward and side scatters) to exclude cell debris and cell aggregates. The green fluorescence was set on a logarithmic scale and the mean value of logarithm of fluorescence was converted in equivalent bound particles using calibrated beads to which a known number of fluorescent particles has been covalently bound (Immuno-Britt, Coulter Counter). Results were expressed as equivalent bound particles after deduction of the value of control. Experimental data were expressed as mean value ± standard variations. Binding capacity and dissociation constants were calculated according to Scatchard using the nonlinear progression data analysis program Enzfitter (Cambridge, UK). For statistical analysis, the Student’s t-test was used. To determine t, the following equation was used,

\[ t = \frac{[kd(1) – kd(2)]/[\sqrt{SV(1)^2 + SE(2)^2}]}{\sqrt{SV(1)^2 + SE(2)^2}} \]

For statistical analysis, the Student's t-test was used. To determine t, the following equation was used, where \( kd(1) \) and \( kd(2) \) are fitted values for the parameters for data sets 1 and 2, \( SE(1) \) and \( SE(2) \) are the standard errors for the fitted values, and \( n1 \) and \( n2 \) are the numbers of datum points in data sets 1 and 2.

**Inhibition experiments**

Competition assays were performed for the binding of fluorescent glycoconjugates bearing Le\(^\alpha\), Gal(x1–2)Gal\(\beta\) and sialyl-Le\(^\alpha\) epitopes. The bacterial suspension was incubated with a 62.5 nM solution of each of the fluorescent glycoconjugates in presence of a 100 molar fold excess of unlabeled polyacrylate derivatives. Bacteria incubated under the same experimental conditions, without any potential inhibitors, are used as controls. After flow
cytometry analysis, the value of mean of fluorescence intensity (expressed as equivalent bound particles) was calculated. The two histograms were different. Incubation with Le<sub>3</sub>-PAA-flu was performed in presence or in absence of unlabeled polyacrylate derivatives bearing Le<sub>3</sub> or Gal(1–2)Galβ, and unlabeled conjugates with Le<sub>4</sub> or Gal(1–2)Galβ epitopes. The same experiments were performed with Gal(1–2)Galβ-PAA-flu, using in this case, unlabeled polyacrylate derivatives bearing Le<sub>3</sub> or Gal(1–2)Galβ epitopes. Competition experiments of the binding of sialyl Le<sub>3</sub>-PAA-flu were performed in presence of the corresponding unlabeled polyacrylate derivative and of unlabeled polyacrylate derivatives bearing Le<sub>3</sub> or Gal(1–2)Galβ epitopes.

In some experiments increasing concentration of Le<sub>4</sub> or sialyl-Le<sub>3</sub> (6.25 to 250 nM) were incubated with bacterial suspension in presence of an 40 molar excess of unlabeled Gal(1–2)Galβ polyacrylate derivative and of free stachyose. After flow cytometry analysis, binding capacities and dissociation constants were calculated as described above.

### Competition binding of Gly-PAA-flu and mucins glycopeptides to P. aeruginosa 1244-NP

Assays were performed in presence of a 100-fold excess of mucin glycopeptides (neutral, mainly sialylated or mainly sulfated).

The bacterial suspension (0.5 ml) was incubated with 62.5 nM solution of Gly-PAA-flu in presence or absence of mucin glycopeptides (100 µg/ml). After flow cytometry analysis, the percentage of inhibition was calculated from the mean values of fluorescence intensity (expressed as equivalent bound particles), obtained after incubation with or without mucin glycopeptides. In some experiments, incubation of sialyl-Le<sub>3</sub>-Gly-PAA-flu with P. aeruginosa was performed in presence of CF or non-CF high molecular weight mucins (50 µg/ml).

### Analytical procedure

Sugar analysis was carried out by gas-liquid chromatography of trimethylsilyl derivatives of methyl-glycosides (Lambin et al., 1984). N-acetylhеuraniminic acid was measured by the thioarbituric acid assay of Aminoff (1961) after hydrolysis with 0.1 M H<sub>2</sub>SO<sub>4</sub> for 30 min at 80°C. Sulfate content was determined by HP AEC after hydrolysis with 1 M HCl as described previously (Lo-Guidice et al., 1994).

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### Abbreviations

CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; asialo-GM1, gangliotetraosyl ceramide; asialo GM2, gangliotriaosyl ceramide; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; NP, nonpililated; Gly-PAA-flu, polyacrylamide-based glycoconjugates labeled with fluorescein; Le, Lewis; Kd, dissociation constant; PA-IL, Pseudomonas aeruginosa (PA-I) lectin; TSB, tryptic soy broth; CFU, colony forming unit; Gal, galactose; Fuc, fucose; GlcNAc, N-acetylgalactosamine; GalNAc, N-acetylgalactosaminic acid.

### References


763


