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Pseudomonas aeruginosa lectins I and II and their interaction with human airway cilia

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Abstract

The bacterium *Pseudomonas aeruginosa* (PA) produces two carbohydrate binding lectins, designated PA lectin-I and lectin-II (PA-IL, PA-IIL). Both lectins are used by the bacterium to adhere to the glycocalyx of mammalian cells. In addition, the lectins immobilize ciliary beat. The kinetics of ciliary beat inhibition by each individual lectin have been analysed; however, their joint action on cilia has not been reported. Here we demonstrate that PA-IL and PA-IIL inhibit ciliary beat in a similar time-dependent manner. If applied simultaneously, ciliary beat inhibition after five hours of incubation was weaker than if each lectin was applied separately. Thus it can be hypothesized that the lectins compete for the same binding site(s) of the glycocalyx. Sugar inhibition experiments demonstrate that D-galactose and L-fucose inhibit both lectins, although clear preferences of D-galactose for PA-IL and of L-fucose for PA-IIL exist. These interactions have to be kept in mind when designing sugar-based therapies.

Key words: Bacterial Adhesion; Cilia; Cystic Fibrosis; Lectins; *Pseudomonas aeruginosa*; Respiratory Tract Infections

Introduction

Respiratory tract infections with *Pseudomonas aeruginosa* (PA) are often life-threatening in patients treated in intensive care units and in cystic fibrosis patients, as this bacterium colonizes compromised respiratory tracts.[1] As an integral part of the colonization process the bacterium interacts with the mammalian host cells. Like any other mammalian cell, airway cells are covered on the outside by a carbohydrate-rich coat, the glycocalyx. *P. aeruginosa* uses some of these carbohydrate residues to attach via two lectins, which represent carbohydrate binding proteins. They are named *P. aeruginosa* lectin-I and lectin-II (alternatively LecA and LecB), and are specific for galactose and fucose, respectively.[2] The structures of both lectins were determined recently.[3–5] Although they were initially thought to be intracytoplasmic, later results showed that they contribute to adhesion[6] and that their expression is controlled by quorum sensing,[7] a process vital for the colonization of surfaces. Within the airways, however, adhesion alone would not be sufficient for the interaction of the bacteria with the respiratory cells as bacteria are trapped within the mucous layer and are subsequently expulsed via the mucociliary elevator. Consequently, the two lectins are also capable of immobilizing the ciliary beat upon binding and thus stop this elevator.[8,9] For PA-IIL it has been shown *in vitro* that this blockage can be reversed by using fucose as a competitive inhibitor,[9] and a proof of principle has been reported in a case report where fucose and galactose inhalation was able to clear the airway infection with *P. aeruginosa*.[10]

While the interaction of both lectins with cilia has been studied individually, their combined interaction with cilia has not been investigated. It is of interest to study this interaction as both galactose and fucose are closely related sugars, with L-fucose being 6-deoxy-L-galactose,[11] and hence they might compete for the same receptor. Furthermore, they also have a different temperature profile for their binding and haemagglutination,[12] which might also be taken into consideration if sugar blocking strategies for these two lectins are to be further developed.

Methods

Materials

PA-IL was obtained from Sigma (Deisenhofen, Germany). PA-IIL was purified by affinity chromatography as previously described.[4] Dialysis of the lectins was carried out with the PlusOne Dialysis

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Kit (Amersham Biosciences, Freiburg, Germany). For histochemical analyses the lectins were biotinylated with biotin (Molecular Probes, Eugene, OR). RPMI-1640 culture medium with L-glutamine, antibiotics and nystatin were obtained from Gibco BRL (Karlsruhe, Germany). L-fucose was from Sigma, and D-galactose from Serva (Heidelberg, Germany). The sugars were dissolved in distilled water. The streptavidin-alkaline-phosphatase complex (ABC) was purchased from Vector Laboratories, Inc. (Burlingame, CA).

Tissue preparation

Experiments were carried out on human nasal mucous membrane obtained from routine surgery. All the patients whose tissue samples were investigated had given informed consent following institutional guidelines. Small pieces (20 mm^2) of the mucosa were transferred to Petri dishes containing modified RPMI-1640 growth medium supplemented with 10 per cent fetal calf serum, 100 units/ml nystatin, 100 µg/ml streptomycin and 100 units/ml penicillin. The specimens were cultured at 37°C in 5 per cent CO_2 for up to two weeks. For histochemical analyses the tissue samples were fixed by 4 per cent paraformaldehyde and embedded in paraplast.

Photoelectrical ciliary beat frequency (CBF) measurements and data analysis

Actively beating ciliated cells were viewed through phase-contrast optics on a Leica DM IRB HC inverted microscope (Leica, Bensheim, Germany) placed on a Micro-g vibration isolation table (TMC, Peabody, MA). During the experiments, the cultured tissue samples were maintained at a constant temperature (35 ± 0.5°C) by a thermostatically-controlled heated stage. The specimens were aligned in such a way that the synchronously beating cilia were in the light path, thus causing refraction of the light as they beat. This light modulation was detected by using a Leica MPV photometer (Leica) attached to the camera port of the microscope. The photometer converted the light refraction into electrical signals, which were measured and analysed by a computer system incorporating a special-purpose MPV-CBF software (Software-Entwicklung Radmann, Solms, Germany). The CBF was calculated by collecting data from 512 readings (10 s) and by performing Fourier analysis. Three of these Fourier transforms taken from the same area were averaged to provide one reading. The CBF determined was considered acceptable when a single predominant frequency was obtained.

The CBF was monitored over a 24 hour period. Control samples were maintained in medium with no additions made. PA-IL and PA-IIL were added to the culture dishes separately or in combination at a final concentration of 1 µM each. In further explants the same concentration of the lectins was co-incubated with 10 µM D-galactose or 10 µM L-fucose, respectively.

Each experiment was carried out on 3–9 tissue cultures obtained from just as many patients. Data were expressed as a percentage of the basal CBF recorded prior to lectin or sugar application. Results were averaged and are displayed as means ± SEM using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA), with n = number of experiments. Statistical significance was determined by Student's two-tailed t-test for paired observations, with $p < 0.05$ being considered significant.

Lectin histochemistry

Histochemical analyses of lectin binding were performed, essentially as described previously.[13] In brief, deparaffinized sections (5 µM) were incubated in 0.1 per cent trypsin (Biochrom KG, Berlin, Germany) dissolved in tris-buffered saline (TBS) with $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) added ('lectin buffer', pH 7.6) for 15 min at 37°C. After the trypsin digestion the slides were incubated with the biotinylated lectins (PA-IL and PA-IIL, 10 µg/ml each) for one hour at room temperature. An incubation with a streptavidine-alkaline-phosphatase complex (ABC) followed. For visualization of the alkaline phosphatase enzyme reactivity, Naphtol-AS-biphosphate and hexatosized New Fuchsin were used as a substrate. The slides were then counterstained in Mayer's hemalum solution (Merck, Darmstadt, Germany), blued under running tap water and finally mounted with Crystal Mount (Biomeda, Foster City, CA).

For negative controls, control sections were used in which the lectin incubation had been omitted. The sugar specificity of the lectins was tested by incubating the slides with D-galactose and L-fucose (10 µM each) before the corresponding lectin staining. All the experiments were done in duplicate.

The slides were examined under a Zeiss Axioplan 2 microscope fitted with a Zeiss AxioCam MRc 5 digital camera (Care Zeiss Jena GmbH, Jena, Germany).

Results

CBF modulation by PA lectins

The CBF of control cultures was within the normal range of between 10 and 16 Hz. The effects of the PA-IL and PA-IIL on the CBF are illustrated in Figure 1. Remarkably, the sole addition of PA-IL (1 µM; $n = 9$) to the cultures caused an initial increase of CBF by 10.0 ± 4.4 per cent ($p = 0.05$) after one hour. Thereafter the beat frequency gradually decreased to 17.7 ± 11.9 per cent ($p < 0.005$) within five hours and then significantly ($p < 0.005$) increased again, reaching 26.7 ± 14.3 per cent of the basal value after 24 hours. In contrast, the application of PA-IIL (1 µM) resulted in a continuous decline and complete cessation of CBF within 24 hours ($n = 9$; $p < 0.005$) without the initial increase of beat frequency characteristic for PA-IL. Of particular significance, the long-term inhibitory effects of PA-IIL on CBF were abolished when co-incubated with PA-IL. Figure 1 shows that the simultaneous addition of the lectins also resulted in a significant ($p < 0.005$) decline in CBF within five hours and 24 hours (67.1 ± 13.1 per cent and 67.6 ±

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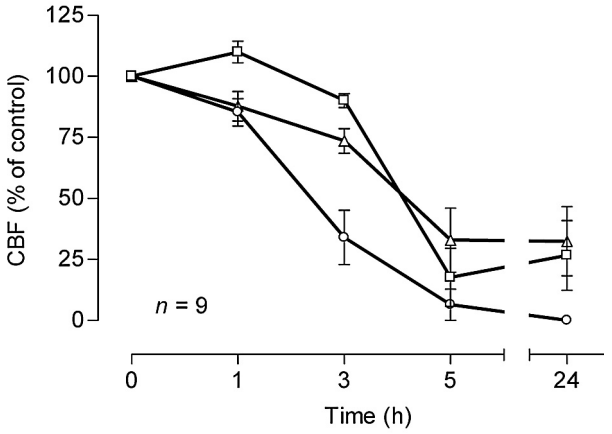

FIG. 1

Effects of PA lectins on nasal ciliary beat frequency (CBF) over a 24 hr period. The effects of solely given PA-IL (□) and PA-IIL (O) as well as the effects of both lectins in combination (Δ) on the CBF of nasal mucous membrane explants are shown. The lectins were added to the medium at a final concentration of 1 μM. Each value represents the mean ± SEM averaged over nine experiments.

14.15 per cent, respectively; $n = 9$); however, the decline was weaker than with PA-IL alone (82.3 ± 11.9 per cent and 73.3 ± 14.3 per cent, respectively).

The CBF-decreasing effects of PA-IL and PA-IIL could be effectively reduced when the lectins were co-incubated with the PA-specific sugars D-galactose and L-fucose (10 μM each) (Figure 2). In comparison to the control values, both sugars caused a marked decrease in the PA-IL- and PA-IIL-induced decline of CBF within five hours ($n = 3$ each). However, only D-galactose in the case of PA-IL and L-fucose in the case of PA-IIL ($n = 3$ each) efficiently inhibited the lectin-induced suppression of ciliary beat within 24 hours (Figure 2). These results suggest that both sugars inhibit both PA lectins when added

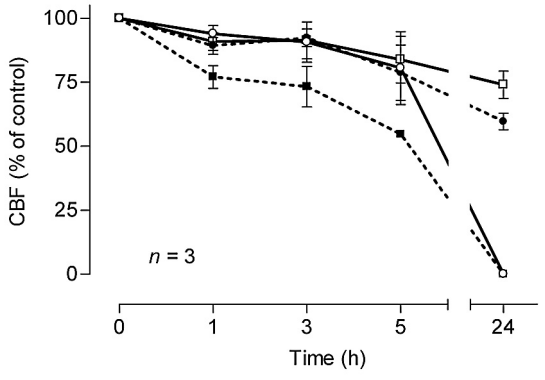

FIG. 2

Modulation of ciliary beat frequency (CBF) by PA lectins co-incubated with their specific sugars. The effects of PA-IL (*open symbols and straight lines*) and PA-IIL (*solid symbols and dotted lines*) co-incubated with D-galactose (*squares*) and L-fucose (*circles*) on the CBF of nasal mucous membrane explants are shown. The lectins and sugars were added to the medium at a final concentration of 1 μM and 10 μM, respectively. Each value represents the mean ± SEM averaged over three experiments.

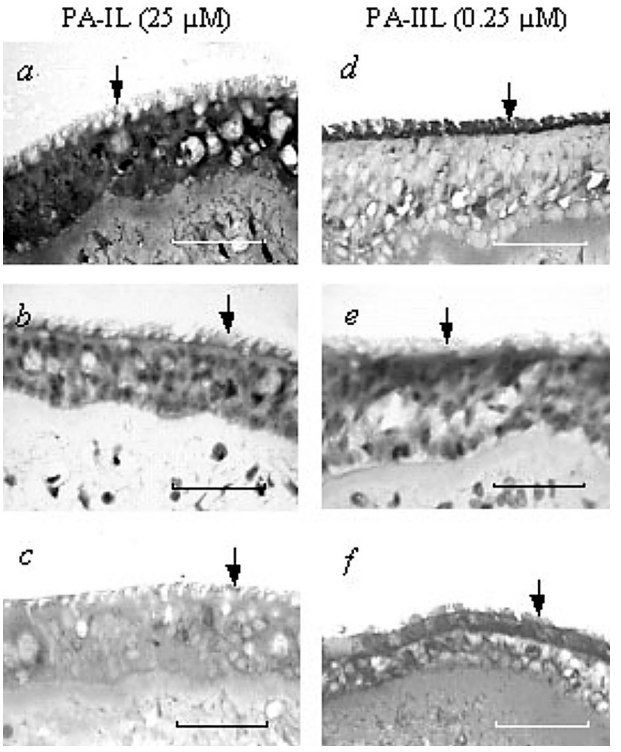

FIG. 3

Comparative histochemical analyses of the binding suppression of biotinylated PA lectins by their specific sugars in human nasal epithelium. (a) and (d): control without sugar treatment; (b) and (e): lectin binding after treatment with 100 μM L-fucose; (c) and (f): lectin binding after treatment with 100 μM D-galactose. Note the weakening of the PA-IL staining by L-fucose (b) and, even more distinctly, by D-galactose (c). In contrast, the binding of PA-IIL was more efficiently suppressed by D-galactose (f) than by L-fucose (e), which was particularly conspicuous on examination of the cilia. Arrows indicate ciliated borders. Bars represent 100 μm.

simultaneously, although preferences of D-galactose for PA-IL and of L-fucose for PA-IIL seem to exist. Apart from its CBF inhibitory effects, PA-IIL, unlike PA-IL, induced heavy mucus secretion in the tissue cultures. This additional effect of PA-IL was suppressed, when D-galactate was co-applied.

Ciliary lectin binding

Binding sites for both biotinylated lectins, PA-IL and PA-IIL, all around the exposed surface of the explant were observed (Figures 3a and d). In particular, an intense staining of the cilia with PA-IIL (0.25 μM) could be detected (Figure 3d). The ciliary staining by PA-IIL, could almost be completely inhibited by co-incubation of the lectin with its specific sugar L-fucose (100 μM; Figure 3e), and clearly weakened by simultaneous addition of D-galactose (100 μM) to the lectin buffer (Figure 3f).

Biotinylated PA-IL (25 μM) could also be visualized on the explants (Figure 3a). However, the cilia were less intensively stained by PA-IL than by PA-IIL. Very little or no reaction product was observed when PA-IL was co-incubated with its specific sugar D-galactose (100 μM; Figure 3c). In contrast, the binding capacity of PA-IL was only

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slightly reduced by simultaneous application of L-fucose (100 μM; Figure 3b).

Discussion

The opportunistic pathogen *P. aeruginosa* synthesizes and releases a wide spectrum of noxious toxins and hydrolytic enzymes as well as ciliostatic factors which could have cytotoxic effects on respiratory epithelial cells and facilitate the bacterial colonization by reducing mucociliary clearance.[14–16] *P. aeruginosa* also produces two lectins, PA-IL and PA-IIL, which function as adhesins[6] and represent virulence factors, as at least suggested for PA-IL.[8] As part of their pathogenetic mechanisms, the two lectins inhibit the ciliary beat, which has been analysed qualitatively for PA-IL[8] and quantitatively for PA-IIL.[9] In this study, the kinetics of the ciliary beat inhibition by PA-IL in human airway epithelia were elucidated quantitatively for the first time and compared with those of PA-IIL. Moreover, the joint action of these two lectins on ciliary beat was investigated. The ciliotoxic action of the two PA lectins is of particular interest for therapeutic purposes as the interaction of the lectins with cilia can be interfered with by the use of simple sugars as exemplified by PA-IIL.[9] The therapeutic value of blocking lectin-cilia interactions by simple sugars had already been demonstrated in one case as a proof of the principle.[17]

The present experiments demonstrate that the action of PA-IL (1 μM) on ciliary beat is triphasic. The short-term action (up to five hours) of this lectin was characterized by an initial acceleration of CBF (up to one hour) followed by an increasing reduction in CBF until five hours after application. After 24 hours a slight increase in CBF could be observed again. Due to the initial acceleration of CBF a PA-IL-induced cross-linking of the cilia, which would be an immediate effect, appears unlikely. Presumably the CBF-modulating effects of PA-IL are caused by binding of the lectin to carbohydrate residues of transmembrane proteins, leading to conformational changes in one or more receptor glycoprotein(s), thereby triggering a (patho)physiological response. It could be speculated that the CBF-accelerating action of PA-IL is based on excitatory mechanisms like an increase of $[Ca^{2+}]_i$[18] or an activation of cAMP-dependent protein kinase (PKA) or cGMP-dependent protein kinase (PKG)[19] in the ciliated cells. Interestingly, earlier studies revealed no cilio-inhibitory effect of PA-IL at concentrations ≤ 10 μg/ml after a 24 hour incubation period, whereas PA-IL at higher concentrations (≥ 50 μg/ml) seemed to interfere with normal ciliary function by toxicity for epithelial cells or by epithelial shedding and loss of cilia.[8] As PA-IL causes severe respiratory epithelial damage and may facilitate bacterial persistence during *P. aeruginosa* infection it was hypothesized that this lectin represents a virulence factor.[8]

In contrast to PA-IL, which initially increases CBF, the action of PA-IIL (1 μM) was characterized by an immediate and continuous reduction in CBF in the present study. This is in accordance with earlier findings.[9] The CBF-accelerating net effect of PA-IL observed during the first hour of incubation could not be demonstrated in the case of PA-IIL and may indicate different short-term effects of the two lectins.

The present experiments also demonstrate that the CBF-reducing effects of PA-IL are weaker at any time than those of PA-IIL. Apart from the different mechanisms of action this may be caused by the different temperature profiles of the lectins. It has been shown that the haemagglutinating activities of PA-IL decrease, while those of PA-IIL increase on an increase in temperature, which points to a preferential adaptation of *P. aeruginosa* to a saprophytic rather than to a parasitic designation.[12] As the present CBF measurements were carried out at a relatively high temperature (35°C) this could also explain the higher degree of effectiveness of PA-IIL in reducing CBF than PA-IL. This observation is corroborated by the results of the histochemical stainings, which were carried out at 21°C, where a much weaker binding of PA-IL in comparison to PA-IIL to the cilia was observed. A 100-fold higher concentration of PA-IL was necessary to clearly visualize a staining of this lectin.

The lectins PA-IL and PA-IIL are similar in subunit size, composition and properties, but differ in their respective saccharide specificities. PA-IL is D-galactophilic, whereas PA-IIL is L-fucose-specific.[2] Accordingly, the long-term effects (24 hours) of PA-IL in this study could be effectively inhibited by its specific sugar D-galactose. However, the short-term inhibitory effects of PA-IL could also be reduced by L-fucose. Correspondingly, a reduction of the short-term CBF-modulating effect of PA-IIL by co-incubation with L-fucose and D-galactose could be observed, whereas the long-term effects were only inhibited by L-fucose. The latter observation agrees with earlier findings that the inhibitory effects of PA-IIL can be abolished *in vitro* by pre-incubating the lectin with its specific sugar, namely fucose.[9] The present results indicate that both closely related sugars compete for the same, lectin-relevant receptors although preferences of D-galactose for PA-IL and of L-fucose for PA-IIL seem to exist.

Our results demonstrate that PA-IL and PA-IIL, if applied simultaneously, modulate the ciliary beat of normal human airway epithelium in a similar time-dependent manner as the individual lectins. Moreover, the CBF reduction after five and 24 hours was even less than with each lectin individually. This confirms our hypothesis that the lectins compete for the same binding site(s) in the glycocalyx of cilia. Interestingly, the initial rise in CBF by PA-IL was also abolished when co-applied with PA-IIL. It can be hypothesized that the CBF-accelerating (intrinsic) effect of PA-IL after one hour is masked by the inhibitory action of PA-IIL, or that binding of PA-IL to the corresponding carbohydrate residues is prohibited by conformational modulations induced by PA-IIL.

Some of the *P. aeruginosa* products such as proteases and rhamnolipids have been shown to induce mucus hypersecretion.[20–22] In the present study, inductions of heavy mucus secretions by PA-IIL could be observed.[8] This hypersecretion could be effectively prevented by co-application of D-galactose, indicating a PA-IIL-specific secondary effect dependent on prior galactose binding. PA-IIL binding to galactose

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residues in the glycocalyx possibly facilitates the production and/or secretion of mucus via the binding to regulatory proteins of globlet cells, as already hypothesized earlier.[9]

- ***Pseudomonas aeruginosa* is the most important bacterial pathogen associated with respiratory tract infections, especially in patients with cystic fibrosis**
- **The present paper was designed to study the joint action of the lectins associated with this organism, namely PA-IL and PA-IIL, on the ciliary beat frequency of nasal mucous membrane explants. The binding suppression of these lectins to the nasal epithelium was examined by sugar inhibition experiments**
- **Simultaneous application of the lectins resulted in a weaker inhibition of beat frequency than did each lectin separately. The lectin-specific sugars galactose and fucose inhibited the action of both lectins although preferences of D-galactose for PA-IL and of L-fucose for PA-IIL existed**
- **These data suggest that PA-IL and PA-IIL compete for the same binding site(s). These interactions have to be taken into account in designing sugar-based strategies with *P. aeruginosa* infections**

Conclusions

In conclusion, the comparative study of PA-IL and PA-IIL elucidates competition for the same binding sites and, at least in part, different actions of the investigated lectins. These new findings might be included in the further development of therapeutic strategies in the case of *P. aeruginosa* infections. Moreover it seems reasonable to test the lectin-inhibitory effects of the 'specific' sugars when applied in combination.

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