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The Role of the Cell Surface Components of *Pseudomonas aeruginosa* in Virulence

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Summary

A variety of roles in virulence, both direct and indirect, can be ascribed to the outer membrane components of *Pseudomonas aeruginosa*. These include the endotoxic nature of *Pseudomonas* lipopolysaccharide (LPS) owing to a lipid A region which cross-reacts antigenically with other gram-negative lipid As. In addition, LPS composition has a direct influence on serum susceptibility of *P. aeruginosa*. A variety of outer membrane proteins are mitogenic for murine B lymphocytes and immunogenic in both animals and humans. Presumably, these properties could be involved in modulation of virulence. A major role in the virulence of *P. aeruginosa* in antibiotic-treated humans is afforded by the low outer membrane permeability and consequent intrinsic antibiotic resistance of this organism. Some of these properties have now been manipulated to provide new methods of anti-pseudomonal therapy.

Introduction

In the past few years, considerable attention has been focused on the role of extracellular products and excreted cellular components as virulence determinants for gram-negative bacteria. For example, Young [33] summarized work describing no fewer than 15 different excreted products implicated as virulence factors in *Pseudomonas aeruginosa* pathogenicity. However, if we take the broadest definition of pathogenicity, that is, the ability of a bacterium to grow in a host and produce sufficient quantities of toxic or harmful products to cause damage to the host, it becomes clear that many different cellular components can be defined as virulence factors. In particular, cell surface (outer membrane) components are important since they are involved in primary interactions with the host immune system and have also been implicated in resistance to the serum bactericidal response, to phagocytosis, and to antibiotics (which could be considered the major handicap to the growth of a gram-negative bacterium in a human host). In this paper, I will expand on this concept with specific reference to our own research on *P. aeruginosa*.

In the past 30 years, *P. aeruginosa* has emerged as a primary opportunistic pathogen in immunocompromised patients. It is now recognized as one of the three major causative agents of gram-

negative septicemia in North America [34]. In patients who develop *P. aeruginosa* bacteremia, the onset of disease can be rapid and often leads to death [34]. *Pseudomonas* is also the major pathogen associated with chronic and fatal lung disease in patients with cystic fibrosis. In these clinical situations, two factors are of major importance, the failure of the host immune system to counteract the bacterial infection (which may be caused, in part, by the fact that some of these patients are immunocompromised) and the poor success of antimicrobial chemotherapy. In each case, the cell surface (outer) membrane is of major importance.

P. aeruginosa, being a gram-negative bacterium, has two cell envelope membranes separated by a single layer of peptidoglycan [26]. The outer membrane is an asymmetric bilayer containing phospholipids on the inner leaflet of the outer membrane and lipopolysaccharide (LPS) as the major lipidic molecule of the outer (cell surface) monolayer. LPS is an amphiphilic molecule containing a hydrophobic region (lipid A, also known as endotoxin) which has five or six fatty acids linked to diglucosamine phosphate. Covalently attached to this is the rough oligosaccharide core containing in its proximal portion an unusual sugar, 2-keto-3-deoxyoctanate (KDO), as well as a variety of heptose and hexose residues [26]. The rough oligosaccharide core may be substituted with a variable number of repeated trisaccharide to pentasaccharide units called the O-antigen [26].

In addition to LPS and phospholipids, the *P. aeruginosa* outer membrane contains between five and nine polypeptides (depending on growth conditions) which are present in high copy number (10^4-5×10^5 copies per cell) and thus are called major outer membrane proteins. Three of these major proteins, F, D1, and P, have been shown to form hydrophilic channels of defined size through the outer membrane [1, 2, 11] and thus bear the common name porins. In addition, protein F as well as the outer membrane lipoproteins H2 and I, are strongly noncovalently bound to the underlying peptidoglycan [13] and may have structural roles. Protein H1 has been implicated in the development of resistance to polycationic antibiotics and chelators in media containing low concentrations of divalent cations [24]. This paper will focus upon the role of specific outer membrane components of *P. aeruginosa*, namely LPS and porin protein F, as virulence determinants, using this broad definition of virulence.

Lipopolysaccharide

Increasing attention has been focused on the LPS of *P. aeruginosa* in recent years (see [32] for review). However, although the composition has been partially defined, the structure of some portions of the LPS, especially the rough core and lipid A portions, is poorly understood. There are a number of reasons for this, including the high molecular weight and substantial variety of constituents of LPS [32], the complexity and lack of available standards for some of these constituents [32], the heterogeneity within given populations of LPS [7, 32], and the fact that many

of the more common analytic procedures cause degradation and loss of some of the components of LPS [7, 32]. Three recent developments may have a major impact on LPS analyses: the development of an LPS isolation procedure which allows recovery of about five times as much LPS from *P. aeruginosa* strains as previously used methods [7], the developing use of nuclear magnetic resonance as a nondegradative method for analyzing sugar compositions [17, 31] and preliminary studies of monoclonal antibodies as specific probes for assisting the analysis of LPS composition [18, 21]. Since this work is in its early stages, the discussion below will consider LPS as a simple tripartite structure (lipid A, rough core, O-side chain).

Antigenicity: Common and Unique Determinants

LPS from *P. aeruginosa*, like other bacterial LPS, has a variety of interesting biologic properties, including mitogenicity towards mouse B lymphocytes [27], pyrogenicity in rabbits, tumor-nectrotizing activity, endotoxicity, interferon-inducing activity, adjuvanticity [29], and strong immunogenicity [27]. With the exception of this latter trait, most of these properties are thought to be caused by the lipid A portion of the LPS [27, 29]. Because lipid A from *P. aeruginosa* has similar biologic properties to the lipid A of *E. coli*, *Salmonella*, and other bacteria [3], we investigated the antigenic similarities between the lipid A moieties of these organisms. To do this, we obtained monoclonal antibodies specific for the lipid A of *E. coli* [22]. These monoclonal antibodies were isolated by Centocor Corporation, Pennsylvania, after the fusion of myeloma cells with the B lymphocytes of mice injected with intact *E. coli* J5 cells. Two of these antibodies, 5E4 and 8A1, reacted with purified lipid A from *Salmonella minnesota* and *P. aeruginosa*; in addition, they cross-reacted with outer membrane and LPS preparations from 36 *P. aeruginosa* strains and 22 other gram-negative bacteria from the families *Vibrionaceae*, *Enterobacteriaceae*, *Rhizobiaceae*, and *Pseudomonadaceae*, but not with the cell walls of three gram-negative bacteria. One of the monoclonal antibodies, 8A1, interacted on Western blots with a similar fast migrating band corresponding to the rough LPS (rough core plus lipid A) from strains of *E. coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, *Salmonella minnesota* (Fig. 1), and with strains representing all 17 serotypes of *P. aeruginosa* [23].

This extensive cross-reactivity of the lipid A-specific monoclonal antibodies explains, in part, the protective effects of antisera to *E. coli* J5 organisms against bacteremia caused by other gram-negative bacteria including *P. aeruginosa* [4]. The strong conservation of these antigenic determinants throughout gram-negative bacteria suggests that lipid A has an important role in these bacteria and may indicate a common evolutionary lipid A molecule.

In contrast, we have shown that monoclonal antibodies against the LPS rough core of our wild-type *P. aeruginosa* strain interact only with LPS strains representing 4-6 of the 17 serotypes [15, 21], suggesting that the rough core of *P. aeruginosa* is heterogeneous. A monoclonal antibody against the O-antigen of serotype 5 LPS is relatively specific for this LPS, in agreement with the

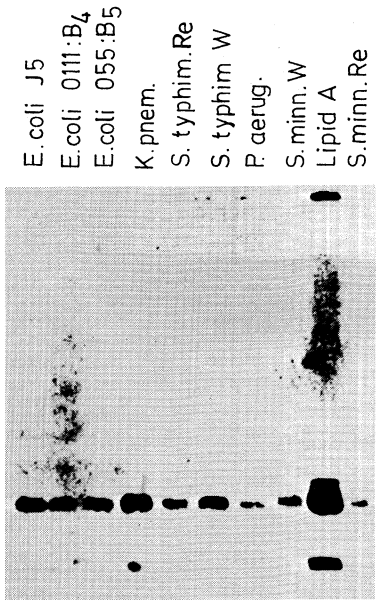

Fig. 1. Reactivity of a monoclonal antibody to lipid A with various lipopolysaccharides (LPS) and lipid A. Purified LPSs were separated by SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose by the Western blotting procedure. They were than interacted with monoclonal antibody 8A1 followed by ^{125}I-labeled protein A and autoradiography to reveal the position of the monoclonal antibody-reactive material. The LPSs used were derived from the strains indicated above the respective lanes. The lipid A was derived from *S. minnesota*. The band stained in all preparations is equivalent to rough LPS (i.e., rough core plus lipid A), except, of course, in the lipid A lane

concept that LPS O-antigen heterogeneity is the basis for serotyping in *P. aeruginosa* [32].

Rough, LPS-Altered Strains Associated with Disease

The overwhelming majority of *E. coli* and *Salmonella* strains isolated from human infections are O-serotypable, suggesting that they possess an O-antigen attached to the lipid A-rough core regions of their LPS. Such strains are called smooth strains owing to their characteristic colony morphology. When LPS is isolated from such strains and separated according to molecular size on SDS-polyacrylamide gel, extreme heterogeneity is observed in the length of the O-side chain (i.e., number of repeating trisaccharide units); however, a large number of LPS molecules contain less than four repeating units and somewhere between 5% and 62% of the LPS is rough, i.e., lacking O-side chains (e.g., [9]). Despite this, the rough cores of the LPS are masked as judged by the resistance of these smooth strains to phages which use this portion of the LPS as their receptor.

P. aeruginosa also shows substantial LPS heterogeneity, although a larger amount of rough LPS, estimated as between 75% (A. Kropinski, personal communication) and 90% [15, 32] of the total LPS, is present. We studied a series of *P. aeruginosa* strains isolated from patients with cystic fibrosis [15]. Many of these strains were not serotypable and exhibited two patterns; failure to agglutinate with any typing sera, or polyagglutinability with more than one typing serum. We were able to demonstrate that these abnormal typing patterns, occurring in up to 60% of cystic fibrosis isolates [35], correlated with deficiencies in the amounts of O-antigen in the LPS of these strains [15]. Admittedly, such strains exist in a unique ecological niche (the lungs of patients with cystic fibrosis), but these observations have

several important implications. First, they call into question the broad application of O-antigen-based antisera for serotyping *P. aeruginosa* [35]. Second, they tend to suggest that immunotherapy using the recent LPS-based vaccines will not be successful in some disease situations. Third, they demonstrate that there is no obligate association between possession of an O-antigen and ability to persist in a host. While other studies have suggested this for intracellular pathogens like *Yersinia pestis* [8] and *Neisseria* [30], this has not been widely recognized among organisms which do not protect themselves by internalization in host cells. Significantly, *P. aeruginosa* infections can persist in the lungs of cystic fibrosis patients for more than 10 years and are the primary infections associated with terminal lung disease in these patients [21].

Role in Serum Resistance

Using these rough strains from the lungs of patients with cystic fibrosis as well as rough, LPS-altered, phage-resistant derivatives of our laboratory wild-type *P. aeruginosa* strain PAO1, we were able to demonstrate a dramatic enhancement in susceptibility to killing by normal human serum of these rough strains when compared with smooth strains, including both patient isolates and strain PAO1. Indeed, smooth strains were able to grow in concentrations of serum exceeding 40% (vol/vol), whereas rough strains were killed by 2% serum [15]. Similar observations have been made in other organisms [28] and it has been proposed that this result reflects blocking of some components by the O-side chains of LPS. Alternatively, I recently proposed [10] that differences in the relative affinities of smooth and rough LPS for Mg^{2+} can explain these phenomena, given evidence that complement attacks an LPS divalent cation binding site on the surface of the outer membrane. In any case, the high susceptibility to serum of the rough *P. aeruginosa* strains from the lungs of cystic fibrosis patients may help explain why these strains are apparently non-invasive and cystic fibrosis patients rarely, if ever, succumb to *P. aeruginosa* bacteremia.

Outer Membrane Proteins

Mitogenic Response

Unlike LPS, none of the *P. aeruginosa* outer membrane proteins appears to be toxic for mice. For example, injection of 5 mg/kg protein F into mice causes no lethality and no obvious clinical symptoms (E.C.A. Mouat and R.E.W. Hancock, unpublished work). Outer membrane proteins are, however, like LPS [5] capable of interacting with host cells, namely B lymphocytes. Proteins F, H2, and I in picomole amounts were each capable of stimulating resting murine B lymphocytes to proliferate [5]. This capability was not diminished in C3H/Hej mice which did not interact with *P. aeruginosa* LPS [5]. T cells were not mitogenically stimulated by these proteins. Interestingly, two of these proteins are outer membrane lipoproteins and their mitogenic effects can be compared

to that of Braun's outer membrane lipoprotein of *E. coli* [19]. The significance of these observations has not, as yet, been elucidated.

Antigenicity

We have demonstrated that outer membrane proteins E, F, H2, and I are strongly antigenically conserved in *P. aeruginosa* strains by using polyclonal rabbit antisera and the Western immunoblotting technique [22]. In addition, we have demonstrated, using monoclonal antibodies, the conservation, among 16 clinical isolates and the 17 serotypes of *P. aeruginosa*, of single antigenic sites on proteins H2 and F [13, 21]. In the case of the two protein F antigenic sites recognized by two separate classes of monoclonal antibodies, indirect immunofluorescence studies have shown that both of these common antigenic sites are surface located; in contrast, the epitope of protein H2 recognized by monoclonal antibody MA1-6 is buried in smooth *P. aeruginosa* strains, but exposed in rough LPS O-antigen-deficient mutants [21]. One of the monoclonal antibodies against protein F, MA4-4, protects mice against subsequent lethal challenge by a virulent *P. aeruginosa* strain PA103 (Hancock and Mouat, manuscript submitted).

These data pose a major question; given the existence of conserved, surface-exposed antigenic sites on protein F of *P. aeruginosa* strains, why are the *P. aeruginosa* strains not rapidly cleared? One possible answer might be that *P. aeruginosa* protein F is weakly immunogenic in vivo or that these antigenic sites are masked. In agreement with this, we have been able to isolate insertion sequence mutants of *P. aeruginosa* strain PAO1 in which these antigenic sites are masked in whole cells, but not in membrane preparations (Angus and Hancock, unpublished work). Furthermore, the sera of four cystic fibrosis patients which were collected shortly after their first episode of *P. aeruginosa* lung infection, had antibody titers against outer membrane proteins that did not differ from those of uninfected patients (although multiply infected patients had extremely high antibody titers to these proteins [16]). Also, four patients with bacteremic *P. aeruginosa* infections did not have antibodies against protein F. In contrast, the sera of rats infected for only seven days with *P. aeruginosa* using the agar bead model demonstrated antibodies against protein F [18]. Thus, while protein F clearly has the potential to be immunogenic in vivo, as demonstrated by our studies using chronically infected rats [18] and cystic fibrosis patients [16], it is uncertain as yet whether antibodies to protein F contribute to clearance of *P. aeruginosa* or whether they are produced too late or in too small quantities to influence these infections.

Restricted Outer Membrane Permeability as a Virulence Factor

The outer membrane shields gram-negative cells against many toxic agents and potentially harmful enzymes in the host. For example, intact *P. aeruginosa* cells are resistant to bile salts, proteases, lysozyme, and phospholipases ([10]; unpublished work). In addition, *P. aeruginosa* demonstrates substantial resistance to antibiotics and disinfectants. This provides an explanation for its rise in prominence as a pathogen over the past 30-40 years.

Treatment of patients with antibiotics would tend to select, over time, for more resistant microorganisms. Significantly, *P. aeruginosa* infections are often hospital-acquired complications of other underlying diseases [34]. The high intrinsic resistance of *P. aeruginosa* to germicidal agents would favor its persistence in hospital environments.

The primary reason for the high intrinsic resistance of *P. aeruginosa* to most commonly used antibiotics is the low permeability of its outer membrane [10, 25]. This low outer membrane permeability appears to be due to the low efficiency in pore formation across the outer membrane of protein F, the major porin of *P. aeruginosa* [25]. We have estimated that despite the larger channel size of porin protein F pores (which are threefold greater in area than *E. coli* porin pores), that only 1 in 400 of the 100 000 trimers in the outer membrane forms active functional pores. The resultant decrease in the rate of permeation across the outer membrane decreases the exposure of the interior of the cell to antibiotics and increases the efficiency of other defense systems (e.g., the low level of constitutive β-lactamase in the periplasm of *P. aeruginosa*). Interestingly, it could be argued that the large channel size of *P. aeruginosa* porin protein F 2.3 nm diameter [1]) is itself significant in pathogenesis. Thus, one could consider a scheme whereby the excreted proteases of *P. aeruginosa* digested host proteins into large peptides. These large peptides could be taken up through protein F channels and serve as substrates for peptidases located on the periplasmic surface of the cytoplasmic membrane [20]. In agreement with this, proteolytic *P. aeruginosa* strains grow more rapidly on burnt skin extracts than nonproteolytic strains [6]. Similarly, one might argue that large channels would enhance the uptake of hydrophobic compounds produced as a result of the action of the excreted lipases of *P. aeruginosa*.

Conclusions and Perspectives

With the exception of the lipid A (endotoxin) portion of LPS, it is hard to argue that outer membrane components are classical virulence factors. However, in the broadest definition of virulence, outer membrane components contribute significantly to the ability of *P. aeruginosa* to grow in a host. Thus, since outer membrane proteins and LPS are present on the surface of *P. aeruginosa*, they are the major structures which interact with the host immune system and consequently help determine the outcome of the infection. In addition, the low permeability of the *P. aeruginosa* outer membrane to extrinsic agents like antibiotics and disinfectants, and to host factors like serum (for smooth strains only), lysozyme, proteases, lipases, and bile salts, etc., can be a major factor in determining the virulence of *P. aeruginosa* during bacteremic infections. Having identified two major contributions of the outer membrane to the course of *P. aeruginosa* infections, we have begun to design therapeutic strategies against this organism. One of these strategies involves the use of monoclonal antibodies against protein F as passive vaccines (see the section on antigenicity of outer membrane proteins) in an attempt to bolster the host immune system. A second strategy consists of our recent

finding that a group of diverse compounds, which we have given the group name "permeabilizers", will increase the permeability of the outer membrane of *P. aeruginosa* to β-lactams, lysozyme, and hydrophobic compounds [12]. Since low outer membrane permeability is a significant impediment to antimicrobial therapy, we hope that one of these permeabilizer compounds, given together with an antibiotic, will provide a more successful method of therapy.

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