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Carbohydrate Research 338 (2003) 2549–2556
CARBOHYDRATE RESEARCH
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Perspective/Review

Promises and pitfalls of *Pseudomonas aeruginosa* lipopolysaccharide as a vaccine antigen

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Received 2 April 2003; accepted 17 June 2003

Abstract

Antibodies directed to the *Pseudomonas aeruginosa* lipopolysaccharide (LPS) O-antigens have clearly shown to mediate the most effective immunity to infection caused by LPS-smooth strains. Such strains are major causes of disease in immunocompromised hosts such as burn or cancer patients, individuals in intensive care units, and those who utilize extended-wear contact lenses. Yet producing an effective vaccine composed of non-toxic, immunogenic polysaccharides has been challenging. The chemical diversity among the different O-antigens representative of the 20 major serotypes, plus additional diversity among some O-antigens representing variant subtype antigens, translates into a large degree of serologic variability that increases the complexity of O-antigen specific vaccines. Further complications come from the poor immunogenicity of the major protective epitope expressed by some O-antigens, and a large degree of diversity in animal responses that preclude predicting the optimal vaccine formulation from such studies. Nonetheless human trials over the years of vaccines eliciting O-antigen immunity have been encouraging, though no vaccine has yet been fully evaluated and found to be clinically efficacious. Newer vaccine approaches such as using polysaccharide–protein conjugates and passive therapy with monoclonal or polyclonal immune sera offer some additional means to try and produce an effective immunotherapeutic reagent for this problematic pathogen.

Keywords: *Pseudomonas aeruginosa*; Lipopolysaccharide; Vaccine; Monoclonal antibody; Immunochemistry

Contents

1. Introduction

Since the advent of modern medical practices that have made nosocomial infections an ongoing problem, *Pseudomonas aeruginosa* has been one of the major pathogens responsible for a wide variety of infections and illness.[1–5] It is particularly problematic for patients in

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doi:10.1016/S0008-6215(03)00312-4

intensive care units, where ventilator-associated pneumonia remains a serious complication of therapy,[3,4,6] for those with an underlying immunocompromised state such as arises from severe burns, human immunodeficiency virus infection or chemotherapy for cancer,[2] for patients with indwelling urinary catheters and for patients with venous-access catheters or other artificial medical devices.[3] In the community, *P. aeruginosa* can be a serious cause of ulcerative keratitis in individuals who wear contact lenses[7] and its notoriety as the major reason for chronic morbidity and the ultimate early mortality in patients with cystic fibrosis is well established.[1,8] Given this broad-ranging ability to cause infection and severe disease, finding immunotherapeutic approaches to *P. aeruginosa* infections is clearly justified.

1.1. *P. aeruginosa* LPS structure and vaccine potential of LPS components

In spite of over 30 years of active research[9,10] finding an effective *P. aeruginosa* vaccine is not imminent. A major part of the problem is that it has long been known that the most effective antigenic target of immunity is the O polysaccharide portion of the organism's cell surface lipopolysaccharide (LPS).[11] *P. aeruginosa*, like many Gram-negative bacteria, also has a conserved core LPS region consisting of two parts (Fig. 1) but antibodies to these structures have never shown any potential for protective efficacy. The inner core region of the *P. aeruginosa* LPS proximal to the lipid A backbone is highly conserved, consisting of two KDO molecules and two heptose molecules which are substituted by various phosphate groups and by a 7-O-carbamoyl group. There is also a conserved galactosamine residue that is N-acylated by an L-alanyl group[12,13] and links the inner and outer core. The outer core is somewhat more variable among the different serogroups, although glucose and rhamnose are the usual monosaccharides present. The outer core is synthesized as two distinct glycoforms, one of which is able to accept the O side chain substituents and one of which does not.[13] Also, a D-rhamnan polymer, referred to by some as the 'A-band' LPS, is associated with the *P. aeruginosa* LPS.[14–16] Some studies have indicated that the A-band LPS and the O-antigen, also referred to in the literature as the 'B-band' LPS, are on the same molecule[17] whereas other studies suggest they are separate.[18,19] Regardless, neither the inner or outer cores nor the rhamnan polymer have proven to be effective targets for vaccine development.[20]

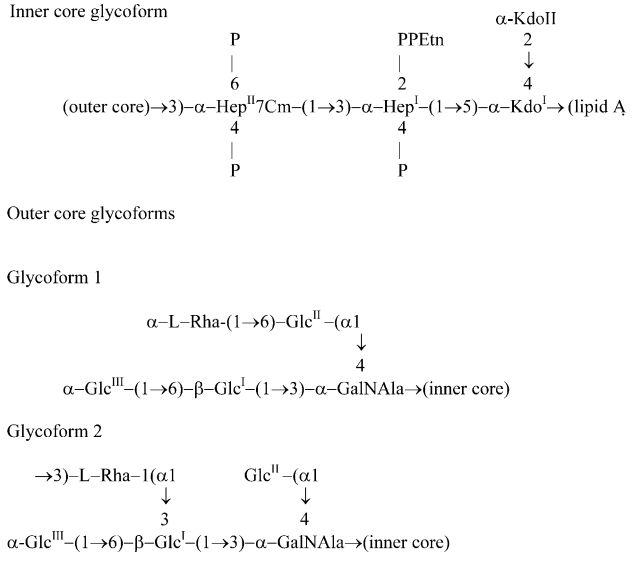

Fig. 1. Structure of the inner and outer core glycoforms of *Pseudomonas aeruginosa* immunotype 1 lipopolysaccharide. All sugars are in the pyranose form and have the D-configuration. The inner core consists of two 2 Kdo (Kdo^I and Kdo^{II}) and 2 Hep residues, one of which, Hep^{II}, is substituted with a carbamoyl residue. The two Hep residues are non-stoichiometrically substituted with phosphates. Linking the outer core to the inner core is a GalN residue N-acylated with alanine. The outer core consists of two glycoforms, both containing rha and glc residues. Only one of these two glycoforms can be substituted by the LPS O-antigen, but it differs depending on the strain. Structures based on studies by Knirel and coworkers.[12,13]

1.2. *P. aeruginosa* O-antigen specific vaccines-the issues

Given the non-protective nature of conserved core epitopes on the *P. aeruginosa* LPS, the focus of vaccine development has been on immunity to the O-antigen.[21] But turning this knowledge into an effective vaccine has been fraught with difficulty. Antibody to the O side chain mediates high levels of opsonic killing activity in vitro, which can be a strong correlate of an effective immune mediator.[22] Such antibody is also protective in almost all animal models of *P. aeruginosa* infection[23–27] and patients with higher levels of antibody to the LPS O-antigen of their infecting *P. aeruginosa* strain during the acute phase of their infection have a better survival probability.[28] But there is considerable chemical, and hence serologic, complexity to the *P. aeruginosa* O-antigen[29] which has made it difficult to develop highly immunogenic, broad based immunotherapeutic reagents.

It is not just the number of important serotypes that is challenging but even more problematic has been the finding that in many cases antibodies to the protective epitopes on the O side chains cannot be readily elicited by immunization.[30] The protective epitopes are not very immunogenic. Finding the means to make the protective epitopes immunogenic is not something that can be predicted from detailed knowledge of chemical structure. Furthermore, the immunogenicity of protective epitopes will vary depending on the species and genetic

background of the animal being used to evaluate immunogenicity.[30] Nonetheless, immunogenicity issues must first be worked out in laboratory animals with no idea of how this will predict the human immune response to O-side chain antigens.

2. Immunologic considerations for use of LPS-specific *P. aeruginosa* vaccines

2.1. Immunochemical properties of the *P. aeruginosa* lipopolysaccharide

Most of the details of the chemical structures of the *P. aeruginosa* LPS are now worked out due to the work of Knirel and colleagues.[29,31] A variety of immunologic classification schemes have also been published over the years, with the most generally accepted one now being based on the International Antigenic Typing Scheme (IATS) which defines 20 serogroups.[32–34] But structural analyses have revealed that there is a considerably greater degree of chemical variability in the *P. aeruginosa* O-antigens, with probably closer to 30 structures being commonly expressed among clinical isolates.[29,31] Contributing more to this confusion are findings that some of the strains of *P. aeruginosa* expressing closely related chemical structures are grouped within the same serogroup, while other strains with closely related O-antigens are grouped into different serogroups.[34–36] In some cases the strains grouped together exhibit cross protection in animal studies, perhaps justifying their grouping.[37] In other cases strains within a serotype do not give rise to much cross protection in spite of having closely related O-antigens.[38,39] As these conclusions are drawn from animal studies, principally involving immune responses of mice, rats and rabbits, we do not know whether the diversity of protective epitopes displayed on *P. aeruginosa* O-antigens would impact human immunity in a comparable way.

Several examples of these situations have been documented experimentally. In most studies reported over the past 3 decades the most common *P. aeruginosa* serotype is the IATS O6 serotype, equivalent to the Fisher immunotype I group defined in the early 1970s.[40] Chemical analysis by Knirel and co-workers found 5 variant O6 structures (Fig. 2).[41] A series of monoclonal antibodies to this O-antigen made by Pier and co-workers[27] showed there were shared epitopes that were targets of opsonic killing antibodies as well as epitopes unique to the subtype-variant structures. However, for the IATS O6 serotype strains, it was possible to find both murine monoclonal and human polyclonal sera obtained from volunteers immunized with the high molecular weight O-antigen of the Fisher IT-1 strain of the O6 serogroup that opsonized all serogroup members and provided protection in mice against infection caused by all serogroup members.

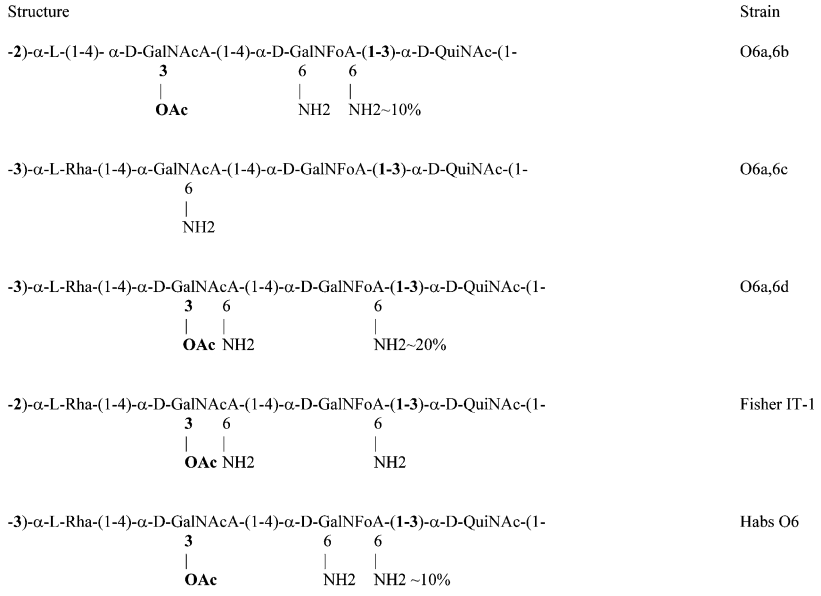

Fig. 2. Structural variation in the tetrasaccharide repeat unit of the serogroup O6 strains of *P. aeruginosa*. Parts of the structure in bold-faced type indicate places where chemical differences exist among the different structures. Some of the structures have been correlated with subtype epitopes, as indicated by the O6 designation listed under strain, based on serologic classification of Lanyi and Bergan.[34] It should also be noted that linkage of the first D-quinvosamine residue to the acceptor rhamnose residue in the outer core is in the β configuration, whereas within the tetrasaccharide repeat it is linked to the neighboring rhamnose via an α linkage.[13] D-GalNAcA (2-acetamido-3-*O*-acetyl-2-deoxygalacturonic acid); D-GalNFoA (2-formamido-2-deoxygalacturonic acid); D-QuiNAc(2-acetamido-2,6-dideoxyglucose).

Strain	Subtype Epitope	Structure
170003	O2a,2b	-4)-β-D-ManNAc3NA-(1-4)-β-D-ManNAc3NacA-(1-3)-β-D-FucNAc-(1- 3 | CH3=NH
IATS O16	O2a, 2b,2e	-4)-β-D-ManNAc3NA-(1-4)-β-D-ManNAc3NAc-A-(1-3)-β-D-FucNAc-(1- 3 **4** | | CH3=NH **OAc**
Fisher IT-3	O2a,2c	-4)-β-D-ManNAc3NA-(1-4)-α-**L-GulNAc**3NAcA-(1-3)-β-D-FucNAc-(1- 3 | CH3=NH
PAO1	O2a,2d	-4)-β-D-ManNAc3NA-(1-4)-β-D-ManNAc3NAcA-(1-3)-**α**-D-FucNAc-(1- 3 | CH3=NH
170006	O2a,2d,2e	-4)-β-D-ManNAc3NA-(1-4)-α-**L-GulNAc**3NAcA-(1-3)-**α**-D-FucNAc-(1- 3 | CH3=NH
Fisher IT-7	O2a, ?	-4)-α-**L-Gul**NAc3N)A-(1-4)-α-D-Man(NAc)2A-(1-3)-α-D-FucNAc-(1- 3 | CH3=NH
170007	O2a,2d,2f	-4)-α-D-ManNAc3NA-(1-4)-α-D-ManNAc3NAcA-(1-3)-α-D-FucNAc-(1- 3 **4** | | CH3=NH **OAc**
Also part of O-antigen of strain 170007; about 2:1 ratio of upper and lower structures		-4)-α-**L-Gul**NAc3NA-(1-4)-β-D-ManNAc3NAcA-(1-3)-α-D-FucNAc-(1- 3 **4** | | CH3=NH **OAc**

Fig. 3. Structural variation in the trisaccharide repeat unit of the *P. aeruginosa* serogroup O2/O5 strains. Parts of the structure in bold-faced type indicate places where chemical differences exist among the different structures. These have been correlated with subtype epitopes, as indicated, based on serologic classification of Lanyi and Bergan.[34] ManNAc3NAmA, (2-acetamido-3-acetamidino-2,3 dideoxymannuronic acid); ManNAc3NAcA, (2,3 diacetamido-2,3-dideoxymannuronic acid); GulNAc3NAcA, (2,3-diacetamido-2,3-dideoxyguluronic acid); FucNAc (2-acetamido-2,6-dideoxygalactose or *N*-acetyl fucosamine).

At the other end of the spectrum is the IATS O2/O5/O16 serogroup, which in fact also includes strains in serogroups O18 and O20.[35] Seven different structures have been found for these strains, which are related because they all produce an O-antigen with a comparable basic trisaccharide core unit.[42] Recent genetic studies indicate that strains that make these O-antigens have a highly conserved set of genes encoding the biosynthetic enzymes capable of producing these polysaccharides, and thus some of the structural variation may in fact arise from post-synthetic changes of the basic trisaccharide unit.[43] As seen in Fig. 3 the differences are due to changes in substituents on the hydroxyl groups such as acetates, the use of monosaccharide isomers for the first or second sugar in the repeat unit, and changes in the linkages among the monosaccharides. The fact that two of the monosaccharides are substituted aminouronic acids also raises the potential for random migration of acetyl groups between O-2 and O-3 carbons, potentially giving rise to antigenic variation. Some strains (i.e., *P. aeruginosa* 170007) are known to produce at least two different variant antigens with a high likelihood that this situation may be common in clinical isolates.

Attempting to correlate antibody-mediated protective efficacy with O-antigen structure has not been very meaningful. In the IATS O2/O5 group the LPS O-antigens from two strains can show high levels of cross protection but have more disparate chemical structures than two structurally closer antigens with low cross protective efficacy. For example, *P. aeruginosa* strain PAO1, whose genome has been sequenced, is classified as an O5 strain, which corresponds to the Fisher IT-7 classification. Yet the O-antigen structure of the PAO1 and IT-7 strains differ in the configuration and linkage of the first monosaccharide in the trisaccharide repeat

unit (Fig. 3); for PAO1 it is a β-(1→4)-linked D-ManNAc3NA unit whereas for strain IT-7 it is an α-(1→4)-linked L-GulNAc3NA unit. Interestingly, immunization against the LPS of one of these strains is highly protective against infection with the heterologous strain. In contrast, the same epimeric change occurs in the middle residue of the trisaccharide repeat of the Fisher IT-3 strain, classified as a serogroup O2 strain, wherein the α-(1→4)-linked L-GulNAc3NA unit is found instead of the β-(1→4)-linked D-ManNAc3NA unit of PAO1. A second difference is that the middle monosaccharide of the trisaccharide repeat unit is linked β-(1→3) to the neighboring D-FucNAc in the IT-3 strain and α-(1→3) to the D-FucNAc unit in strain PAO1 (Fig. 3). Yet the LPS O-antigens from these two strains are not cross protective. Immunization against strain PAO1 elicits high levels of cross protection to strain 170003,[38] which differs from strain PAO1 in the linkage between the middle β-(1→4)-linked D-ManNAc3NA unit and the terminal D-FucAc unit (Fig. 3*). But in strain IATS O16, the terminal D-FucNAc of the trisaccharide repeat has a 4-linked O-acetate, and immunization with strain PAO1 provides only minimal protection against this strain.[38] So overall, there is no clear-cut pattern of the relationship of structure to protective efficacy.

Using the opsonic killing assay as a surrogate measure of the potential protective efficacy of a multivalent *P. aeruginosa* LPS-specific vaccine, Hatano and coworkers[39] made a heptavalent vaccine consisting of high molecular weight O-polysaccharides of seven different *P. aeruginosa* strains. While the individual components were immunogenic and elicited opsonic antibody against the strain from which the vaccine was derived, there was a highly variable level of cross opsonic activity elicited to *P. aeruginosa* strains not used to make the vaccine antigens. In particular, within certain serogroups the vaccine antigen included in the formulation did not elicit antibodies to strains expressing variant, subtype epitopes but were still classified as being within the same serogroup. In addition, immune responses in mice and rabbits to the heptavalent vaccine were disparate,[39] indicating an inability of animal immunogenicity studies to predict what might likely occur in vaccinated humans. Thus the biology of the immune response to the purified *P. aeruginosa* O-antigens is species, and perhaps individual-animal specific, not predictable from structural information and thus not amenable to production of a vaccine with which one could anticipate high levels of efficacy in humans based on results from animal studies.

2.2. Human trials of *P. aeruginosa* LPS-based vaccines

Some of the earliest trials predicated on the observation of the high protective efficacy of antibody to LPS against *P. aeruginosa* infection use semi-purified LPS usually containing other bacterial components.[10,44–46] In the late 1960s, investigators from Parke–Davis company in the United States produced an LPS vaccine extracted from the 7 Fisher IT strains that was evaluated in burn patients[10,45,46] and children with leukemia or cystic fibrosis.[47,48] While there was evidence of efficacy in some of these trials, the vaccine ultimately proved to be too toxic for routine use. Indeed, in the trial in children with cystic fibrosis steroids had to be mixed in with the vaccine to reduce the otherwise unacceptable local toxic effects.[47,48]

Another similar vaccine was produced and tested in the 1970s and early 1980s by Burroughs-Wellcome in England.[49] This vaccine was prepared as an extract from the culture broth of multiple strains of different LPS serogroups, and the active immunogenic component was determined to be the LPS.[44,50] Efficacy trials, principally in burn patients, were encouraging,[51–53] with a high level of efficacy found in patients both actively immunized with the vaccine and passively injected with a hyperimmune globulin derived from sera of healthy individuals given the vaccine. However, after these trials the further development of this vaccine was not pursued.

A conjugate vaccine composed of the purified O-antigen from eight different *P. aeruginosa* serogroup strains covalently coupled to the exotoxin A antigen of *P. aeruginosa* was developed and tested in the 1980s through the 1990s by Swiss Serum and Vaccine Institute (now Berna Biotech Ltd.). A pivotal clinical trial was carried out in the early 1990s evaluating a hyperimmune globulin (IVIG), derived from donors immunized with the octavalent *P. aeruginosa* conjugate vaccine along with a 24-valent *Klebsiella* capsular polysaccharide and compared to outcomes from patients given an albumin placebo.[54] The trial was carried out in the intensive care units of 16 U.S. Veterans Affairs and Department of Defense Hospitals but found no significant difference in the overall severity and incidence of infection among treated and placebo groups.[54]

The same vaccine was found to be safe and immunogenic when given to cystic fibrosis patients without a history of *P. aeruginosa* colonization.[55] Published reports of observations of efficacy based on unblinded, non-randomized, and retrospective observations suggested some efficacy[56,57] but these were not properly designed and blinded clinical trials. The vaccine is currently being evaluated in a phase III clinical trial involving some 470 patients in 48 cystic fibrosis centers located in four European countries (http://www.berna-biotech.com/news/archive/article/20020205_01.html).

Phase I safety and immunogenicity trials of monovalent preparations of two different purified high molecular weight versions of the O-polysaccharide have been conducted.[58,59] The vaccines were safe and immunogenic, but in further pre-clinical trials of a heptavalent vaccine there were inconsistent immune

responses in animals and evidence of antagonism between some of the individual components.[39]

Several limitations of the O-antigen vaccine approach have arisen. In addition to the extensive serologic variation and incomplete immunogenicity of protective epitopes noted earlier, several O-antigens cannot be incorporated into a conjugate or high molecular weight O-antigen vaccine because they contain internal ketosidic linkages and thus cannot be adequately separated from the toxic lipid A component using acid hydrolysis.[29,31] Thus, IATS serogroup O3 and O7/O8 O-antigens are not included in the Berna Biotech vaccine, yet these strains do occur as clinical isolates in patients. If an effective vaccine is eventually produced that incorporates a limited number of *P. aeruginosa* O-antigens, there is the possibility that such immunologic selective pressure will cause strains expressing non-vaccine O-antigens to replace those that the vaccine can control. Such a situation has been observed with nasal carriage of *Streptococcus pneumoniae* in children given a heptavalent conjugate vaccine.[60]

2.3. Passive therapy and monoclonal antibodies to LPS O-antigens

As active immunization of patients at risk for *P. aeruginosa* infection is not always feasible due to the precipitous circumstances under which they frequently become more susceptible to infection, passive therapy with either IVIG or monoclonal antibodies represents a potential solution. In addition to the trial with hyperimmune antibody derived from the sera of healthy individuals immunized with the octavalent O-antigen-conjugate that was not successful,[54] a number of human monoclonal antibodies to *P. aeruginosa* O-antigens have been investigated for safety profiles in humans.[61–63] However, none have been evaluated in a full-fledged clinical trial whose results have been published. These antibodies tend to be highly protective in animal models[64–71] confirming the high level of immunity against *P. aeruginosa* mediated by antibody to LPS O-antigens. Currently production of a sufficiently multivalent set of human monoclonal antibodies to the major serogroup O-antigens of *P. aeruginosa* would be technically and economically daunting, if not prohibitive. One potential solution is the use of transgenic mice with human immune systems to produce these antibodies.[72] Another potential solution might lie in use of large transgenic animals genetically engineered to express a human immune system that can be immunized with something as simple as heat-killed bacteria or purified LPS which might have acceptable toxicity in such animals. Recovery of human antibodies from milk or serum might then be used to produce a passive therapy reagent.[73]

3. Implications

3.1. The future

Are the serologic variability, immunogenic properties and difficulties in bringing a *P. aeruginosa* LPS-based vaccine to clinical use going to prevent this from happening. While there are ongoing clinical trials based on such approaches and many funded investigators pursuing this by a variety of approaches, they are large hurdles to surmount to achieve this goal. For example, the clinical trial ongoing in cystic fibrosis patients has to confront the fact that the clinical disease is associated with LPS-rough, non-O-antigen producing strains that instead overproduce a different polysaccharide antigen, alginate[74,75] which gives rise to the typical mucoid morphology of these strains on agar medium. Vaccine efficacy will depend on preventing sufficient infection with the LPS smooth strains that initially colonize these patients and prevent their developing into the LPS rough, mucoid variants. One worry is that immunologic pressure from the patient's antibody response to the O-antigen selects out for the LPS rough phenotype. However, some data have suggested that the natural antibodies that cystic fibrosis patients develop in response to *P. aeruginosa* infection are of low affinity and not opsonic, whereas the vaccine-induced antibodies are of high affinity and mediate opsonic killing.[57]

Other concerns are not specific to *P. aeruginosa* LPS vaccine but the more general issue of how to develop a vaccine for a mostly nosocomial pathogen. At-risk populations such as police officers, fire fighters and military personnel could be vaccinated but it is not clear there is sufficient disease in this population to carry out a clinical trial. Patients most in need of an effective vaccine are primarily found in intensive care units and are not generally identifiable prior to this event. In addition, they often have severe underlying disease that could preclude achieving effective immunogenicity in these patients. Also, active vaccination would have to engender protective antibody before the major time period for the onset of development of clinical infection and disease.

Passive therapy offers a means for potential proof of principle that antibody to O-antigens will ameliorate *P. aeruginosa* infection, but the expense of both polyclonal and monoclonal antibodies could be prohibitive. In addition to transgenic mice expressing human immune systems for production of monoclonal antibodies,[72] development of larger transgenic animals such as goats with human immune systems could potentially be a means to produce cost effective polyclonal, multivalent passive therapy reagents as could transgenic animals producing antibodies in their milk.[73]

Which, if any, of these pathways will ultimately prove to be useful for producing an effective vaccine to *P.*

aeruginosa based on immunity to the LPS O-antigens is completely speculative. The inability to turn the knowledge of the efficacy of anti-O-antigen immunity into an effective vaccine in over 35 years highlights the challenges and difficulties faced in this arena. The ongoing persistence of *P. aeruginosa* as a major human pathogen, however, underscores the need for the development of effective immunotherapeutics. Perhaps other *P. aeruginosa* antigens, some perhaps not yet investigated to any degree, will be able to provide immunity comparable to that achieved with antibody to O-antigens without the same degree of problems. Advances in genomic analysis of bacterial antigen production, immunologic and biotechnological knowledge, and the increasing problem of antibiotic resistance will surely drive the future research paths towards achieving the goal of an effective *P. aeruginosa* vaccine.

Acknowledgements

Much of the work carried out by the author on *P. aeruginosa* LPS vaccines has been supported by NIH grant AI 22535 from the National Institutes of Allergy and Infectious Diseases.

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