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PSEUDOMONAS IMMUNOTHERAPY: A HISTORICAL OVERVIEW*

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SUMMARY

The historic development of vaccines to be used as immunotherapy for *Pseudomonas aeruginosa* infections, in various patient populations, is reviewed. Commentary is offered concerning the relevance of each approach in light of our current understanding of the pathological process of these infections.

INTRODUCTION

With the widespread use of penicillin and other antibiotics to control Gram-positive organisms in the 1950s, there was a shift in the type of microorganisms causing severe infections in a variety of patient populations. At this time the Gram-negative bacteria, particularly *Pseudomonas aeruginosa*, emerged as the greatest infectious threat to hospitalised patients. Although the introduction of new antibiotics with anti-pseudomonal activity showed initial promise in the control of *Pseudomonas*, the innate capacity of this organism to become resistant to newly introduced antibiotics soon became a problem in treating these infections. Thus, alternative means were sought to treat and prevent *Pseudomonas* infections. Extensive research studies on the mechanisms of

Table 1: *Pseudomonas* virulence associated and cellular factors used or suggested as antigens for immunotherapy

Lipopolysaccharide
Exotoxin A
Ribosome
Flagella
Pili
High-molecular-weight polysaccharides
Alginate/mucoid exopolysaccharide
Outer membrane proteins
Multicomponent/conjugate
DNA
Type III secretion/intoxication proteins

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pathogenesis of *P. aeruginosa* infections led to the discovery of a variety of virulence-associated factors that lent themselves to the possibility of an immunologic approach to the prevention and treatment of *Pseudomonas* infections. Some of the factors that have been tested in this regard are listed in Table 1.

Although many factors are listed, they may not all be equally effective as universal immunogens. Data have accumulated that some virulence factors may be associated with certain infection processes but not others. Proteases seem to be important virulent factors in patients with cystic fibrosis, whereas exotoxin A is not. Others have compared virulence products produced by *P. aeruginosa* isolated from patients with different site infections and suggest from their results that "(1) elastase, phospholipase C, toxin A and exo-enzyme S are produced by *P. aeruginosa* isolates from different sites of infection; (2) the production of higher levels of elastase and phospholipase C is important in all types of infections, while the production of toxin A and exo-enzyme S is important in wound infections; (3) persistent infection with *P. aeruginosa* may enhance exo-enzyme S production." Thus, neutralisation of any onespecific virulence factor by immunologic or other means may not be successful in preventing, eliminating, or improving morbidity or mortality in *P. aeruginosa* infections in all of the forms they are seen in patients. This caveat should be kept in mind when reading this review. Since the literature on immunologic approaches to control *Pseudomonas* infections is vast, this review will give only an abbreviated overview of a variety of approaches that have been attempted to prepare *Pseudomonas* vaccines over the years.

REVIEW

In the 1960s, when *P. aeruginosa* had started to replace Gram-positive cocci as the organism causing the most mortality from sepsis, a variety of immunologic approaches to the prevention and treatment of these infections was attempted. Because of the lack of knowledge of the virulence factors associated with this organism and a lack of understanding of its mechanisms of pathogenesis, most of these attempts at immunotherapy relied on cell wall components (lipopolysaccharides; LPS) as antigens. It was believed that the generation of opsonising antibody against the infecting strain would clear the bacteria from the host, thereby aborting the infection. As knowledge of the various 0 serotypes of *P. aeruginosa* became better known, multivalent LPS vaccines were developed (*Feller* et al., 1964; *Millican* et al., 1966; *Alms* and *Bass*, 1967; *Alexander* et al., 1971; *Haghbin* et al., 1973; *Young* et al., 1973; *Alexander* and *Fisher*, 1974; *Pennington* et al., 1975; *Miler* et al., 1977; *Jones* et al., 1980; *Pennington* and *Pier*, 1983). These were tested not only in animal models [1-3,9,11] and patients, especially burned patients (*Alexander* et al., 1971; *Alexander* and *Fisher*, 1974; *Jones* et al., 1980), where the incidence of lethal *P. aeruginosa* infection was very high but also in patients with various forms of cancer (*Young* et al., 1973; *Haghbin* et al., 1973; *Pennington* et al., 1975) and acute and chronic lung disease (*Pennington* et al., 1975) (Table 2). Although research and testing of these types of vaccines went on for at least two decades and in spite of positive results in animal and patient testing, especially in burns, these vaccines, because of the LPS nature and the potential

problems involved with LPS, never gained clinical acceptance.

Early investigations into virulence factors associated with *P. aeruginosa* infections described an ADP-ribosylating toxic substance that was designated exotoxin A. Some studies of *Pseudomonas* infection suggested that animals infected with *P. aeruginosa* would die a "toxic" death even in the face of treatments which reduced the infecting bacterial load to a significant degree. This suggested to some investigators that as an alternative to immunologic approaches that functioned to protect by reducing the microbial load, one might be able to enhance survival even in the face of ongoing infection by neutralisation of the toxic exoproduct, exotoxin A (*Pavlovskis* et al., 1977, 1981; *Snell* et al., 1978; *Cryz* et al., 1983) (Table 3). There were mixed results using this approach; at best, antitoxin treatment alone only increased survival time ((*Pavlovskis* et al., 1977, 1981; *Snell* et al., 1978). Only in the presence of additional treatment that simultaneously reduced microbial load did antitoxin treatment enhance long-term survival (*Snell* et al., 1978). Despite encouraging studies, mostly using burned mice, there are no clinical studies using this approach to *Pseudomonas* immunotherapy. However, exotoxin A toxoid is being used in combination with other potential protective immunogens and in multicomponent and conjugate vaccines. More about this will be described later.

For a time, ribosomes and ribosomal RNA vaccines were evaluated for their ability to enhance survival from *P. aeruginosa* infection in a variety of animal studies using unburned (*Smith* et al., 1974; *Gonggrijp* et al., 1981; *Lieberman* and *Ayala*, 1983) and burned rodents (*Lieberman* et al., 1986) (Table 4). Although some success in animal models was presented, questions about LPS contamination of ribosomal preparations cast doubt on these results, and RNA and ribosomal vaccine development fell out of favour.

When it was demonstrated that motility and attachment were associated with *P. aeruginosa* virulence, particularly in burn wound infections, many studies were directed at the study of flagella and pili as protective immunogens (*Holder* et al., 1982; *Holder* and *Naglich*, 1986; *Montie* et al., 1987; *Sato* et al., 1988; *Ochi* et al., 1991) (Table 5). Several studies showed the efficacy of flagella immunisation in a variety of burned animal studies (*Holder* et al., 1982; *Holder* and *Naglich*, 1986; *Montie* et al., 1987; *Ochi* et al., 1991). The appeal of flagella immunisation was that there are only two immunotypes of flagella in *P. aeruginosa*, thus a successful divalent vaccine could be uniformly protective (*Holder* and *Naglich*, 1986). Flagella as protective immunogens are still being investigated not only for protection against burn wound infections but also in the prevention of *P. aeruginosa* infection in cystic fibrosis patients. Pili, bacterial appendages used for attachment, have been shown to be virulence-associated factors in *P. aeruginosa* as well. Flagellated and piliated strains of *P. aeruginosa* have a 10-fold lower LD_{50} than their isogenic non-piliated mutants, and non-piliated strains lose their ability to adhere to epidermal cells *in vitro* (*Sato* et al., 1988) Antipilin serum inhibited piliated strains from adhering to these same cells, suggesting that pili, as well as flagella, were appropriate proteins to be considered as protective vaccines against *P. aeruginosa* infection. Although they have not reached points where they can be used as successful vaccines, studies on how portions of the pilin adherence binding domain and how peptides to the c-terminal receptor binding regions of four strains of *P. aeruginosa* pilin may be used as vaccines have been reported.

Table 2: Features of some early immunotherapeutic approaches to treat *Pseudomonas aeruginosa* infections using whole cells or cell wall-associated materials

Immunogen	Tested in	Active	Passive	Effect of immunisation	Reference
Heat-killed phenol preserved whole cells - one strain	Rabbits	+	+	Significant ↑ survival	*Feller* et al., 1964
Heat or formalin killed whole cells - one strain	Unburned and burned mice	+	-	Significant ↑ survival; ↓ pathologic findings in organs	*Millican* et al., 1966
Alcohol precipitate of "slime" fraction-one strain	Mice	+	+	Significant ↑ survival	*Alms* and *Bass*, 1967
LPS prepared from seven 0 serotype strains combined to make a heptovalent vaccine (Pseudogen®)†	Burned patients Burned patients	+ +	- +*	↑ survival ↑ survival	*Alexander* et al., 1971 *Alexander* and *Fisher*, 1974
Heptavalent LPS vaccine (Pseudogen®)	Cancer patients	+	-	Significant but limited ↓ in fatal infection high incidence of untoward side effects	*Young* et al., 1973
Heptavalent LPS vaccine (Pseudogen®)	Paediatric acute leukaemia patients	+	-	No control of infection observed	*Haghbin* et al. 1973
Heptavalent LPS vaccine (Pseudogen®)	Acute leukaemia and cystic fibrosis patients	+	-	Leukaemia: possibly fewer *Pseudomonas* infections; cystic fibrosis - no clinical benefit in spite of high antibody titres	*Pennington* et al., 1975
EDTA-glycine extraction of viable cells from each of 16 distinct 0 serotype strains combined to make polyvalent vaccine (later called PEV-01)	Mice Burned patients;	+ +	+ +	Significant ↑ survival ↓bacteraemia	*Miler* et al., 1977 *Jones* et al., 1980
Pseudogen and PEV-01	Acute pneumonia/ Guinea pigs			Significant protection - both vaccines	*Pennington* and *Pier*, 1983

* Given to patients who arrived or became bacteraemic within 5 days of hospital admission.
† Parke Davis and Co., Detroit, MI

Table 3: Protection studies using exotoxin A immunisation

Type of immunisation	Results	Reference
Passive	↑ Survival time (burned mice)	*Pavlovskis* et al., 1977
Passive	↑ Survival time; long term survival ↑ with additional antibiotic treatment (burned mice)	*Snell* et al., 1978
Active	↑ Survival time; lower viable bacterial counts in blood and liver (burned mice)	*Pavlovskis* et al., 1981
Passive	No effect on survival or number of bacteria found in blood, liver or skin (burned mice)	*Cryz* et al., 1983

Table 4: Protection studies using ribosomes

Immunogen (type of immunisation)	Results	Reference
Ribosomes/1 strain (active)	Significant homologous but not heterologous strain protection (mice)	*Smith* et al., 1974
Purified ribosomes prepared from crude ribosomal fractions from two strains and RNA extracted from these purified preparations (active)	Cross-protection (mice)	*Gonggrijp* et al., 1981
Ribosomal fractions one strain (active and passive)	Significant protection (C3H/HeJ and ICR mice)	*Lieberman* et al., 1983
Ribosomal fractions, two strains (active and passive	Cross protection when immunisation was prior to infection; post infection protection was time interval dependent between immunisation and infection (scald burned rats)	*Lieberman* et al., 1986

Table 5: Protection studies using flagella or pili immunisation

Immunogen (Type of Immunisation)	Results	Reference
Purified flagella (active)	Flagella antigen specific ↑ survival; uniform ↑ survival with divalent immunisation (burned mice)	*Holder* et al., 1982
Partially purified flagella (active)	Flagella antigen specific *in vitro* inhibition of motility using antiflgella	*Holder* et al., 1986
Highly purified flagella (active and passive)	Flagella antigen specific *in vitro* inhibition of motility using antiflagellar scrum; flagella antigen specific ↑ survival (burned or scalded mice)	*Montie* et al., 1987
Pili (active)	↑ Survival with challenge strain from which pili isolated (scalded rats)	*Sato* et al., 1988
Monoclonal antibody to partially purified flagella (passive)	Flagella antigen specific *in vitro* inhibition of motility; flagella antigen specific ↑ survival (burned mice)	*Ochi* et al., 1991

Table 6: Protection studies using high-molecular-weight polysaccharide (HMWP) immunisation

Valency (Type of Immunisation)	Results	Reference
Active and passive (monovalent)	↑ Protection, serotype specific (mice)	*Pier* et al., 1978
Active and passive (monovalent)	↑ Survival, homologous protection (mice)	*Pier* et al., 1981
Active and passive (divalent)	↑ Survival; some but not complete cross-protection (mice)	*Pier*, 1982
Active and passive (divalent)	Strong, serotype specific and weakly cross-reactive antibody response to active immunisation; cross protective ↑ LD_{50}; serotype specific ↑ survival with passive immunisation (mice)	*Pollack* et al., 1984
Active (monovalent)	↑ Serotype specific protection; 1000-fold more HMWP needed for protection compared to LPS (mice)	*Cryz* et al., 1984

The success or failure of these efforts remains to be seen.

In another effort to avoid some of the pitfalls of the use of LPS vaccines to prevent and treat *P. aeruginosa* infections, several investigators turned to the use of high molecular weight polysaccharides as potential vaccine candidates using both active and passive immunological procedures (Table 6). Several animal studies attest to the potential efficacy of this type of immunisation (*Pier* et al., 1978, 1981; *Pier*, 1982; *Pollack* et al., 1984; *Cryz* et al., 1984). Despite this, there does not seem to be any contemporary interest in this type of immunotherapy for the prevention and treatment of *Pseudomonas* infections.

Because of the association between mucoid *P. aeruginosa* and the pathogenesis of these infections in cystic fibrosis patients, interest was generated in using *Pseudomonas* alginate (*Woods* and *Bryan*, 1985; *Pier* et al., 1990) and mucoid exopolysaccharide (*Pier* et al., 1990, 1994; *Johansen* et al., 1994) as immunogens to prevent and treat *P. aeruginosa* infections in this patient population (Table 7). Despite some encouraging results in animal studies, the clinical application of these vaccines has not been realised.

For at least two decades there has been considerable interest in the use of a variety of outer membrane proteins as immunogens for the prevention of *P. aeruginosa* infections (*Gilleland* et al., 1984; *Hancock* et al., 1985; *Matthews-Greer* and *Gilleland*, 1987; *von Specht* et al., 1995,1996a,b; *Finke* et al., 1990; *Fox* et al., 1994; *Hughes and Gilleland*, 1995; *Mansouri* et al., 1999; *Lee* et al., 1999,2000; *Knapp* et al., 1999; *Jang* et al., 1999; *Kim* et al., 2000). Part of the reason for this is that outer membrane proteins are exposed on the *Pseudomonas* cell surface, and at least one, protein F, is conserved and antigenically related in all serotype strains. Encouraging results have been obtained in a wide variety of animal studies using either intact animal models (*Gilleland* et al., 1984; *Hancock* et al., 1985; *von Specht* et al., 1996a; *Finke* et al., 1990) or animal models that represent a number of clinically relevant circumstances: Burns (*Matthews-Greer* and *Gilleland*, 1987; *von Specht* et al., 1996a; *Jang* et al., 1999) and other immunosuppressed patient populations (*von Specht* et al., 1995; *Knapp* et al., 1999) and acute (*Fox* et al., 1994) and chronic lung disease (*Hughes and Gilleland*, 1995) (Table 8). More progress has been made in translating outer membrane protein vaccines successes in animal models to the human circumstance than any other *P. aeruginosa* virulence factor. Immunisations with outer membrane proteins have been shown to cause large, long-lived increases in antibody titre (*Lee* et al., 1999; *Mansouri* et al., 1999; *Jang* et al., 1999) and to be safe (*Kim* et al., 2000) and well tolerated (*von Specht* et al., 1996; *Mansouri* et al., 1999). Further, outer membrane proteins were shown to be generated after immunisation in burn patients (*Kim* et al., 2000). IgG prepared from these burn patients increased protection when used for passive immunisation of normal and burned mice infected with *P. aeruginosa*. Current interest in these types of vaccines continues.

From the earliest days of research into immunotherapy for *P. aeruginosa* infections, multicomponent (*Kawaharajo* and *Homa*, 1977; *Okada* et al., 1980; *Holder* and *Neely*, 1989; *Gilleland* et al., 1993; *Matsumoto* et al., 1998) or conjugate vaccines (*Tsay* and *Collins*, 1984; *Cryz* et al., 1986; *Gilleland* et al., 1993) have been developed (Table 9). The fact that development of multicomponent and conjugate vaccines continues to the present day speaks to the relevance that many investigators see in this approach. Many of the earlier

Table 7: Protection studies using alginate/mucoid exopolysaccharide immunisation

Type of Immunisation	Results	Reference
Active	↑ Antibody production; ↑ bacterial clearance associated with ↑ in alginate antibody, inconsistent results (rat agar bead, chronic lung infection model)	*Woods* and *Bryan*, 1985
Active	↑ Growth of bacteria in lung but only with immunising dose which generated opsonising antibody (rat agar bead, chronic lung infection model)	*Pier* et al., 1990
Active	Significantly fewer % of lungs infected - bacteria completely cleared; pathologic changes from acute to chronic-type inflammatory response in lungs (rat agar bead, chronic lung infection model)	*Johansen* et al., 1994
Active	Immunogenic, well tolerated; elicited long lived opsonic antibodies; mediated opsonic killing of heterologous mucoid strains (human volunteers)	*Pier* et al., 1994

Table 8: Protection studies using outer membrane protein (OMP) immunisation

Immunogen (Type of Immunisation)	Results	Reference
Protein F (active and Passive)	3-fold ↑ LD_{50} (mice)	*Gilleland* et al., 1984
Monoclonal antibody to protein F (passive [pre-infection])	2-3 fold ↑ LD_{50} (mice) 8-fold ↑ LD_{50} (burned mice)	*Hancock* et al., 1985
Proteins F and H (active)	Significant ↑ survival with protein F but not protein H immunisation (scalded mice)	*Matthews-Greer* and *Gilleland*, 1987
Proteins F, H_2 and I mixture (active)	2-26-fold ↑ LD_{50} with different challenge strains due to 30-60% variation in animals response to immunisation (mice)	*von Specht* et al., 1996a
Lipoprotein I (active)	4-5-fold ↑ LD_{50} (mice)	*Finke* et al., 1990

Table 8 (continued): Protection studies using outer membrane protein (OMP) immunisation

Immunogen (Type of Immunisation)	Results	Reference
Protein F (active)	Significant ↓ in severe pulmonary lesions - significant ↓ in lung compliance (rat agar bead, chronic lung infection model)	*Fox* et al., 1994
Recombinant F and I fusion proteins (active and passive)	Significant ↑ in LD_{50} (cyclophosphamide immunosuppressed and SCID (mice)	*von Specht* et al., 1995
Synthetic peptides of protein F (active)	Significant ↑ survival with 2/3 peptides tested - intranasal immunisation (mouse acute pneumonia model)	*Hughes* and *Gilleland*, 1995
Protein I – express in *E. coli* (active)	Immunisation well tolerated; significant ↑ in titres; long lived (human volunteers)	*von Specht* et al., 1996b
Protein F: protein I hybrid (active)	Significant ↑ in antibody; elevated antibody still measurable 6 months after last vaccination; well tolerated (human volunteers)	*Mansouri* et al., 1999
Mixed OMP (passive)	Affinity purified anti-OMP from pooled human IgG using mixed OMP; antibody purified from burn patient sera, as well. Both antisera enhanced opsonic phagocytosis of *P. aeruginosa*, *in vitro*. Passive administration of IgG ↑ LD_{50} in mice-IP challenge	*Lee* et al., 1999
Mixed OMPs (active [humans], passive [mice])	Phase I/IIa clinical trial in healthy male volunteers and significant ↑ in OMP-specific antibody; higher in IM than SC immunised; ↑ protection in normal and burned mice	*Jang* et al., 1999
Protein F: protein I hybrid expressed in *E. coli* (active and passive)	↑ Protection by both active and passive immunisation (SCID mice—IP challenge)	*Knapp* et al., 1999
Mixed OMPs (active [humans], passive [mice])	Active immunisation in burn patients; serum from immunised patients showed high opsonophagocytic activity; ↑ survival in mice - IP challenge	*Lee* et al., 2000
Mixed OMPs (burned patients)	Tested immunisation schedules found 1.0 mg doses at 3-day intervals safe and effective in conferring protection against *P. aeruginosa* bacteraemia	*Kim* et al., 2000

Table 9: Protection studies using multicomponent/conjugate preparation immunisation

Immunogen (type of Immunisation)	Results	Reference
OEP: toxoids of elastase and protease; alone and in combination (active)	↑ Survival; combined immunisation no better than single component immunisation (tail-burned mice)	*Kawaharajo* and *Homa*, 1977
OEP: elastase, protease and exotoxin A toxoids (active)	↑ Survival; challenge strain dependent (burned mice)	*Okada* et al., 1980
Low molecular weight polysaccharide: albumin (active and passive)	↑ Survival with homologous strain infection (burned mice)	*Tsay* and *Collins*, 1984
Monovalent high molecular weight: polysaccharide: exotoxin A conjugate (active)	↑ Circulating homologous 0 serotype LPS+ exotoxin A antibody; ↑ survival (burned mice)	*Cryz* et al., 1986
Hyperimmune globulin: antitoxin: protease inhibitors (passive)	↑ Survival when used together compared to individual treatments (burned mice)	*Holder* and *Neely*, 1989
Octavalent HMWP: exotoxin A conjugate (active)	↑ Circulating IgG to exotoxin A + all serotypes contained in vaccine; ↑ protection after infection with all serotypes contained in vaccine (mice)	*Cryz* et al., 1989
Elastase: exotoxin A: outer membrane protein F (active)	Reduced severe pulmonary lesions–no better than protein F vaccine alone (rat agar bead, chronic lung infection model)	*Gilleland* et al., 1993
Alginate: toxin A conjugate (acute)	Significantly fewer % of lungs infected—bacteria completely cleared; pathologic changes from acute to chronic-type inflammatory response in lungs (rat agar bead, chronic lung infection model)	*Johansen* et al., 1994
Octavalent 0 polysaccharide: toxin A conjugate (active)	High titre antibody response associated with lower incidence of infection (six year follow-up in non Pseudomonas colonised CF patients)	*Cryz* et al., 1997
Toxoids of elastase: alkaline protease: exotoxin A (active)	No protection when used separately; ↑ survival in combination (gut derived sepsis in cyclophosphamide immunosuppressed specific pathogen-free mice)	*Matsumoto* et al., 1998

Table 10: DNA vaccine approaches to *Pseudomonas aeruginosa* immunisation

DNA encoding gene (type of immunisation)	Results	Reference
Type B flagellin (active)	↑ Survival in burned *P. aeruginosa* infected mice using gene gun to immunise; no ↑ when immunisation via IM route	*Baker* et al., 1999
Outer membrane protein F (active)	↑ In opsonic activity in immune sera; ↓ in macroscopic lung lesions and bacteria in immunised mice (agar bead chronic lung infection model)	*Price* et al., 2001
Mutated exotoxin A gene producing immunologically active but non-lethal toxin (active)	Neutralisation of exotoxin A *in vitro*; significant survival in immunised (gene gun) mice challenged with 13 x LDs exotoxin A	*Denis-Mize* et al., 2000
Modified exotoxin A gene (active)	Protection of mice from intoxication with lethal dose of exotoxin A	*Shiau* et al., 2000

Table 11: Additional immunological approaches

Immunogen	Results	Reference
Pooled monoclonal antibodies directed against 0-saccharide of *Pseudomonas aeruginosa* serotype E, core saccharide of LPS from *P. aeruginosa* serotypes A, G, F, H, K, and flagellin type b (passive)	Safe and well tolerated by 8 pneumonia, 4 burns and 8 patients with both burns and pneumonia. Too few patients to assess efficacy but clinical impression of improvement	*Harrison* et al., 1997
Two epitopes from *P. aeruginosa* elastase (active)	50-70% reduction in lung histopathologic changes with one of the two peptides. Protection when lung challenged with *Burkholderia cepacia* as well as with *P. aeruginosa* (rat agar bead, chronic lung infection model)	*Sokol* et al., 2000

vaccines consisted of toxins of known *Pseudomonas* virulence factors, e.g., proteases, elastases, and exotoxin A, together with some form of cell wall-associated materials (*Kawaharajo* and *Homa*, 1977; *Johansen* et al., 1994; *Okada* et al., 1980; *Holder* and *Neely*, 1989; *Gilleland* et al., 1993; *Cryz* et al., 1997; *Matsumoto* et al., 1998). Many of these early studies were done in mice, burned mice, in particular (*Kawaharajo* and *Homa*, 1977; *Okada* et al., 1980; *Tsay* and *Collins*, 1984; *Cryz* et al., 1986; *Holder* and *Neely*, 1989). More recently, conjugate vaccines were shown to be effective in animal models of chronic lung infections (*Gilleland* et al., 1993) and gut-derived infection in immunosuppressed mice (*Matsumoto* et al., 1998). A recent clinical study follow-up has shown promising results, with high titre antibody response associated with a low incidence of infection in cystic fibrosis patients (*Cryz* et al., 1997). Current interest in this type of approach to the prevention and treatment of *P. aeruginosa* infection remains high.

In recent years a novel approach to anti-infectious agent vaccinology has been described: DNA vaccines and genetic immunisation. Genetic immunisation uses plasmids that express bacterial proteins in eucaryotic cells, and this eliminates the laborious methods of protein purification and potential for LPS contamination. In genetic immunisation, the gene for the target protein is cloned into a eucaryotic expression plasmid, usually under the control of a viral promoter such as the cytomegalovirus promoter. Cells that take up the expression plasmid produce the target protein. The predominant immune response induced depends on the target protein, the construct, the route of immunisation, and the amount of plasmid injected. Most genetic immunisation studies have been targeted toward viruses, intracellular bacterial pathogens, and pathogenic protozoa and thus have focused on stimulating strong cellular responses. However, some DNA vaccine studies have been directed to the elaboration of antibodies to protein virulence-associated factors from *P. aeruginosa* (Table 10). DNA immunisation has been shown to be effective in enhancing protection in burned mice using flagellin B as the immunogen (*Baker* et al., 1999) and in a model of chronic lung infection using outer membrane protein F as the immunogen (*Price* et al., 2001). Further, results from DNA immunisation studies using exotoxin A as the immunogen, demonstrated that antisera from the immunised mice neutralised the activity of exotoxin A, *in vitro* (*Denis-Mize* et al., 2000) and protected the animals from death by intoxication when given lethal doses of toxin (*Denis-Mize* et al., 2000; *Shiau* et al., 2000). Some difficulties in DNA vaccination derive from finding appropriate virulence factor genes to clone and choosing the best way to administer the vaccine. Results have varied according to whether DNA vaccines were delivered by gene gun or intramuscularly (*Baker* et al., 1999; *Price* et al., 2001). While this approach is intriguing and has great potential, its practical application in the clinical arena remains to be seen.

Some additional, novel immunologic approaches have been tested recently (Table 11). These include preliminary testing, in pneumonia and burn patients, of the use of combined monoclonal antibodies to a variety of *P. aeruginosa* virulence antigens (*Harrison* et al., 1997) and an animal study which showed reduced severity of experimental lung infection in animals immunised with an to epitope of *Pseudomonas* elastase (*Sokol* et al., 2000). Although the monoclonal antibodies were shown to be well tolerated and safe in a few pa-

Table 12: Type III secretion/intoxication proteins

Immunogen (Type of Immunisation)	Results	Reference
Purified translocation protein PcrV (active and passive)	Decreased lung inflammation and injury; significant ↑ survival (mouse acute lung infection model)	*Sawa* et al., 1999
Purified translocation protein PcrV (active)	Significant ↑ survival; O serotype non-specific; supplemental antitoxin treatment necessary for significant enhanced long-term survival when challenge made using very high exotoxin A generating strain (burned mice)	*Holder* et al., 2000, 2001
Anti PcrV ab (passive)	Complete survival; lethal airspace infection (mice); ↓ lung injury; bacteraemia and plasma TNF-α; significant improvement in haemodynamic parameters associated with shock (rabbit model of septic shock)	*Shime* et al., 2001
Monoclonal antibody to PcrV (passive)	Prevented sepsis and death (acute lung infection model in mice)	*Frank* et al., 2002

Table 13: Immunisation via the mucus membrane route

Immunogen used (route)	Results	Reference
Serotype specific LPS (GI tract)	↑ Survival in both burned mice and chronic lung infection models	(*Holder* et al., 1992)
Killed serotype specific whole cells (GI tract)	↑ Serotype specific survival; antibody to exotoxin A necessary for ↑ survival when challenge was with high exotoxin A producing strain (burned mice)	(*Schryvers* et al., 1987)
Live attenuated aroA deletion mutant (nasal)	↑ Protection (acute pneumonia model; mice)	(*Priebe* et al., 2003)
hybrid outer membrane protein F-1 vaccine (nasal)	Induction of IgG and IgA in sera; safe and well tolerated (human volunteers)	(*Larbig* et al., 2001)

tients with pneumonia and burns (*Harrison* et al., 1997), the full efficacy of such immunologic treatment has yet to be demonstrated in large, double-blind clinical trials.

Besides the novel immunologic approaches cited above, research in recent years has shown that the type III secretion and intoxication system is a virulence factor for *P. aeruginosa*. Type III-mediated intoxication consists of three functional sets of genes encoding secretion and chaperone proteins, proteins involved in the translocation of effectors to the cytoplasm of eucaryotic cells, and the effector toxic proteins themselves. Type III proteins have been shown to be necessary for *P. aeruginosa* virulence in mouse models, both of acute lung injury and burn wound infection. Further in both of these models, immunisation using the purified type III translocation protein, PcrV, enhanced survival when mice were challenged with lethal doses of *P. aeruginosa* (Table 12). In both the mouse-infected lung model (*Sawa* et al., 1999) and the burned mouse model (*Holder* et al., 2000, 2001), passive and active immunisation proved effective. However, in the burned mouse model, supplemental immunisation using antitoxin was necessary for full protection in PcrV immunised burned mice infected with a strain producing high amounts of exotoxin A (*Holder* et al., 2000). Additionally, passive treatment with anti-PcrV antibody improved several physiological parameters of septic shock in a study which used a *Pseudomonas* induced lung injury model in rabbits (*Shime* et al., 2001). Further, monoclonal antibody generated against PcrV protein prevented sepsis and death when used as a passive treatment for infected mice (*Frank* et al., 2002). The success of both active and passive PcrV immunisation in enhancing survival and reducing negative consequences of infection in animal models of very diverse *P. aeruginosa* infections - lung, burn wound and septic shock - suggest that further exploration of type III proteins as immunogens against *P. aeruginosa* infections in human patients is warranted.

In reviewing proposals for immunological approaches to the prevention/treatment of *P. aeruginosa* infections, one additional aspect, not related to the specific immunogens used, should be considered. Over a decade ago, it was demonstrated, and more recently substantiated, that immunological protection could be obtained by presenting *Pseudomonas* antigens to the host via mucus membranes (*Holder* et al., 1992; *Schryvers* et al., 1987; *Larbig* et al., 2001; *Priebe* et al., 2003) (Table 13). Results from the earlier studies demonstrated that feeding serotype - specific killed *P. aeruginosa* cells (*Holder* et al., 1992) or LPS (*Schryvers* et al., 1987) conferred protection in both burned mouse and chronic lung infections. Further, incorporating the protein immunogen, exotoxin A into food pellets fed to mice lead to increases in circulating anti-exotoxin IgG and IgM and the mice were protected against lethal exotoxin A challenge (*Holder* et al., 1992). Recently, nasal immunisation using an attenuated aro A deletion mutant enhanced protection in an acute pneumonia model in mice (*Priebe* et al., 2003) and the safety and immunogenicity of an outer membrane protein vaccine was demonstrated, in humans, via this route of immunisation (*Larbig* et al., 2001). Thus, it appears that *Pseudomonas* immunisation can be achieved by presenting a variety of *Pseudomonas* protective antigens to the host via its mucus membranes located in the GI tract or nasal passages. Because of that, this novel means of establishing immunological protection against various

Pseudomonas infections, apparently regardless of the immunogen used, should be investigated further.

This review gives some insight into the interest that has been engendered in immunologic approaches to the prevention and treatment of *P. aeruginosa* infections in a wide variety of clinical circumstances. The search has gone on for several decades and continues today. Some approaches have been discarded as unfruitful, e.g.: those that used cell wall components, LPS, individual exoproducts, ribosomes, high molecular muco-exopolysaccharides, alginate, etc. On the other hand, some approaches are being pursued, actively, today. For example, a flagella vaccine study has just been completed in cystic fibrosis patients and results should be available in the next several months (Gerd Doring, personal communication). Further, outer membrane protein immunisation has moved out of laboratory and animal studies into safety, efficacy and tolerance studies in human volunteers (*von Specht* et al., 1996b; *Mansouri* et al., 1999; *Jang* et al., 1999; *Kim* et al., 2000) and limited trials in some patient populations (*Lee* et al., 1999, 2000). In addition, a conjugate polysaccharide, toxin A, vaccine has shown promise in reducing *P. aeruginosa* infection in cystic fibrosis patients (*Cryz* et al., 1997). These studies should and are being pursued further.

Future development of vaccines for the prevention of *Pseudomonas* infections looks promising with clearer understanding of the role that Type III intoxication proteins play in the pathogenesis of *Pseudomonas* infections and the fact that some of these proteins may be used as immunogens for a vaccine (*Sawa* et al., 1999; *Holder* et al., 2000, 2001). Further, the new use of DNA vaccines holds great hope in the future development of immunotherapy for the prevention of *P. aeruginosa* infections in a variety of patient populations (*Baker* et al., 1999; *Denis-Mize* et al., 2000; *Shiau* et al., 2000; *Price* et al., 2001). An additional consideration for future *Pseudomonas* vaccine development would be studies on how, best, to present different immunogens to the host by the route which is the safest and provides the highest and most protective antibody titre, as well. Studies on the presentation of immunogens via the mucus membrane route should be high on this "to study" list.

In conclusion, although no *Pseudomonas* vaccine has made its way into common clinical use yet, the search continues and, currently, the goal seems more attainable.

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