Study to Determine the Safety and Immunogenicity of an Oral Inactivated Whole-cell *Pseudomonas aeruginosa* Vaccine Administered to Healthy Human Subjects

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ABSTRACT

This study examines the safety and immunogenicity of an oral, whole-cell *Pseudomonas aeruginosa* vaccine administered to healthy volunteers. Thirty subjects received an oral dose of Pseudostat^{TM} in two timed, measured, doses with serological follow-up to 56 days post vaccinination.

Following vaccination several individuals were identified as antibody responders for all three immunoglobulin isotypes tested, specifically against whole-cell *P. aeruginosa* extract and outer membrane proteins F and I. The mean pooled lipopolysaccharide (LPS) antigen specific IgA showed the most significant and constant increases in titer post dose, with a similar increase in titer for whole-cell *P. aeruginosa* extract specific IgA. The results demonstrated an increased phagocytic ability of the selected macrophage cell line in post vaccination sera. Furthermore a significant increase in intracellular macrophage killing of opsonized *P. aeruginosa* was also demonstrated (82% on Day 14 post dose) in the presence of the post vaccination sera. The safety component of the study did not show any vaccine-attributable adverse effects in any of the subjects as documented by clinical evidence, haematology and biochemistry profiles. We conclude that Pseudostat^{TM} is safe and immunogenic in humans at this dose, and that further studies to determine the appropriate dosage and efficacy are needed. In our study, we have shown that the most significant and sustained responses to oral vaccination in human adult volunteers was serum IgA and that pooled sera collected post immunization has an increased capacity to promote opsonophagocytotic killing of *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is a Gram negative bacterium with a ubiquitous distribution within the biosphere. In the compromised host, it is capable of establishing opportunistic infections and this is particularly common in subjects with lung dysfunction. *P. aeruginosa* is particularly well adapted to the conditions found in the lungs of cystic fibrosis (CF) patients, where a defective chloride channel transport protein results in an increased viscosity of secretions making it difficult to clear airway mucous. Colonization takes place at an early age often in the absence of any overt clinical presentation or culture positive sputum and throat swabs (3). Because the bacteria are not effectively eradicated from the CF lungs, chronic colonization occurs. *P. aeruginosa* adapts through gene switching to undergo a number of phenotypic changes. These include the loss of lipopolysaccharide (LPS) O antigen, which renders the strain non-typeable or polyagglutinating, and the production of excessive amounts of an alginate polysaccharide capsule (14), that allows the microbe to exist in microcolonies (biofilms) within the lungs. In addition these non-typeable, mucoid colonies exhibit a reduced susceptibility to antibiotics and 'frustrated phagocytosis', where the excessive amounts of alginate prevent phagocytosis by polymorphonuclear neutrophils and macrophages. The resulting excessive production of proteases, superoxide radicals and inflammatory mediators contribute to the subsequent destruction of normal lung tissue. The major antigen of immune complexes in the sputum of CF patients has been shown to be LPS (17). Outer membrane protein F (OprF) and outer membrane protein H2 (OprH2) in particular have been shown to induce strong antibody activity, while OprI, OprF and OprH2 are highly conserved in *P. aeruginosa* (31, 33).

Current therapies with antibiotics are targeted at controlling bacterial load of *P. aeruginosa* and other bacteria. These frequently fail to adequately clear established infections, while low antibiotic concentrations in the airways are ineffective and may lead to the development of resistant bacterial strains. A vaccine which could prevent or delay initial colonization with *P. aeruginosa* in the lungs may have a positive impact on CF patients, and contribute to improvement in quality of life and survival in these patients. In addition, it is also feasible that immunization may reduce bacterial load of patients who have become chronically colonized with *P. aeruginosa*. Vaccine candidates for *P. aeruginosa* have been under study for some 30 years or more but progress has been slow (9). The potential to vaccinate against *P. aeruginosa* infection has been recently reviewed and a number of exciting opportunities have been identified including mucosal immunization (26). Most studies have focused on burns patients and CF patients, and many have not progressed beyond the initial proof of concept stages. A Cochrane review in 1999 (16) concluded that there was a paucity of randomized clinical trials assessing the effectiveness of vaccination against *P. aeruginosa* in CF patients. The only trial to meet their inclusion criteria, was one evaluating a blended LPS administered to children, which showed no clinical benefit at the 10 year follow-up. There was also a suggestion that the vaccine may have been detrimental with the immunized group appearing to have more severe pulmonary exacerbations than the control group (18). Although this does not preclude a vaccine approach to *P. aeruginosa* infection in the management of CF, it may have added to the reluctance and slow progress of developing a vaccine.

This study reports on a phase 1 safety and immunogenicity study using an oral whole-cell inactivated *P. aeruginosa* vaccine administered to healthy volunteers. The vaccine has previously been shown to protect against acute *P. aeruginosa* challenges to the lungs of rodents

(2, 7) and may be suitable for the development of an oral formulation for CF infants to prevent or delay colonization by *P. aeruginosa* (7). Successful mucosal and systemic immune stimulation mediated via gastrointestinal associated lymphoid tissue would allow for the development of oral vaccines to be used as a prophylactic and therapeutic tool against chronic *P. aeruginosa* infections.

MATERIALS AND METHODS

Clinical Trial Rationale

The aim of the study was to assess the safety of Pseudostat™ as an oral vaccine in humans and to obtain preliminary information on antibody responses to *P. aeruginosa* following oral vaccine administration. For the safety study treatment was selected as the lowest scaled-up by weight dose derived from mice studies which had induced a protective response against a *P. aeruginosa* lung challenge and statistically significant increases in serum *P. aeruginosa* specific antibody titers. Based on animal data a "Priming" and "Stimulatory" dose regime was chosen as it could produce a greater response than a single exposure.

Study Design

This was a single center open-label phase 1 study to assess the safety and immune response to Pseudostat™, an oral preparation of whole-cell, formaldehyde-inactivated *P. aeruginosa*, in healthy human volunteers. Thirty (30) healthy subjects between the ages of 18 and 50 years who met the inclusion criteria were recruited for the study. Subjects were excluded if they had any clinically relevant medical condition including a past history of Pseudomonas infection requiring medical treatment, a history of alcohol or drug abuse, any allergic sensitivity or intolerance to vaccines, impaired oral absorption, were immune-compromised, or had participated in any study during the last 2 months, were pregnant or of child bearing age and had not been taking the combined oral contraceptive pill for at least 3 months.

Vaccination and Safety

The Pseudostat™ vaccine consisted of enteric-coated hard gelatine capsules each containing 150 mg of lyophilized formaldehyde-inactivated *P. aeruginosa* strain 385 serotype 2, phage type 21/44/109/110X/1214 which was equivalent to 2 x10^{11} bacteria per capsule. The vaccine was manufactured for the trial by Boehringer-Ingelheim. Strain 385 is a clinical isolate of a mucoid phenotype. In extensive preclinical animal experiments immunization with this strain gave optimal and reproducible responses, and immune protection was observed against challenge with other serotypes (7). All subjects were dosed over a 3 day period to avoid any possibility of seasonal effects. All subjects were asked to fast six hours prior to the clinic visit for dosing and one hour after. Eligibility of subjects was established at screening (Day –14) and each subject received a single 150mg dose of Pseudostat™ on Day 0 and Day 28 with follow-up visits two weeks after each dose (Day 14 and Day 42 respectively) and a final visit on Day 56. At each of the five visits, and prior to dosing, 30 ml of blood and 10 ml of saliva were collected into plain tubes. Serum prepared from blood samples and saliva were stored at -80°C until analyzed. At each visit, vital signs, concomitant medications and any adverse events were noted, and on Day 0 and Day 56 a clinical examination was undertaken by a qualified physician and blood samples collected for routine hematology and biochemistry analysis at an accredited pathology laboratory. An adverse event was recorded for any change from the subject's baseline (pretreatment) condition, including any clinical or laboratory test value abnormality of clinical significance which occurred after the start of the study, whether it was considered related to the study medication or not.

Determination of Antibodies to whole-cell extract of *P. aeruginosa*, and Outer Membrane Proteins (OprF and OprI)

Enzyme linked immunosorbant serum assays (ELISAs) were developed and validated for measuring antibody responses to whole-cell *P. aeruginosa* extract and outer membrane proteins, OprF and OprI. Microtiter plates were coated with 100 μl of a 10 μg/ml solution of either soluble whole-cell *P. aeruginosa* extract, or recombinant His tagged OprF or OprI, in 50 mM sodium bicarbonate buffer at pH 9.6. Plates were then blocked with 0.1% v/v Tween 20 in phosphate buffered saline (PBS) at pH 7.0. Serum samples were diluted at 1:50 for IgA, 1:200 for IgM and 1:2000 for IgG. Triplicate samples were dispensed into microwells for incubation overnight at 2-8°C. After incubation, plates were washed twice in PBS containing 0.05% v/v Tween 80, and incubated for a further 1 hour at 37°C with 100 μl of the appropriate peroxidase conjugated anti-human immunoglobulins diluted in PBS containing 0.05% v/v Tween 20 (1:10,000 for IgG, and 1:2000 for IgM, and IgA). After washing with PBS in 0.05% v/v Tween 80, the plates were developed by addition of 100 μl of tetramethyl benzidine chromogen for 10 minutes at room temperature (controlled at 22°C). Reactions were stopped by the addition of 50 μl of 25 % v/v phosphoric acid, and absorbency values were measured at 450 nm in a microtiter plate spectrophotometer. Endpoint titers were calculated as the mean absorbencies against the baseline with co-efficients of variations typically less than 5% for all triplicate determinations and trend analysis at both dilutions.

Determination of Antibodies to Lipopolysaccharide

LPS was prepared from the *P. aeruginosa* vaccine strain by the method of Westphal and Jann (32). Microtiter plates were coated with 100 μl of 25 μg/ml LPS in 10 mM PBS at pH 7.4 containing 20 mM $MgCl_2$ and incubated overnight at 37°C. The plates were then blocked with 200 μl of 10 mM PBS (pH 7.4) containing 5% w/v bovine serum albumin and 0.05% v/v Tween

20 for 15 min at 22°C. Plates were washed twice in PBS containing 0.05% v/v Tween 80, and 100 μl of pooled serum diluted in PBS (1:200 for IgA and IgM, and 1:2000 for IgG) were added to each well and incubated overnight at 4°C. After washing in PBS containing 0.05% v/v Tween 80, the plates were incubated with the appropriate peroxidase conjugated anti-human immunoglobulin (1:2500 dilution with PBS containing 0.05% v/v Tween 20) for 1 hour at 37°C and developed as above.

Measurement of Total Serum Immunoglobulins

The total concentration of non-specific IgG, IgA, and IgM were determined in the serum and saliva of each individual by the use of a Beckman Array 360 nephelometer and standard reagents (Beckman Coulter Inc, Fullerton California).

Detection of Saliva Antibody Responses

Measurement of antibody responses were determined in saliva using essentially the same ELISAs as above for whole-cell *P. aeruginosa* extract, OprF and OprI, but with lower sample dilutions. Total salivary albumin was also measured by nephelometry.

Opsonization and Phagocytosis Assays

The sera of all subjects were pooled for each of the time points during the study and used to determine opsonophagocytosis of live *P. aeruginosa* 385 with a human monocytic cell line (THP-1). The THP-1 cell line ($5x10^4$) was seeded into tissue culture flasks and grown in RPMI-1640 media containing 10% v/v fetal calf serum and 1% w/v Penicillin-streptomycin for 3 days. The cells were then activated to produce a more macrophage-like phenotype by the addition of 50

ng/ml of myristyl phorbol ester for 24 hours. After activation non-adherent cells were removed by washing and the adherent cells harvested using a cell scrapper and suspended in Hanks Balanced Salt Solution (HBSS). Bacteria were opsonized by combining 50 µl of bacteria (equivalent to $1x10^9$/ml) to 20 µl of pooled serum for each of the time points and incubating for 30 min at 25°C. Cells were washed twice in PBS and made to 1 ml, of which 50 µl was added to 500 µl of activated THP-1 cells and again made to 1 ml with HBSS. The cells were incubated for 30 min at 37°C, washed in HBSS containing 250 µg/ml of gentamycin sulfate, and further incubated for 10 min to kill adherent non-phagocytosed bacteria. Cells were then washed in PBS, and a 50 µl sample removed and lysed in 450 µl of water. A further 10-fold dilution of the sample was made and 50 µl of this dilution plated onto nutrient agar to determine the number of colony forming units. The remaining macrophages with phagocytosed *P. aeruginosa* were incubated for a further 60 min (total time 90 min) and plated as above to determine the extent of killing of *P. aeruginosa* by the macrophages. Non-sera samples were used as negative controls, and a control sample of pooled serum from cystic fibrosis subjects known to be colonized with *P. aeruginosa* was also included to represent a high titer sample to *P. aeruginosa* antigens. The assay was repeated for each time point on five occasions and the results meaned.

Statistical Analysis

Serum and saliva specific antibody levels as determined by ELISA absorbance at 450 nm were compared at each time point against Day 0 using the paired two-tailed t-Test. Statistical significance was defined as occurring when P values were <0.05. Salivary antibody data was also expressed as a ratio of salivary albumin levels to adjust for possible salivary flow-rate differences between individual subjects. For the purpose of the immunogenicity study,

responders were defined as those showing at least two or more postdose antibody titer increases with at least one greater than 15% of the predose baseline levels.

Opsonization was extrapolated from colony counts immediately after phagocytosis. Bacteriocidal effect was measured one hour postphagocytosis and expressed as a percent difference in the final colony counts.

For the purpose of analyzing the serum IgM specific antibody response to whole-cell extract one subject was removed as an outlier. For this subject, values determined on Day 42 and 56 were three to six times the group mean. Removal of this subject from the IgM analysis did not alter the statistical significance observed for the group mean.

No significant differences were observed for any of the parameters determined between Day -14 and Day 0, therefore Day 0 was taken as baseline measure throughout.

RESULTS

Safety

Thirty (30) subjects were recruited as eligible for the study, of which 28 subjects received the first dose and 24 subjects completed the study to Day 56. Subjects were removed from the study if they received routine or prohibited medications, did not receive both doses or did not attend key visits. Twenty one subjects were males (70%) and nine females (30%). The mean age $\pm$SD was 23.1 $\pm$4.2 years, with a range of 18 years to 35 years of age, and the mean weight $\pm$SD was 69.9 $\pm$12.0kg. Pseudostat™ was well tolerated by all subjects, although, during the study 14 subjects reported 20 adverse events, none of which were considered to be possibly or probably related to the use of Pseudostat™. Adverse events were grouped by body systems and the preferred terms used the WHO classification system. These identified two individuals with musculo-skeletal system disorders, two individuals with central nervous system disorders (headache and convulsion), three with gastrointestinal disorders (diarrhea, dyspepsia, vomiting), and 13 with respiratory system disorders (pharyngitis, rhinitis, upper respiratory tract infection). Only one adverse event was considered clinically significant, though not related to the study drug. Two reported adverse events (gastritis and upper respiratory tract infection) occurred prior to treatment, while among the posttreatment adverse events twice the number of upper respiratory tract infections followed the second dose versus the first dose. There was no close temporal relationship to either day of dosing, or any consistency between adverse event reports with regards to time of occurrence (ranging 9-21 days post Day 0 and 4-22 days postdose Day 28). This was also true of other adverse event reports except for the "headache" which occurred one day after the initial dose. One serious adverse event involved a subject who had an epileptic seizure (described as of 'moderate' severity) and was hospitalized overnight for treatment. This

subject was subsequently found to have had a seizure some months previously, not reported to the investigators, and a family history of epilepsy. No abnormal hematological or biochemical test results of clinical significance were found at screening or at the Day 56 follow-up visit for any subjects. There were no clinically significant changes in vital signs observed throughout the course of the study. All patients felt well at the completion of the study.

Total Serum Immunoglobulins

Total serum immunoglobulins showed no statistically significant changes or trends throughout the course of the study. Values for IgA and IgM are presented in Table 1A.

Specific Antibody Responses to Whole *P. aeruginosa*

Serum whole-cell *P. aeruginosa* extract-specific IgA increased by Day 14 after the first dose, and remained above the Day 0 level for the remainder of the observation period (Table 1B). The group mean showed a statistically significant increase in *P. aeruginosa* specific IgA titer when compared to Day 0, for Day 14 (p=0.009), Day 28 (p=0.017) and Day 42 (p=0.010) but failed to reach significance on Day 56 (p=0.053). Whole-cell *P. aeruginosa* extract specific IgM antibody titer also reached a statistically significant group mean increase from baseline at Day 14 (p=0.019) (Table 1B). No statistically significant changes were observed for whole-cell *P. aeruginosa* extract specific serum group mean IgG (data not shown).

Of the subjects tested, 39-65% were positive responders for all antibody classes and proteins tested. The least number of responders was to OprI at 39% and 48% for IgA and IgM respectively. All other categories showed over 50% responders, however, for the purpose of this analysis responders were defined as those showing at least two or more postdose antibody titer

increases with a least one greater than 15% of the predose baseline levels. Hence, of the 24 volunteers who completed the study, 29% were considered IgA responders, 25% IgM responders and 33% IgG responders against whole *P. aeruginosa* cell extract (Table 2).

There were no significant changes in the levels of *P. aeruginosa* specific IgA salivary antibody levels following immunization. Salivary IgG antibodies against whole-cell *P. aeruginosa* extract demonstrated an increase from baseline levels at Day 14 which waned by Day 42 before a more significant second increase at Day 56 ($p=0.027$) (data not shown). No statistically significant changes were observed for either salivary IgA or IgG antibodies against any of the *P. aeruginosa* antigens tested when the antibody data was expressed as a ratio of the salivary albumin levels. Salivary IgM specific antibodies were not measured.

Outer Membrane Protein (OprF and OprI) specific IgA Responses

No significant group mean IgA, IgM or IgG serum responses were observed for either OprF or OprI. Of the 24 volunteers 46% were OprF specific IgA responders and 33% were OprI specific IgG responders. To a lesser degree and in decreasing order were OprF specific IgM responders (29%), OprI specific IgA responders (21%), OprI specific IgM responders (17%) and OprF specific IgG responders (4%) (Table 2).

LPS

LPS specific serum IgA titers increased significantly ($p<0.05$) from the baseline levels on Day 14, Day 28, Day 42 and Day 56 with maximum increases occurring 28 days post first dose (Figure 1). LPS specific serum IgM titers showed no significant change from baseline on Day 14, however there were statistically significant ($p<0.05$) decreases in responses for Days 28, 42

and 56. LPS specific serum IgG titers showed a similar pattern to LPS specific serum IgA, when compared to Day 0, achieving statistically significant increases ($p<0.05$) on all days postdose except Day 14 (Figure 1C).

Opsonophagocytosis Assays

The number of colonies grown after opsonization and phagocytosis by activated THP-1 monocytes in the presence of pooled serum from Pseudostat™ vaccinated individuals is represented in Figure 2. The results show an increase (34-62%) in the number of colonies when compared to Day 0 with the highest macrophage capture of live *P. aeruginosa* in the presence of CF sera which was used as a control. After a further 60 minute incubation period the percent of opsonized colonies killed ranged from 45-82% compared to 40% on Day 0 (Figure 3). Killing of bacteria was particularly enhanced on Day 14 (82%) at which maximal anti-*P. aeruginosa* IgA responses in serum were also observed. After Day 14 the levels drop progressively but were still maintained above baseline values.

DISCUSSION

Studies in a rodent model of acute infection have demonstrated that mucosal immunization with a whole killed cell *P. aeruginosa* vaccine results in enhanced clearance of the bacteria from the lung as well as reduced mortality (2, 7). In a preliminary study of nine bronchiectasis patients, oral immunization with an enteric coated whole-cell killed *P. aeruginosa* vaccine resulted in the detection of circulating antigen reactive peripheral blood leukocytes as well as a significant reduction in the levels of *P. aeruginosa* in the sputum (8). Further evidence to support the development of a whole killed cell vaccine and an oral immunization strategy for *P. aeruginosa* comes from previous studies on non-typeable *Haemophilus influenzae* (NTHi) where patients with recurrent acute exacerbations of chronic bronchitis were orally immunized with a whole killed cell NTHi vaccine (4, 5, 6, 20, 28). A recent Cochrane Review of 6 NTHi trials of 440 subjects reported that oral immunization significantly reduced the number and severity of acute exacerbations (13). In those studies bacterial load was reduced, as determined by the incidence of throat colonization and/or quantitative or semi-quantitative bacteriology of sputum samples. In addition, NTHi-specific cellular responses were detected in peripheral blood lymphocytes following oral immunization with whole killed cell NTHi vaccine.

This study represents a clinical pilot study designed to demonstrate the immunogenicity and safety of an oral vaccine against *P. aeruginosa* in healthy volunteers. The dose chosen, was the lowest dose expected to induce an immune response in humans based on a scaled-up dose from successful dose-ranging studies in rodents. Future placebo controlled studies will determine efficacy and optimal dose and treatment regimes in human subjects.

Safety of the vaccine in healthy individuals was confirmed in the absence of any probable or possible adverse events, and no hematological or biochemical profile derangements were recorded for any subjects throughout the study. While 20 adverse events were recorded, these were not considered of clinical significance (the exception being one case of epilepsy missed on initial screening) and none were attributable to the study vaccine being administered. Upper respiratory tract infection represented 60% of the adverse events reported with twice the number of upper respiratory tract infections following the second dose compared to the first dose. However, there was no temporal relationship to either the day of dosing or in the time of occurrence.

The vaccine was demonstrated to be immunogenic. Of particular note is the significant increase in IgA specific antibody responses against both whole-cell extract and LPS in the post immunization observation period. Confidence in the consistency and specificity of these changes was further supported by comparison with group mean total serum IgA, which did not follow a similar trend. A sustained specific IgG LPS antibody response was also observed post immunization. The opsonization results clearly showed a substantial increase in ability of pooled sera from the study volunteers to promote phagocytosis of *P. aeruginosa* by a human macrophage cell line. The results confirm the specificity of the response observed, and that antibodies induced were opsonizing for *P. aeruginosa*. Opsonizing antibody responses have been shown to be important for protection against *P. aeruginosa* infection in CF patients (24, 30). Hostoffer et al. (15) reported on the importance of IgA receptors (Fc alpha R) on mucosal phagocytes and suggested an important role in defense of mucosal surfaces. They demonstrated high levels of expression of Fc alpha R on neutrophils obtained from bronchoalveolar lavage fluid of cystic fibrosis patients and showed that neutrophil superoxide production was enhanced

by IgA. Significant IgA responses following pneumococcal polysaccharide vaccination showed that the IgA induced a comparable receptor mediated phagocytosis response as did IgG, and that the IgA holds an important leukocyte receptor which provides immunity against *Streptococcus pneumoniae* (25). In this study, both opsonization and killing peaked at Day 14 post first immunization, and although there was a decline in functional activity after this observation point, functional activity remained greater than the preimmunization levels. The two booster immunizations did not enhance either opsinization or killing. Further studies are required to investigate the kinetics of the functional antibody data, particularly in response to booster immunization.

The overall salivary immune responses did not reach statistical significance and were variable at each time point of the study. These observations probably reflect the inherent difficulties of measuring antibody responses in mucosal secretions particularly following oral immunization. Despite the absence of a detectable and consistent salivary antibody response to the vaccine this does not preclude the possibility of a mucosal immune response in the lungs or on other mucosal surfaces. If memory T-cells and B-cells are migrating to other mucosal surfaces from the gut, a measurable response may not be observed until booster encounter with antigen at these surfaces. IgA can reach external secretions by passive paracellular diffusion and may have a role in local defense on mucosal surfaces in addition to locally produced IgA antibodies. Further more, leakage of serum IgA onto mucosal surfaces is suspected to increase in inflammatory situations such as those that exist in the CF lung (22). In the context of the results of this study, Berstad et al. (1) noted an absence of salivary antibody response to a whole-cell pertussis vaccine administered nasally to human volunteers, despite strong serum antibody responses for IgA and IgG. Recently a hybrid OprF-I *P. aeruginosa* vaccine was tested in human volunteers by

intranasal application which resulted in serum IgA and IgG responses in 75% of the volunteers (19). Although saliva and nasal washings were taken for analysis in that study, the results were not reported, implying no responses may have been detected. Studies with oral whole-cell vaccination in an animal model have shown that protection against *P. aeruginosa* challenge can be observed in the absence of any significant serum or BAL antibody response (7). These findings are also consistent with results observed for whole killed cell NTHi oral immunization of subjects with chronic bronchitis, which did not stimulate a specific antibody response in saliva, although the vaccine was highly efficacious (6). Dose ranging studies in mice (Dunkley, unpublished) have shown that enhanced clearance at the lowest dose was achieved in the absence of any detectable serum antibody response. Increasing the dose by one log, which is equivalent to the single doses given in this study (2.6 mg/kg vs 2.1 mg/kg), resulted in better clearance in the mouse and the detection of statistically significant increases in serum IgA and IgM, as indeed observed in this trial. These results may suggest an important role for serum IgA in the protection against *P. aeruginosa* lung infections.

Studies of this vaccine in an animal model have indicated that both antibody and T cells are important in protecting rodents from an acute lethal challenge of *P. aeruginosa* to the lungs (2, 7, 10, 11). However, some evidence points to a detrimental effect, at least in chronically colonized CF patients where increasing serum IgG antibody levels to *P. aeruginosa* LPS, exotoxin A and the resulting immune complexes formed, have been associated with poor prognosis (21). Kronborg *et al.* (17) showed that in sputum, immune complexes were almost entirely composed of LPS specific IgG. Studies have shown that the sera of colonized patients have high titers of poorly opsonizing antibodies (27) and that the opsonizing ability of sera decreases upon conversion to the mucoid form and begins the onset of chronic colonization (30). Of importance,

the results from this study clearly demonstrate that the antibody response induced was opsonogenic. The specificity of opsonizing antibodies and their protective role requires further study (12, 23, 24).

In a recent review (26) we concluded that mucosal immunization was a potential viable option for protection against *P. aeruginosa* infections in at risk patients. This study has demonstrated that oral immunization against *P. aeruginosa* is safe and induces a significant serum antibody response, notably of the IgA isotype. In addition an increase in functional opsonization and killing by macrophages was demonstrated *in vitro* using postvaccination serum. Proof of concept in human subjects, based on the extensive preclinical studies with animal models, has been established. Further studies will involve dose ranging studies and characterization of the immune response induced with respect to serotype specificity, cell mediated immune mechanisms, the identification of the functional opsonins and their antigenic specificity, and the kinetics of the response.

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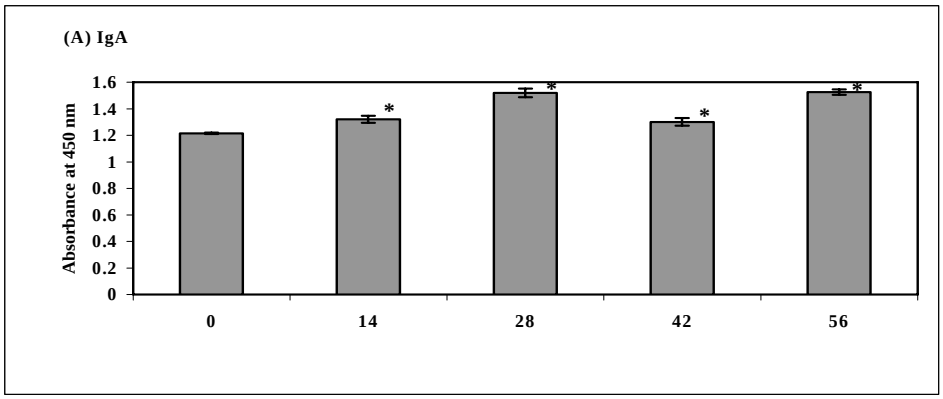
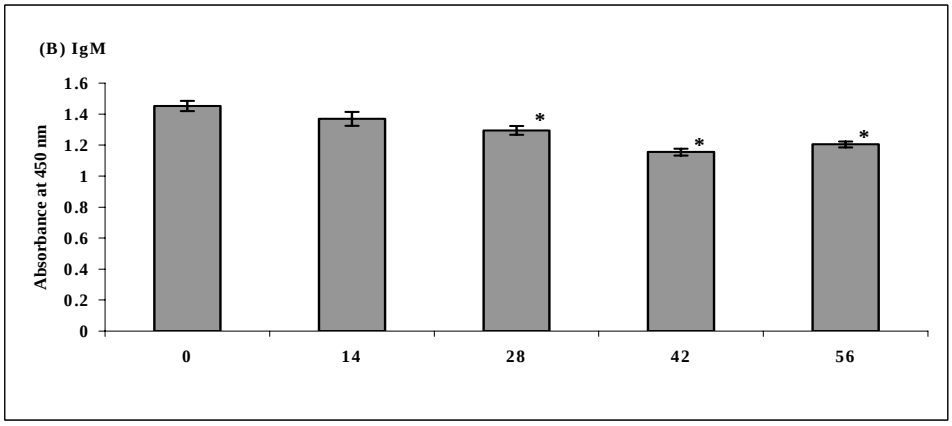
Figure 1: LPS specific serum antibodies (A) IgA, (B) IgM and (C) IgG. Values presented are the mean ± SEM of the absorbance at 450nm. * indicates values significant difference ($p<0.05$) from Day 0 value. 24 subjects were observed.





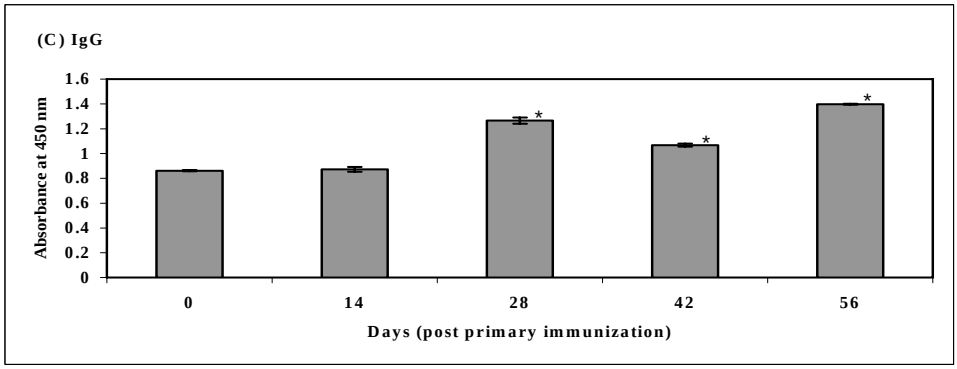


Figure 2. Phagocytosis of *P. aeruginosa* by activated monocytic cell line (THP-1) in the presence of pooled sera obtained from Pseudostat™ immunized volunteers. Values illustrated represent the mean total viable colonies (TVC) captured of 5 assay replicates for each observation group. CF = cystic fibrosis serum.

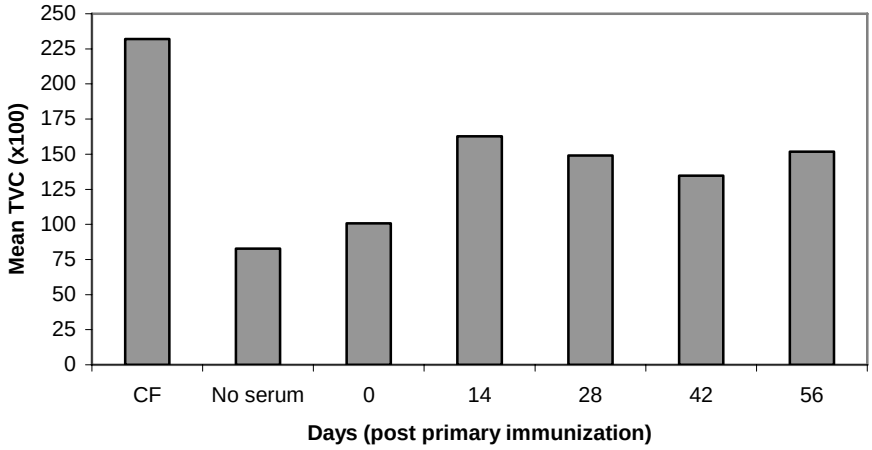


Figure 3. Percent of *P. aeruginosa* killed by monocytic cell line (THP-1) in the presence of pooled sera from Pseudostat™ immunized volunteers. Values illustrated represent the mean of 5 assay replicates for each observation group. CF = cystic fibrosis serum.

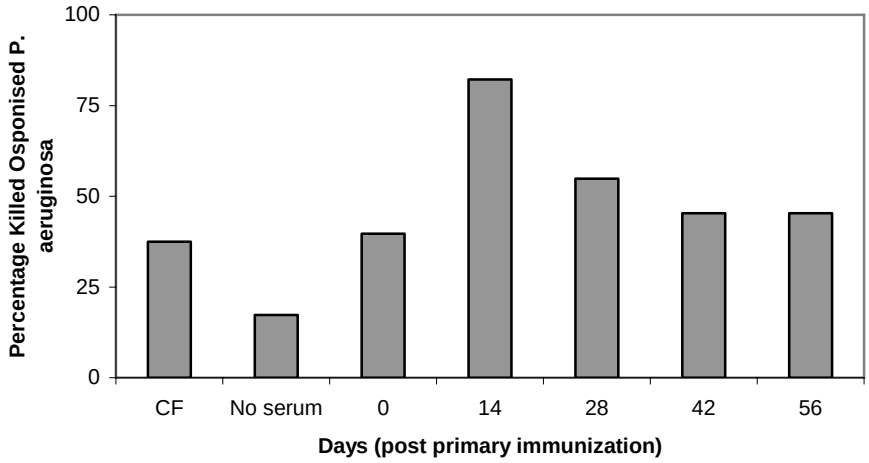


Table 1. IgA and IgM total serum immunoglobulin concentrations (A) and specific whole-*P. aeruginosa* cell extract serum antibody levels (B). Values presented are Mean ± SEM, * indicates value significantly different ($p<0.05$) from Day 0. 24 subjects were observed.

	(A) Total Serum Immunoglobulins (g/L)		**(B) Whole-cell Extract Specific Antibodies (absorbance at 450 nm)**	
	IgA	**IgM**	**IgA**	**IgM**
Day	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**	**Mean ± SEM**
0	2.23 ± 0.47	1.28 ± 0.27	1.17 ± 0.12	0.77 ± 0.07
14	2.28 ± 0.48	1.36 ± 0.28	1.30 ± 0.13*	0.82 ± 0.07*
28	2.28 ± 0.48	1.33 ± 0.28	1.22 ± 0.12*	0.80 ± 0.06
42	2.24 ± 0.47	1.30 ± 0.27	1.23 ± 0.12*	0.76 ± 0.06
56	2.28 ± 0.48	1.33 ± 0.28	1.21 ± 0.12	0.78 ± 0.07

Table 2: Percent responders in each category of antigen specific antibody as determined for any individual showing in serum at least two or more postdose antibody titer increases with at least one greater than 15% of predose base-line levels, *n* = number of subjects who responded of the 24 subjects who completed the trial.

	Whole cell Extract % (*n*)	**OprF % (*n*)**	**OprI % (*n*)**
IgA	29% (7)	46% (11)	21% (5)
IgM	25% (6)	25% (6)	17% (4)
IgG	33% (8)	4% (1)	33% (8)