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A protein-based pneumococcal vaccine protects rhesus macaques from pneumonia after experimental infection with *Streptococcus pneumoniae*

Philippe Denoël¹, Mario T Philipp², Lara Doyle², Dale Martin², Georges Carletti¹, and Jan T Poolman¹

¹GlaxoSmithKline Biologicals, Rixensart, Belgium

²Tulane National Primate Research Center, Tulane University Health Sciences Center, Covington, LA, USA

Abstract

Infections caused by *Streptococcus pneumoniae* are a major cause of mortality throughout the world. Protein-based pneumococcal vaccines are envisaged to replace or complement the current polysaccharide-based vaccines. In this context, detoxified pneumolysin (dPly) and pneumococcal histidine triad protein D (PhtD) are two potential candidates for incorporation into pneumococcal vaccines. In this study, the protective efficacy of a PhtD-dPly vaccine was evaluated in a rhesus macaque (*Macaca mulatta*) model of pneumonia. The animals were immunized twice with 10 µg of PhtD and 10 µg of dPly formulated in the Adjuvant System AS02 or with AS02 alone, before they were challenged with a 19F pneumococcal strain. The survival was significantly higher in the protein-vaccinated group and seemed to be linked to the capacity to greatly reduce bacterial load within the first week post-challenge. Vaccination elicited high concentrations of anti-PhtD and anti-Ply antibodies and a link was found between survival and antibody levels. In conclusion, AS02-adjuvanted PhtD-dPly vaccine protects against *S. pneumoniae*-induced pneumonia. It is probable that the protection is at least partially mediated by PhtD- and Ply-specific antibodies.

Keywords

Streptococcus pneumoniae; pneumonia; PhtD; pneumolysin; rhesus macaque; vaccine

Introduction

Streptococcus pneumoniae is one of the leading causes of infectious morbidity and mortality in the world, being responsible for a large spectrum of infections such as otitis media,

Corresponding author: Jan T. Poolman, Research & Development, GlaxoSmithKline Biologicals, Rue de l'Institut 89, B-1330 Rixensart, Belgium, Phone: +32-(0)2-656 9847, FAX: +32-(0)2-656 8436, jan.poolman@gskbio.com.

Trademark disclosure

OptEIA is a trademark of Becton, Dickinson and Company. C ytospin is a trademark of Thermo Electron.

Authorship disclosure

All authors approved the final version of the manuscript. PD, MTP and JTP were more particularly involved in the conception and design of the study, LD and DM in the acquisition of data, GC in the analysis of data.

pneumonia, bacteremia and meningitis [1,2], and affecting mainly high-risk populations such as infants < 2 years of age, the elderly and immuno-compromised individuals. The emergence of antibiotic-resistant pneumococcal strains has further emphasized the need for providing effective prophylactic vaccination [3,4].

Current vaccines are composed of epidemiologically dominant serotype-specific pneumococcal capsular polysaccharides, conjugated or not to a carrier protein [5–8]. They have greatly helped to reduce the burden of pneumococcal diseases, but limitations in their use have appeared, mainly due to the fact that the dominant serotypes may depend on geography and vary over time.

An alternative approach involves the development of vaccines that target common pneumococcal protein antigens. Multiple candidates have been envisaged, including the cholesterol-binding cytotoxin pneumolysin (Ply). Ply is an interesting candidate for pneumococcal vaccine. It is produced by virtually all pneumococcal strains [9] and has been long known to be immunogenic [10]. Pneumolysin is a key virulence factor, exerting cytotoxic effects on epithelial cells through its membrane pore-forming activity [11], thereby facilitating carriage and disease. There are indications that anti-Ply antibodies may be protective. For instance, IgG antibodies to Ply that are transferred from mother to child have been associated with delaying the age of first pneumococcal carriage in high-risk infants [12]. Furthermore, patients with acute pneumococcal infection have significantly lower anti-Ply IgG than healthy controls [13], and low natural anti-Ply levels were shown to be associated with higher incidence of bacteremic pneumococcal infections among HIV patients [14]. Due to its hemolytic effects, Ply cannot be used as such in vaccines, but non-toxic genetically derived pneumolysin toxoid mutants (dPly) have been generated and shown to be immunogenic [15–17].

Among the more recently described pneumococcal proteins, the pneumococcal histidine triad (Pht) protein family deserves attention. Four members of this family have been described so far. Of those, PhtA, PhtB and PhtD share up to 81% sequence identity, whereas PhtE shares only up to 35% identity. All four Phts, but particularly PhtD, are well conserved across the pneumococcal species [18–20]. They are expressed at the surface of the bacterial membrane, which may explain why they are antibody targets in infected individuals [21]. These proteins, described as lung-specific virulence factors [22], are characterized by a histidine triad motif repeated five to six times in their amino acid sequences, such motif affording affinity for divalent cations, particularly zinc and manganese [23,24]. We recently suggested that the Pht proteins may serve as cation storage molecules [19]. In mouse immunization studies, all members of the Pht family have been shown to afford a high level of protection to subsequent pneumococcal infection with a number of different strains/serotypes [18,20,25–28]. However, due to its better phylogenetic conservation and protective effects, PhtD appeared as the best vaccine candidate in the Pht family, deserving further investigation.

Although mouse studies undoubtedly demonstrated the potential of PhtD and dPly to induce protection against *S. pneumoniae* infection, the protective potential of these proteins against pneumococcal pneumonia has not been evaluated yet. Recently, a *S. pneumoniae* infection

model was established in the rhesus macaque (*Macaca mulatta*) [29]. In this model, the clinical course of the disease mimicked the aspects of human pneumonia and was accompanied by elevated levels of neutrophils and pro-inflammatory cytokines in the broncho-alveolar lavage fluids. This model was chosen to assess the efficacy of a PhtD-dPly-based vaccine.

Materials and methods

Animals

The animals used in this study were 3- to 7-year-old male rhesus macaques (*Macaca mulatta*) of Chinese origin. They were housed in an animal holding room isolated from other project animals. All were singly caged, fed commercial monkey chow (Purina Mills 5037 R, PMi Nutrition International, LLC, St Louis, MO, USA), had water available ad libitum, and were free of simian retrovirus infection. Practices in the housing and care of animals conformed to the regulations and standards established to implement the Animal Welfare Act. Animal care facilities were fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care-International. All animal-related protocols were approved by the Institutional Animal Care and Use Committee of the Tulane National Primate Research Center. The animals were selected for having low concentration of anti-19F IgG antibodies (<0.5 µg/ml). The concentration of anti-19F antibodies was determined as described earlier [30].

Antigens

PhtD was cloned from the N4 strain (serotype 4) and produced in *Escherichia coli*. The non-His-tagged protein was purified from bacterial lysate through multiple chromatography steps. Pneumolysin was cloned from the 6B 493/73 strain, produced in *E. coli* and also purified from bacterial lysate through multiple chromatography steps. Further, pneumolysin was detoxified by formol treatment to obtain dPly. Detoxification was ascertained by the absence of residual hemolytic activity *in vitro*, the absence of local reactogenicity after i.m. injection in rats and the absence of *in vivo* toxicity after intranasal challenge in mice.

Immunizations

The animals were immunized twice intra-muscularly, at day 0 and at day 28, with 10 µg of PhtD and 10 µg of dPly formulated in AS02. AS02 is an Adjuvant System containing 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) and QS21 in an o/w emulsion [31].

Bacterial culture

The *S. pneumoniae* serotype used in this study was 19F (ATCC No. 6319, American Type Culture Collection, Manassas, VA, USA). For the preparation of the animal inocula, 100 µl of frozen stock bacteria suspension was inoculated into 100 ml of Todd-Hewitt broth (THB; Becton Dickinson, Sparks, MD, USA), and then incubated in a 5% CO₂ atmosphere at 37 °C for 15 hours. Bacteria were pelleted by centrifugation at 3500 × *g* for 30 min at 4 °C and resuspended in 3 ml of THB. A 2 ml aliquot of the saline suspension was used for each animal, which correspond to 10⁸–10⁹ cfu, as determined by previous quantifications of similar cultures. The remaining volume was used for a precise quantification of the actual

inoculum, by serial dilution and colony counting (see Table 1). To this end, 250 µl of a 10^7 -fold and a 10^8 -fold dilution of each inoculum was added to blood agar plates (trypticase soy agar with 5% sheep blood; BD Diagnostic Systems) and alpha-hemolytic colonies, recorded as streptococci, were counted after an overnight culture.

Challenge

At day 42, the *S. pneumoniae* inocula were instilled into the lower respiratory tract of the animals via bronchoscopy. After pre-medication with a combination of acepromazine and glycopyrrolate, the animals were anesthetized with tiletamine and zolazepam. Isoflurane was administered via a pediatric nasal cannula, when needed, to maintain a steady state of anesthesia. For the inoculation, a 3.5-mm pediatric bronchoscope (Karl Storz, Culver City, CA, USA) was advanced into the proximal trachea down to the main stem bronchi. The bronchoscope was then directed to a distal bronchus of the left caudal lung lobe, and 2 ml of sterile saline followed by 20 ml of air were instilled into the bronchus as the 'uninoculated' control. Subsequently the bronchoscope was directed to the right caudal lung lobe, and the appropriate *S. pneumoniae* cfu count contained in 2 ml of saline, followed by 20 ml of air were administered into a distal bronchus. The bronchoscope was allowed to remain in place for 10 s and was then removed. The animal was placed in right lateral recumbency post-inoculation in an attempt to contain the *S. pneumoniae* inoculum predominantly in the right caudal lung lobe.

Broncho-alveolar lavage (BAL) fluid collection

A 20 ml aliquot of normal saline was instilled into the chosen subsegmental bronchus of the left lung through the bronchoscope and immediately aspirated. The procedure was repeated into a subsegment of the right lung lobe. After the broncho-alveolar lavage, the animal was carefully monitored by manual observation of the respiratory rate and effort, and evaluation of the mucous membrane colour. A pulse oximeter, blood pressure monitor, and/or electrocardiograph were employed when clinically indicated. Oxygen was provided via a pediatric nasal cannula throughout the procedure.

For a limited number of monkeys (3/group), aliquots of BAL were frozen at -80°C until use for the determination of inflammatory cytokine concentration.

Titration of *S. pneumoniae* in BAL fluid

Collected BAL fluids were extensively stirred. A 0.25 ml volume was added to a blood agar plate (trypticase soy agar with 5% sheep blood; BD Diagnostic Systems) and cultured in 5% CO_2 at 37°C for 21–24 hours. In addition, 0.5 ml was added to 4.5 ml saline for 10-fold serial dilutions up to 1×10^6 -fold. A 0.25 ml volume of each 10-fold dilution was cultured on blood agar plates. Alpha-hemolytic colonies were counted and recorded as pneumococci.

Inflammatory cytokines in broncho-alveolar lavages

Concentrations of TNF- α , IL-1 β , IL-6 and IFN- γ were determined by enzyme-linked immunosorbent assay (ELISA; OptEIA™ ELISA kit, Pharmingen), according to the manufacturer's instructions.

Neutrophil counts in broncho-alveolar lavages

BAL fluid cell monolayers were obtained on microscope slides using a Shandon Cytospin™ 3 device (Shandon Scientific, Chechire, UK). For that, 100 µl of BAL fluid from right lung was applied into the sample chamber and filter card assembly of the cytospin device before being centrifuged for 7 min at $72.3 \times g$. Cells were formerly concentrated or diluted, depending on the sample, in order to obtain a suitable cell monolayer. After centrifugation, the slides were air-dried and stained with Wright-Giemsa stain. Neutrophils were counted in relation to the total leucocyte population, which included monocyte, macrophage, mast cells, eosinophils and lymphocytes.

Pneumonia score

The severity of the pneumonia was scored according to a ranking from 1 (very mild pneumonia) to 6 (severe pneumonia), depending on the size of the cell infiltrate as evaluated on lung X-rays.

Serology

Blood was obtained by venous puncture at the beginning of the study (before immunization), and after the second immunization (before the challenge). The concentration of anti-PhtD and anti-dPly antibody was measured by ELISA. Briefly, microtiter plate was coated overnight at 4 °C with either PhtD or dPly (1 µg/ml in phosphate-buffered saline [PBS]). After washing, two-fold serial dilutions (in PBS-BSA 0.2%-Tween-80 0.05%) of the animal sera were added to the wells and incubated for 1 h at 20 °C with shaking. After washing, the presence of PhtD or dPly-specific antibodies was detected by addition of horseradish peroxidase-conjugated mouse anti-human IgG Fc PAN (HP6043; Hybridoma Reagent Laboratory), diluted 1/4000 in PBS-BSA 0.2%-Tween-80 0.05%, followed by a washing step and an incubation in o-phenylenediamine in citrate buffer 0.1M pH 4.5. The reaction was stopped by addition of HCl 1N and the plate was read at 490 nm (620 nm for the reference filter) in a microplate reader.

Statistics

The Fisher's exact test was used to compare the death rate between the two groups at day 7 and at day 14. ANOVA was used to compare the pneumonia scores between groups. A Log-rank survival test was done to compare the percentage of survival between the 2 groups, taking into account the day of death during an experimentation of 63 days. Finally, multiple logistic regression was used to evaluate the survival rate as a function of anti-PhtD and anti-Ply titers.

Results

Efficacy of the vaccine

After immunization, the animals were challenged with *S. pneumoniae* at Day 42. The efficacy of the vaccine was evaluated in all animals up to 9 weeks after challenge. BAL fluid was collected periodically from both the left and right lungs for assessment of the presence of *S. pneumoniae*. Animals were also assessed clinically and lung X-rays were taken when

necessary to evaluate the extent of pneumonia. The animals were humanely euthanized when showing clinical signs of severe pneumonia. The date of death was recorded.

Quantification of *S. pneumoniae* in BAL fluid—BAL fluids were collected serially, at the time points listed in Table 2. At day 1 post-challenge, all animals but one in the AS02 group and three in the PhtD-dPly/AS02 group had detectable *S. pneumoniae* cfu in BAL fluids from the right lung, where the challenge took place. BAL fluids from left lungs were often positive, but to a much lesser extent than the right BAL fluid. Bacteria in the left lung resulted probably from some inevitable contamination during the challenge procedure or a quick redistribution of live bacteria.

A large disparity in the numbers of cfu at day 1 could be noticed, but no direct link was found between cfu number and mortality. However, death seems to be associated with the highest values of cfu at day 3, reflecting that the animal has difficulty to rapidly alleviate the infection. All animals with cfu number $> 10^6$ at day 3 in the right lung had to be euthanized shortly thereafter because they showed clinical signs of severe pneumonia. In both groups, the bacterial load progressively decreased over time in surviving animals. In the vaccine group, two animals (EB41 and ER53) did not eliminate the bacterial infection after 5 or 7 weeks (days 35 or 49, respectively) post-challenge. At day 63, EB41 was still infected, particularly in the left lung, while the right lung was almost free of cultivable organisms.

Inflammatory reaction—The concentrations of TNF- α , IL-1 β , IL-6, and IFN- γ in the right (inoculated) and left BAL fluids of 3 animals/group were determined by specific ELISAs (data not shown). For both groups, levels of TNF- α , IL-1 β , IL-6 and IFN- γ in the right lung peaked 24 h after inoculation, independently of the group. After 3 days (except 7 days for TNF- α in monkey EC42), all cytokine levels returned to baseline values. No increase in cytokine level was evidenced in the left, non-inoculated lung.

Neutrophil infiltration, as a marker of inflammation, was evaluated in the right lung of three animals/group. In both groups, a marked increase in relative neutrophil count was evidenced one day after instillation of the bacterium, reaching 88.5–98.5% of the leukocyte species. The relative number of neutrophils progressively decreased thereafter to return to normal value after two weeks.

Clinical assessment

A subset of animals in each group (10 in the AS02 group and 12 in the PhtD-dPly/AS02 group) was followed by X-Ray photography, in order to evaluate pneumonia score (Table 3). Among the 10 animals from the AS02 group, 4 died before day 7. Among the surviving animals, all had a pneumonia score 1 at day 7 and one of them showed a score 6. In the PhtD-dPly/AS02 group, one animal died before day 7. Among the 11 surviving animals, one showed a pneumonia score = 0, and none of them had a score 6 at day 7.

For each group, we calculated the total pneumonia score, which is the sum of the highest scores of all animals in one group. This was not necessarily the score observed at day 7. Therefore, it also includes the scores reached by the animals that were dead at day 7. Further, the mean pneumonia score is the total score divided by the number of animals taken

into account. The mean pneumonia score was lower in the PhtD-dPly/AS02 group than in the control group but the difference did not reach statistical significance (ANOVA, $P=0.2879$)

Survival—The survival of the animals is illustrated in figure 1. The survival at the end of the study (day 63) was higher in the PhtD-dPly/AS02 group compared with the AS02 group (logrank test; $P=0.0272$).

Survival was also evaluated at days 7 and 14 post-challenge. The results are shown in Table 4. The number of colonized and cleared animals was determined on the basis of the number of cfu in the lungs. Protection from death by PhtD-dPly/AS02 vaccine was observed at day 14 post-challenge ($P=0.0289$, Fisher's exact test, 1-tailed), but protection may already have been manifest by day 7 ($P=0.0644$).

Pre immunization and post-II (pre-challenge) serology

Immunization of PhtD- and dPly-naïve monkeys with AS02-adjuvanted bivalent PhtD-dPly vaccine elicited anti-PhtD and anti-dPly antibody levels ranging from 1323.4 to 7264 and from 184.7 to 1269.4 $\mu\text{g/ml}$, respectively (figure 2), as measured at day 42. There was no aspecific stimulation of the immune system by AS02, indicating that the responses were antigen-specific.

Relationship between antibody titers and protection—A multiple logistic regression was performed to evaluate whether a link exists between antibody titers and protection from death. Results are displayed in table 5 and this analysis shows that a monkey has a greater chance to survive the challenge with higher levels of anti-PhtD and/or anti-dPly antibodies. For instance, an animal with anti-PhtD levels 73 $\mu\text{g/ml}$ or anti-dPly 5 $\mu\text{g/ml}$ has 80% of chance to survive the pneumococcal challenge. One of the two types of antibody was sufficient to protect and no additional or synergistic effect between anti-PhtD and anti-dPly responses was measured.

Discussion

Common pneumococcal proteins are currently being evaluated as vaccine candidates against *S. pneumoniae* infection. In this regard, PhtD, a member of the Pht protein family, and dPly, a detoxified form of pneumolysin, are considered for inclusion in pneumococcal vaccines. In the present study, vaccination with these two proteins was evaluated in a nonhuman primate model of pneumonia. This model was set up recently in rhesus macaques and was shown to mimic the progression of disease in human [29]. An advantage of using this species for the evaluation of vaccine is that rhesus macaques already serve as model to study human immune responses [32–36], which may be used as basis to evaluate the response to the vaccine.

Here, we demonstrate that vaccination with AS02-adjuvanted PhtD-dPly protects from mortality induced by *S. pneumoniae* challenge. Fewer animals died in the group vaccinated with the two proteins, compared with the group receiving AS02 only. Moreover, among the surviving animals, pneumococcal colony counts were slightly lower in the PhtD-dPly group

and pneumonia scores were also lower. The mechanisms by which this is achieved are not known and have not been investigated in this paper. However, our results may give some hints.

The nature and the course of the inflammatory response in the infected lung were similar in both groups, as evaluated by local neutrophil recruitment and inflammatory cytokine production in a limited number of animals. One day after challenge, the concentrations of the inflammatory cytokines TNF- α , IL-1 β and IL-6 were elevated in the inoculated lungs. Before Day 5, most measurements had returned to baseline. There was no such increase in the left, non-inoculated lungs.. In both groups, neutrophil counts peaked the day after challenge and returned progressively to normal values within 3 weeks, which corresponds to the observations made when this model was set up [29]. These results are indicative of no effect of vaccination on the inflammatory response consecutive to pneumococcal infection. However, they need confirmation, as the number of samples was limited in the present study.

Here, the capacity to eliminate rapidly the bacterium in the lungs was revealed as an important factor. Indeed, mortality seemed to be directly related to the incapacity of the animal to reduce rapidly and significantly the bacterial load in the infected lung. All deaths occurred within the first 8 days and concerned monkeys for which bacterial load in the infected lung was still higher than 1 million cfu/mL after 3 days. This also applied to the only monkey who died in the PhtD-dPly/AS02 group. In contrast, surviving animals showed a dramatic decrease in cfu counts within the first 3 days following challenge. They were in majority in the PhtD-dPly/AS02 group, reflecting the effect of vaccination. When using polysaccharide-based pneumococcal vaccines, protection is known to be related to the presence of the antibodies induced to the type-specific pneumococcal polysaccharides [37,38]. The biological function of these antibodies is to bind onto the surface of the pneumococcal cell and in so doing to lead to uptake and killing of the infectious agent by human phagocytic cells in a process called opsonophagocytosis. In our study, the main effect of PhtD-dPly/AS02 vaccination that we evidenced was the production of high levels of anti-PhtD and anti-dPly antibodies, but it has not been directly investigated whether these antibodies were opsonic or whether they neutralized the pathogen through the binding to crucial surface molecules. Nevertheless, in order to unravel whether they might have played a role in protection, a multiple logistic regression was carried out with the aim to highlight a link between antibody levels and survival. By this means, it was indeed demonstrated that the chance to survive correlates with anti-PhtD and anti-dPly antibody levels. For instance, anti-PhtD or anti-dPly > 1147 and 116 μ g/ml, respectively, afford 95% of chance to survive the challenge. Such finding confirms the antibodies as the main actors of the protection against *S. pneumoniae* infection, which entails that in the future, threshold antibody concentrations of vaccine-induced anti-PhtD and anti-Ply might be associated with protective immunity.

Beyond what has already been demonstrated in mouse models [26], this study confirms that vaccination with pneumococcal proteins, and particularly PhtD and dPly, protects against pneumonia. In our model, the role of vaccine-induced antibodies was shown to be critical, which advocates the use in a potential future pneumococcal vaccine of an adjuvant able to stimulate antibody production.

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Conflict of interest

The study was supported by GlaxoSmithKline Biologicals s.a. (GSK). GSK took the decision to submit the paper. PD, GC and JTP are employees of GSK. They own shares and options to shares in GSK. In addition, JP and PD are designated inventors on a variety of patents owned by GSK.

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Figure 1.
Survival of AS02- and PhtD-dPly/AS02- vaccinated monkeys after challenge with *Streptococcus pneumoniae*.

Figure 2. Concentration of anti-PhtD and anti-Ply antibodies before and after immunization (before challenge) with AS02 or PhtD-dPly adjuvanted with AS02.

Table 1

Challenge doses.

Group AS02		Group PhtD-dPly/AS02	
Animal	Inoculum (CPU)	Animal	Inoculum (CPU)
CV28	3.3×10^9	BV72	2.6×10^9
DH89	1.7×10^8	DG44	1.7×10^8
DR41	6.8×10^8	DK46	6.8×10^8
EG23	5.7×10^8	EB41	5.7×10^8
EC75	7.5×10^8	ED33	7.5×10^8
EC42	4.5×10^8	DV95	4.5×10^8
CT13	6.5×10^8	FH71	6.5×10^8
FJ68	6.5×10^8	DV66	6.5×10^8
FG52	7.0×10^8	EP43	6.5×10^8
DH85	1.2×10^9	FJ20	6.5×10^8
FJ62	1.2×10^9	DF9 ⁹	7.0×10^8
GB23	3.3×10^8	FH22	1.2×10^9
DD96	4.9×10^8	EL42	1.2×10^9
FT52	2.6×10^8	GB09	3.3×10^8
GT55	2.0×10^9	ER53	4.9×10^8
FC89	2.6×10^8	FH20	2.6×10^8
		GK62	2.0×10^9
		GB30	2.6×10^8

Table 2

Quantification of *S. pneumoniae* colony-forming units (cfu/mL) in broncho-alveolar lavage fluid specimens collected longitudinally. All values are “measured value” $\times 10^{-4}$.

Animal	Day 1		Day 3		Day 7		Day 14		Day 21		Day 35		Day 49		Day 63	
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
Group AS02																
CV28	0.48	620	0.26	3000	D (6)	D	D	D	D	D	D	D	D	D	D	D
DH89	45	370	0	4	0.0012	0.12	0	0	0	0	0	0	0	0	0	0
DR41	0.0008	120	0	0.079	0	0	0	0	0	0	0	0	0	0	0	0
EG23	2.2	1000	0.008	120	0	19	D (8)	D	D	D	D	D	D	D	D	D
EC75	0.029	3200	0	0	0	0	0	0	0.0064	0	0	0	0	0	0	0
EC42	0.4	280	4.1	64	0.0032	20	0.002	0.004	0.026	0.055	0	0	0	0	0	0
CT13	5.5	8.8	0	7.4	0	0	0	0	0	0	0	0	0	0	0	0
FJ68	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FG52	0	41.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DH85	0	0.818	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FJ62	0	396	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GB23	0.002	700	tmtc	218	D (6)	D	D	D	D	D	D	D	D	D	D	D
DD96	0.019	25200	0.009	16	0	0.0004	0	0	0	0	0	0	0	0	0	0
FT52	12	2720	D (3)	D	D	D	D	D	D	D	D	D	D	D	D	D
GT55	34	tmtc	D (2)	D	D	D	D	D	D	D	D	D	D	D	D	D
FC89	2.4	200	2.4	1.2	D (3)	D	D	D	D	D	D	D	D	D	D	D
Group PhdD-dPly/AS02																
BV72	nd	330	tmtc	0.35	0.008	0.0008	0	0.08	0	0	0	0	nd	nd	nd	nd
DG44	0	660	0	0.0012	0	0	0	0	0	0	0	0	0	0.0004	0	0
DK46	0.0028	100	0.033	16	0.0008	0.002	0	0	0	0	0	0	0	0	0	0
EB41	0	100	0	0	tmtc	tmtc	0.0066	1.5	4.5	2.6	3.8	2.3	0.0012	0.0196	tmtc	0.0188
ED33	0	1.2	0.0020	0.28	0	0	0	0	0	0	0	0	0	0	0	0
DV95	12	420	0	0.0028	0	0	0	0	0	0	0	0	0	0	0	0
FH71	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Animal	Day 1		Day 3		Day 7		Day 14		Day 21		Day 35		Day 49		Day 63	
	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R
EP43	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DF99	0	0.0128	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FH22	0	2.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0
EL42	0	0.6	0	0	0	0	0	0	0	0	0	0	0	0	0	0
DV66	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
FJ20	0	80	0	0	0	0	0	0	0	0	0	0	0	0	0	0
GB09	2.3	2920	0.002	21.6	0.004	40.8	0	0	0	0	0	0	0	0	0	0
ER53	0.0008	2600	0.0052	332	0	0.0008	0	0	0.0024	0.0028	0	tmtc	nd	0.0004	nd	0
FH20	0.18	208	nd	100	0.0008	3.8	0	0	0	0	nd	nd	nd	nd	nd	nd
GK62	100	tmtc	D (2)	D	D	D	D	D	D	D	D	D	D	D	D	D
GB30	0.008	120	0	3	0	0	0	0	0	0	0	0	0	0	0	0

L, left lung

R, right lung

D, dead; the day of death is indicated in parentheses.

nd, not determined

tmtc, too many cfu to count. This qualifies samples for which the counts were >1200 cfu/mL at the dilution factor used.

Table 3

Pneumonia scores of surviving animals on day 7 post-challenge.

	Group AS02	Group PhtD-dPly/AS02
N animals on day 0	10	12
N surviving animals on day 7	6	11
N surviving animals on day 7 with:		
No signs of pneumonia	0	1
Pneumonia score ^a = 1	1	4
Pneumonia score = 2	3	4
Pneumonia score = 3	0	1
Pneumonia score = 4	1	0
Pneumonia score = 5	0	1
Pneumonia score = 6	1	0
Total pneumonia score ^b	45	37
Mean pneumonia score ^c	4.5	3.08

^aThe severity of the pneumonia was scored according to a ranking from 1 (very mild pneumonia) to 6 (severe pneumonia), depending on the size of the cell infiltrate as evaluated on lung X-rays.

^bThe total pneumonia score of a group is the sum of the highest scores reached by all animals of this group.

^cThe mean pneumonia score is the total pneumonia score divided by the number of animals.

Comparison of the two groups: $P=0.2879$; ANOVA)
N, number.

Table 4

Survival and lung infection status of the monkeys on days 7 and 14 post-challenge.

Groups	Number of monkeys (%)						
	Day 0	Day 7		Day 14		Dead ^b	
		Alive	Dead ^a	Alive	Colonized		
AS02	16	7 (43.75)	4 (25)	5 (31.25)	9 (56.25)	1 (6.25)	6 (37.5)
PhlD-dPly/AS02	18	11 (61.11)	6 (33.33)	1 (5.56)	15 (83.33)	2 (11.11)	1 (5.55)

^aComparison dead versus alive ($P=0.0644$; Fisher's exact test)

^bComparison dead versus alive ($P=0.0289$; Fisher's exact test)

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Table 5

Probability of survival as a function of anti-PhtD and anti-Ply antibody concentrations after immunization with PhtD-dPly/AS02 vaccine.

Predicted probability of event dead (P) (%)	Predicted probability of event alive (1-P) (%)	Odds P/(1-P)	Inverse odds (1-P)/P	Anti-PhtD concentration (µg/ml)	Anti-Ply concentration (µg/ml)
1	99	0.010	99	21107	3303
5	95	0.053	19	1147	116
10	90	0.111	9	307	26
20	80	0.250	4	73	5
30	70	0.429	2.3	28	2