A Protein E-PilA fusion protein shows vaccine potential against nontypeable *Haemophilus influenzae* in mice and chinchillas

Short title: PE-PilA as vaccine antigen

Carine Ysebaert\(^a\), Philippe Denoël\(^a\), Vincent Weynants\(^a\), Lauren O. Bakaletz\(^b\), Laura A. Novotny\(^b\), Fabrice Godfroid\(^a\), Philippe Hermand\(^a\)#

Affiliations

\(^a\) GSK, Rue de l’Institut, 89, 1330 Rixensart, Belgium

\(^b\) The Research Institute at Nationwide Children’s Hospital, Center for Microbial Pathogenesis and The Ohio State University College of Medicine, Department of Pediatrics, 700 Children’s Drive, Columbus, OH 43205, United States.

#Corresponding author: Philippe Hermand, GSK, Rue de l’Institut, 89, 1330 Rixensart, Belgium; email: philippe.hermand@gsk.com
Abstract

PE-PilA is a fusion protein composed of immunologically relevant parts of protein E (PE) and the majority subunit of the type IV pilus (PilA), two major antigens of nontypeable *Haemophilus influenzae* (NTHi). Here we report on the preclinical evaluation of PE-PilA as vaccine antigen. The immunogenic potential of PE and PilA within the fusion was compared with that of isolated PE and PilA antigens. When injected intramuscularly in mice, the immunogenicity of PE within the fusion was equivalent to that of isolated PE, except when formulated with alum. In contrast, in our murine models PilA was consistently found more immunogenic as a sub-entity of the PE-PilA fusion protein than when injected as an isolated antigen. Anti-PE antibodies following immunization with PE-PilA, demonstrated the same capacity to inhibit the binding of PE to vitronectin than those induced after PE immunization. Likewise, PE-PilA-induced anti-PilA antibodies inhibited the formation of NTHi biofilms and disrupted established biofilms *in vitro*. These experiments support the immunogenic equivalence between fused PE-PilA and isolated PE and PilA, respectively. Further, the potential of PE-PilA immunization against NTHi-induced disease was evaluated. After intranasal NTHi challenge, colonisation of the murine nasopharynx significantly dropped in animals formerly immunized with PE-PilA, and in chinchillas, signs of otitis media were significantly reduced in animals that have received anti-PE-PilA antibodies.

Taken together, our data support the use of PE-PilA as an NTHi vaccine antigen.

Key-words: *Haemophilus influenzae*; chinchilla; Vaccine; Protein E; PilA;
Introduction

*Haemophilus influenzae* is a gram-negative pathogen able to colonize the nasopharynx and to induce disease in both the upper and lower respiratory tracts. Both encapsulated and non-encapsulated (“nontypeable”; NTHi) forms of the bacterium exist and exclusively colonize the human host. In adults, NTHi is recognized as the major bacterial cause of exacerbation in chronic obstructive pulmonary disease (COPD) (1, 2). The clinical manifestations are more diverse in children, comprising sinusitis, conjunctivitis and pneumonia, but NTHi is best known as the predominant pathogen of chronic and recurrent otitis media (OM) (3-6). It can also be considered as equivalent to the pneumococcus for its involvement in acute OM (7-12).

Different prophylactic vaccines to target this pathogen were developed more than a decade ago and were evaluated in preclinical studies (13). However, none of them was fully satisfactory, mainly due to sequence variation among the various strains of NTHi. The clinical evaluation of an 11-valent polysaccharide pneumococcal conjugate vaccine using *H. influenzae*-derived Protein D (PD) as carrier protein showed 35.3% efficacy against NTHi-induced acute OM episodes (14, 15). Although this result opened interesting perspectives for the control of NTHi-induced diseases, it also highlighted the need for additional NTHi vaccine antigens to reinforce this PD approach. For that, our strategy has been to combine relevant parts of two well-established vaccine candidates, Protein E (PE) and PilA, in a single fusion protein (16).
PE is known as a ubiquitous NTHi adhesive protein, important for adhesion to host epithelial cells (17-19). In this regard, interaction of PE with host laminin plays an important role (20). PE was also shown to bind vitronectin, thereby protecting the bacterium from complement attack (21-23). Host complement system is further dampened by the plasmin that results from the binding of PE to plasminogen (24). PE has recently been proposed as a relevant NTHi vaccine antigen (25). PilA, the second antigen of the fusion protein, is the major subunit of type IV pili (Tfp) (26). Tfp are hair-like filaments, known to be well conserved among H. influenzae isolates (27), binding its target cells through ICAM-1 (28). Tfp are also involved in twitching motility and biofilm formation (29-32). It has been shown that anti-PilA antibodies are able to both prevent the formation of and disrupt established NTHi biofilms (33), which further qualifies PilA as a target vaccine antigen.

In another work, we described and characterized the fusion molecule PE-PilA and demonstrated that the individual structure of each of the two entities is kept within the fusion structure (16). Here, the immunological potential of the fusion molecule is evaluated.

Results

Immunogenicity of PE-PilA

In mice, the humoral responses against PE after PE-PilA immunization were similar to those after immunization with PE alone, as evaluated on Day 42 (1106 µg/mL versus 1273 µg/mL, respectively, when given intramuscularly (IM) and adjuvanted with AS01, and 1349 µg/mL versus 1139 µg/mL when given intranasally (IN) and...
adjuvanted with the heat-labile toxin of *Escherichia coli* (LT), respectively).

However, this was not the case when adjuvanted with alum (Fig. 1). In the latter case, although 100% of mice seroconverted after immunization with PE-PilA, the level of anti-PE antibodies was almost five times lower than after immunization with PE alone (126 µg/mL versus 608 µg/mL, respectively).

In mice, PilA was found not, or very slightly immunogenic when given alone, independently of the adjuvant and the route of immunization (Fig. 1). All anti-PilA levels lay below 0.05 µg/mL. However, PilA within the fusion was found more immunogenic than isolated PilA, and this was particularly remarkable when PilA was adjuvanted with AS01 (IM administration) or LT (IN administration). With alum as adjuvant, PE-PilA IM immunization elicited anti-PilA levels that were 30-fold higher compared with PilA immunization, but when AS01 was used anti-PilA antibody levels were more than 2000-fold higher, reaching 80 µg/mL. After IN immunization in the presence of LT, the differences were even more striking since the anti-PilA antibody levels were 463 µg/mL after PE-PilA immunization, which represents more than 10⁴ times the levels obtained after PilA immunization.

**Inhibition of vitronectin binding by PE with antibodies to PE-PilA fusion**

We aimed to determine whether antibodies from mice immunized with PE-PilA were able to inhibit vitronectin binding to PE (Fig. 2). The sera used for the determination of the humoral responses were used for this experiment, without adjustment for antibody levels. As could be expected, sera from mice immunized with adjuvant alone or PilA alone (negative controls), even if anti-PilA antibodies were generated, were not able to inhibit the binding of vitronectin to PE. When immunized with PE-PilA
admixed with alum, elicited antibodies could inhibit PE-vitronectin recognition, but to a lesser extent than after immunization with PE admixed with alum, reflecting the difference in anti-PE antibody levels between the two groups. When adjuvanted with AS01, PE and PE-PilA gave comparable anti-PE antibody levels after immunization and, accordingly, the levels of vitronectin binding inhibition were similar for the two groups.

Inhibition of biofilm by anti-PE-PilA antibodies

Type IV pili are composed of a majority subunit PilA and play a critical role in biofilm formation and anti-PilA antibodies are known to play a role in biofilm dispersal. This assumption could be verified in our experiments in which chinchilla anti-PilA antibodies produced after immunization with PilA were able to inhibit NTHi biofilm formation in vitro. Three clinical isolates of NTHi were examined, strain 86-028NP (from which PilA in the fusion originates), strain 1714 and strain 1128 (two strains showing the least PilA amino-acid sequence identity compared to strain 86-028P) (Table 1). Whereas biofilms formed by each NTHi strain varied in overall height and architecture, growth in the presence of naive serum did not inhibit the formation of characteristic bacterial towers and intervening water channels (Fig. 3A,D&G). In contrast, incubation in the presence of anti-PilA antibodies that had been arbitrarily diluted 1:50, prevented the formation of comparable biofilm structures (Fig. 3B,C, E,F,H&I). Biofilm thickness and biomass were significantly reduced for all three NTHi isolates after incubation with PilA antibodies, compared with naive serum (Fig. 3J-K; p ≤ 0.01) and anti-PilA or anti-PE-PilA showed comparable efficacy.
As an additional functional assessment of PilA antibodies *in vitro*, NTHi biofilms were first established for 24 h prior to incubation with naive serum or antisera against PilA (again, both sera diluted 1:50 prior to use). Compared to biofilms incubated with naive serum (Fig. 4A,D&G), treatment of pre-formed NTHi biofilms with anti-PilA antibodies induced dispersal of each of the three strains from these structures (Fig. 4B,C, E,F,H&I). A significant reduction in biofilm thickness and biomass was observed for each of the three strains by anti-PilA and anti-PE-PilA antibodies, compared to naive serum (Fig. 4J&K; \( p \leq 0.001 \)). As before, anti-PilA and anti-PE-PilA antibodies induced similar biofilm dispersal efficacy. Collectively, these data demonstrated the ability of anti-PilA antibodies to both prevent the formation and induce the dispersal of biofilms formed by NTHI strains despite amino acid diversity within the PilA subunit.

Protection in the nasopharyngeal colonization model

To assess the protective activity of PE-PilA vaccination against naso-pharyngeal colonization, we used a non-inflammatory nasopharyngeal colonization murine model. In this model, the bacteria colonize locally and do not spread to lungs, due to the small volume of inoculum, and do not infect systemically. Mice were immunized intranasally with adjuvant only (LT), PE alone, PilA alone or PE-PilA, all adjuvanted, before they were challenged via the same route with the 3224A or the 3219C NTHi strain (see Table 1). The intranasal route was used, as pilot experiments in our laboratories showed no protection with the parenteral route in this model.

When cohorts were compared to each other over time, a significant reduction in the number of bacteria (\( p < 0.001 \)) was shown in the groups immunized with PE-PilA and
No protection was observed with PilA alone ($p = 0.9937$), which is in line with earlier observation showing that PilA alone is weakly or non-immunogenic in mice (Fig. 1).

Protection against otitis media in a chinchilla model

Antisera generated in adult chinchillas were titrated for anti-PE and anti-PilA antibodies before they were used for passive transfer. As individual antisera, the midpoint titer for anti-PilA was 1007 and 1661 for anti-PE. These titers were within the same log range as those obtained by immunization with PE-PilA fusion (i.e. 1273 for anti-PilA and 2984 for anti-PE). Therefore, admixing individual anti-PilA and anti-PE antisera prior to passive transfer yielded immune pools that were comparable in titer to those obtained by immunization with the PE-PilA fusion protein. Two days after intranasal challenge with NTHi, control analysis of the naso-pharyngeal lavage fluids indicated that the three groups were equally colonized with the challenge isolate, therefore any differences in protection observed among cohorts could be ascribed to the specific antiserum administered.

Transfer of immune serum pools protected to different extent the chinchilla host against experimental otitis media due to the NTHi 86-028NP strain (Fig. 6A). For the four analysed parameters (e.g. percentage of middle ears that did not develop disease, time to disease onset, time to disease resolution and proportion of animals that developed OM), receipt of serum against anti-PilA mixed with anti-PE was significantly different from the AS04 group and only trends were observed for the PE-PilA group. Whereas 80% of middle ears showed signs of inflammation in the cohort that received anti-AS04 antibodies, significantly fewer (20%) of ears had signs of OM.
after receipt of anti-PilA + anti-PE antiserum, and while not statistically significant, 50% of middle ears in the cohort that received anti-PE-PilA serum remained healthy. The proportion of animals with signs of disease was significantly less in the PilA + PE group than in the AS04 group (p< 0.01). Thus, compared to receipt of anti-AS04 serum, a significantly reduced proportion of middle ears in the PilA + PE cohort had signs of experimental OM (p< 0.01). Further, the time to disease onset was significantly longer for the PilA + PE group compared to the AS04 group (p = 0.005), and whereas a delay was observed in the cohort administered anti PE-PilA, this outcome was not statistically significant (p = 0.07). For those animals in the cohort that received anti-PilA + anti-PE serum that did develop OM, signs of disease resolved significantly earlier than control (p = 0.015). The recovery time in the PE-PilA group was not significantly different from that of control (p = 0.21). When comparing the areas under the curves, as illustrated in Fig. 6, the PilA + PE group was found significantly different from the control (p = 0.0293), but not the PE-PilA group (p = 0.5893). Vaccine efficacy was determined to be 28.9% (CI: 22.5% to 35.3%) for animals administered anti-PilA plus anti-PE antiserum and 14.7% (CI: 9.7% to 19.7%) for antiserum against PE-PilA.

In another chinchilla experiment, antisera generated after PE-PilA/AS04 immunization were compared with antisera generated after AS04 injection (Fig. 6B). It was observed that the proportion of animals with signs of otitis media was not different between the two groups (p = 0.087). However, the time to disease onset was significantly longer in the PE-PilA group (p = 0.0058), and for those animals that developed signs of disease, the recovery time was shorter (p = 0.0025) compared to receipt of anti-AS04 serum. Finally, the area under the curve was lower in the PE-
PilA group (p = 0.0011). Efficacy for the PE-PilA group was 36.7%. These results showed the capacity of PE-PilA immunization to reduce the signs of otitis media.

Discussion

In a previous work, we designed a fusion protein made of PE and PilA for potential NTHi vaccine applications (16). The aim of the present work was to evaluate the immunogenicity of this fusion protein and the functionality of the elicited antibodies.

PE was found immunogenic, producing high levels of antibodies in BALB/c mice when administered IM or IN, and anti-PE antibodies were also generated after immunisation with PE-PilA to similar level as with PE alone, except when formulated with alum, which remains an unexplained observation. In contrast, PilA was found not immunogenic in BALB/c mice when administered as an isolated protein. This was surprising, as PilA demonstrated immunogenicity in other animal models such as chinchillas and rabbits (27, 33). Being incorporated within PE-PilA rendered PilA more immunogenic in these mice, and this was particularly evident when formulated with AS01 for IM injections or when given intranasally in the presence of LT.

Both anti-PE and anti-PilA antibodies generated after immunisation with PE-PilA were functional. Anti-PE antibodies were able to inhibit the binding of PE to vitronectin. Knowing that binding of PE to vitronectin can protect the bacterium from complement attack (21, 22), the presence of inhibiting antibodies may render the bacterium more vulnerable to that killing mechanism. Likewise, anti-PilA antibodies generated by PE-PilA immunisation were functional, as anti-PilA antibodies were shown to prevent biofilm formation. The mechanism in play involve the inhibition of...
Tfp-mediated adherence and blockade of NTHi twitching motility that are foundational steps to initiate, organize and develop these structures (26, 29, 31-33). Further, the elicited anti-PilA antibodies were able to induce the disruption of established biofilms, which is achieved via a ‘top-down’ dispersal event that is dependent on PilA expression and LuxS-mediated quorum signalling (33, 34). This capacity to impede biofilm formation and to disperse already formed biofilms is an important feature in the fight against the pathogen. Indeed, biofilms have been described in children suffering from otitis media with effusion or recurrent acute OM (35-38) and NTHi is a predominant organism associated with OM. In addition, disruption of biofilms releases the bacteria in the planktonic state in which they are more vulnerable to antibodies and antibiotics (34, 39-41).

After the demonstration of its ability to elicit functional antibodies in vitro, we aimed to determine whether PE-PilA was able to induce protection in animal challenge models. Our results showed that immunisation with PE-PilA was able to prevent NTHi colonisation in the mouse nasopharynx, similarly to immunisation with PE alone, which was demonstrated for two different NTHi strains. This model is non-inflammatory, does not engage the innate side of immunity (unpublished information), which highlights the potential role of the vaccine-induced antibodies in preventing colonization. In addition to the NTHi colonisation model in mice, we used the chinchilla model to study the ability of passively transferred anti-PE-PilA antibodies to prevent the occurrence of otitis media. The chinchilla is a well-established and reliable model for otitis media (42). Biofilms form in the middle ear of chinchilla after NTHi challenge (35) and in vivo-formed biofilms are known to contain high amounts of type IV pilin protein (43), making them targets of choice for
anti-PilA antibodies. In earlier chinchilla experiments, anti-PilA antibodies alone were shown to reduce signs of otitis media symptoms by 42%, whereas it was only 21% for anti-PE antibodies (unpublished results). In the present study, anti-PE-PilA antibodies were as efficient as anti-PilA antibodies to impact biofilms in vitro. Therefore, it was particularly interesting to study the behaviour of the PE-PilA-induced antibodies in the chinchilla model. However, the outcome was less striking in chinchillas than in vitro, although it could be concluded from the two chinchilla experiments that PE-PilA-induced antibodies are able to reduce the signs of otitis media. Some discrepancy between in vitro and in vivo experiments, and between different animal models are not uncommon and may rely on intrinsic specificities of the respective animals’ immune systems.

In conclusion, PE-PilA has shown potential as vaccine antigen, more particularly based on the capacity of PE-PilA-induced antibodies to impede NTHi nasopharyngeal colonization, to both prevent and disrupt bacterial biofilms and to inhibit PE-vitronectin binding. Based on these and other results, the development of this vaccine antigen has been pursued. It has been decided to associate it with PD, another NTHi antigen, and this combination has now reached the clinical development phase (44).

Materials and methods

Antigens and Adjuvant Systems

Recombinant PE antigen consisted of amino acids 22-160 of the PE sequence completed by a His-tag. Its size was approximately 18 kDa. The initial gene fragment originated from the NTHi 772 strain. Periplasmic expression was assured by the pelB
signal sequence upstream the PE sequence. The construct was expressed in

Escherichia coli BLR(DE3) cells and the resulting antigen was purified by

immobilized metal affinity chromatography.

The recombinant isolated PilA antigen consisted of amino acids 40 to 149 of the PilA

sequence + 17 amino acids from PilB. The initial gene fragment originated from

NTHi 86-028NP strain and was associated with a sequence for His-tag. The construct

was expressed in E.coli Origami B(DE3) cells and the resulting antigen was purified

by immobilized metal affinity chromatography. His-tag was removed by thrombin

cleavage capture kit (Novagen), yielding an antigen of approximately 12 kDa. In the

biofilm dispersal experiments, antibodies directed against another recombinant PilA

antigen, called rsPilA [described in (27)] were used.

PE-PilA is a 28.8 kDa His-tagged fusion protein encompassing amino acids 19 to 160

of PE and amino acids 40 to 149 of PilA, linked by a GG amino acids linker (16). The

gene construct was expressed in E. coli BLR(DE3) and the secreted peptide was

purified from cell lysate through several chromatography steps.

AS01 is a liposome-based Adjuvant System containing 3-O-desacyl-4'-

monophosphoryl lipid A (MPL) and QS-21 (Quillaja saponaria Molina, fraction 21;

licenced by GSK from Antigenics LLC, a wholly owned subsidiary of Agenus Inc, a

Delaware, USA corporation) (45). AS04 is an Adjuvant System composed of MPL

adsorbed on an aluminium salt (46).

NTHi strains
Different strains of NTHi were used in the study. They are detailed in Table 1.

Animals

The female BALB/c Ola Hsd mice used in this study were purchased from Harlan (Horst, The Netherlands). They were 5-week-old at time of the first immunization. All mouse studies were ethically reviewed (ethical committee protocols n° 04/88/02A, 04/88/03A and 07/136/02A) and carried out at GSK (Rixensart, Belgium) in accordance with European Directive 2010/63/EU, and the GSK Policy on the Care, Welfare and Treatment of Animals. Upon arrival, the mice were acclimated for at least 5 days. Afterwards, they were randomly allocated to the different groups. All animals had free access to food and 0.22 µm-filtered tap water. Nesting material was provided with nonstructural enrichment material. Air supplied in the housing room was 100% fresh air filtered by EPA filter and the ventilation was at least 20 cycles per hour. The animal room conditions were set as follows: temperature: 20-24 °C; humidity: 55% (range from 40 to 65%) and light/dark cycle: 12 h/12 h.

All chinchilla studies were conducted at The Research Institute at Nationwide Children’s Hospital (Columbus, Ohio, United States). Prior to enrolment, juvenile chinchillas (*Chinchilla lanigera*; from Rauscher’s Chinchilla Ranch, LLC, LaRue, Ohio, United States) were nominally bled and individual sera assayed via western blot to confirm that no animal had a significant pre-existing level of antibody against any outer membrane protein of NTHi. Animals were then clustered into cohorts of nine or ten animals each, based on body weight (average weight per cohort = 411 g).

Adult chinchillas (from the Rauscher’s Chinchilla Ranch, LaRue, Ohio) weighed 625 ± 23 g at study start. All procedures with the chinchillas were performed in...
compliance with morbidity/exclusion criteria detailed in a protocol approved by the Nationwide Children’s Hospital Research Institute Animal Care and Use Committee (#01304AR) and in compliance with the United States Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

Immunization of mice

Groups of 33 mice were immunized IM on Days 0, 14 and 28 with 1 µg PilA, 1 µg PE, or 1 µg PE-PilA adjuvanted with alum or AS01. Control injections consisted of alum or AS01 alone. The IgG levels directed against each antigen were determined by enzyme-linked immunosorbent assay (ELISA) in sera collected on Days 28 (Day 14 post-II) and 42 (Day 14 post-III).

ELISA

To determine anti-PE or anti-PilA IgG levels, microtiter plates were coated with PE or PilA (2 µg/mL or 4 µg/mL, respectively, in carbonate buffer) overnight at 4 °C. After washing, serial two-fold dilutions of murine sera (starting at 1/500 for PE assay or 1/20-1/500 for PilA assay, depending on the experiment, in phosphate-buffered saline containing 0.05% Tween-20 [PBS-T]; 1 h at 25 °C). Afterwards, in both assays, peroxidase-conjugated goat anti-mouse IgG antibodies (Jackson laboratories code 115-035-003; 1/2500 or 1/1250 in PBS-Tween) were added for 1 h at 25 °C. The colorimetric reaction was obtained by the addition of o-phenylenediamine dihydrochloride in citrate buffer in the presence of hydrogen peroxide for 15 min and stopped by addition of 1 N HCl. Plates were read in a spectrophotometer at 490 and 620 nm. In both cases, an in-house calibrated reference serum was used and IgG
concentrations (expressed as µg/ml) were calculated by the 4-parameter method using
the Soft Max Pro software.

**Inhibition of vitronectin binding**

The sera collected on Day 42 for the determination of humoral responses were also
used for the inhibition of vitronectin binding assay, which is a method to assess the
functionality of anti-PE antibodies. A pool was made with all sera within each group.

Vitronectin binding assay was carried out in microtiter plates. Plates were coated with
PE (5 µg/mL in PBS) for 2 h at 37 °C. After washing, saturation of the nonspecific
binding sites was done by incubation with PBS-ovine serum albumin (BSA) 1%, and
then two-fold serial dilutions of heat-inactivated immune murine sera (in PBS-T-BSA
0.02%) were added to the wells for overnight incubation at 4 °C. After washing,
vitronectin (Sigma-Aldrich SRP3186; 4 µg/mL) was added and incubated for 1 h at
37 °C. Finally, after another washing step, bound vitronectin was detected by the
addition of horseradish peroxidase-conjugated sheep anti-vitronectin antibodies
(L12050350 C12120412; US Biologicals; 1/1000 in PBS-T for 30 min at 37 °C),
followed by o-phenylenediamine dihydrochloride as described in the former
paragraph. The mid-point titer (corresponding to the first dilution of murine sera able
to inhibit 50% of binding) of each tested pool was determined.

**Effects of antibodies on biofilm**

Biofilm experiments were carried out with three different strains of NTHi: the
homologous strain 86-028NP, and the two heterologous strains 1714 and 1128, for
which PilA shows 88.2 and 81.8% amino-acid sequence identity with PilA the
vaccine antigen, respectively (Table 1).
Dispersal of established biofilm

To obtain biofilms, as previously described (47), overnight cultures of NTHi were resuspended in 5 mL equilibrated (37 °C, 5% CO₂) brain-heart infusion broth supplemented with 2 µg nicotinamide adenine dinucleotide/mL and 2 µg heme/mL (sBHI) so that OD₄₉₀ = 0.65. Bacteria were diluted 1:6 in equilibrated sBHI in a 50 mL non-closed sterile conical tube and incubated at 37 °C in 5% CO₂. After 3 h, bacteria were diluted 1:2500 in equilibrated sBHI and 200 µL of the bacterial suspension were added to each well of an 8-well chambered glass slide (Nunc, Lab-Tek 155411) for an overnight incubation at 37 °C in 5% CO₂ during which biofilms form. Medium was refreshed for an additional 8 h incubation after which immunized or naïve serum pools (1:50 in sBHI; from the adult chinchillas used in the in vivo study; see section 2.9) were added to the chambers. After 16 h at 37 °C in 5% CO₂, biofilms were washed with sterile saline, incubated with Live/Dead BacLight Bacterial Viability Kit (Invitrogen L7007), and fixed for 1 h at room temperature with 10% formalin. Biofilms were immediately viewed on a Zeiss 510 confocal laser scanning microscope and images analysed with COMSTAT software (48).

Inhibition of biofilm formation

The beginning of the procedure is the same as in the former section. After the bacteria have been added to each well of an 8-well chambered glass slide, incubation took place for 1 h. Then medium was replaced by immune or naïve sera (1:50 in sBHI) for 4 h at 37 °C in 5% CO₂. Chambers were rinsed and biofilms allowed to form overnight or longer (medium refresh every 16 h in such case) in sBHI. Biofilms were
stained for viability, fixed and then viewed by confocal microscopy and images analyzed with COMSTAT software.

Nasopharyngeal colonization model

For the nasopharyngeal colonization model, groups of 20 mice were immunized IN (10 µL in one nostril) on Days 0, 14 and 28 with 6 µg PE, PilA or PE-PilA, adjuvanted with LT (50 µg/mL; except at the third immunization). The IgG levels against each antigen were measured by ELISA in sera collected on Day 42 (Day 14 post-III). The animals were also challenged IN on Day 42 with $5 \times 10^6$ colony-forming units (cfu) of 3224A or 3219C NTHi strain (10 µL in one nostril). Full nasal cavities were dissected one and two days after the challenge, homogenized, and the resulting suspension cultured overnight at 37 °C on chocolate agar to determine bacterial load.

Chinchilla model

Cohorts of six adult chinchillas were subcutaneously immunized three times at 28-30-day intervals with 10 µg of PE, PilA or PE-PilA, all adjuvanted with AS04. Control group received AS04 alone. Animals were bled 10 days after receiving the third immunization, and the sera were pooled by cohort before passive transfer to the juvenile chinchillas.

The passive transfer study was carried out as follows: seven days before NTHi challenge, adenovirus serotype 1 strain was intranasally inoculated ($2 \times 10^7\text{ TCID}_{50}/\text{mL}$ in 200 µL, 100 µL per naris) to the young chinchillas (9 or 10/group). One day before the challenge, the chinchillas were intracardially injected.
with (mixes of) adult chinchilla antibodies obtained via immunization with the different proteins (5 mL serum/kg body weight). One group received serum pool from animals immunized with PE mixed 1:1 with that of animals immunized with PilA (PE + PilA group). Another group received serum pool from animals immunized with PE-PilA (PE-PilA group). A third group received serum from adult animals immunized with AS04 alone (AS04 group). The day after, the young chinchillas were intranasally challenged with 10⁸ cfu of NTHi (100 µL per nare). Colonization status was verified two days later by nasopharyngeal lavage and culture of the lavage fluids on chocolate agar supplemented with 15 µg ampicillin/ml to limit growth of nasopharyngeal flora. Further, the general health of the juvenile chinchillas was evaluated and the occurrence of ear infection was monitored by video otoscopy and tympanometry for 35 days after the bacterial challenge (49).

**Statistical analyses**

Inhibition of vitronectin binding was analysed by ANOVA, followed by Tukey adjusted test. Vaccine efficacy in nasopharyngeal model was measured by ANOVA2 with groups and time as factors. Groups were compared to each other by Tukey adjusted test. Biofilms thickness and biomass were analysed by one-way ANOVA. For the passive transfer experiments, a sample size of n=10 allowed detection of 65% difference between two proportions (OM incidences) with a power of 80%, using a χ² test. Four different statistical analyses were performed in each experiment to compare the vaccinated groups and the adjuvant control group, respectively on the proportion of animals developing OM (Fischer’s exact test), the time to OM onset (first day of disease), the recovery time (last day of disease) and the area under the score curve. Results were considered significant when p values were equal to or below 0.05.
Acknowledgments

The authors thank Jan Poolman (GSK at the time of the study), Christiane Feron and Vincent Verlant (GSK) for providing advice on the design and interpretation of the experiments. They also thank Patricia Godissart, Alexia Vermeire, Sereiwaddhana Neou, Marie Laloy, Francesco Galetto, Philippe Lancelot and Dominique Duchêne (all GSK) and Zachary Jordan (The Research Institute at Nationwide Children’s Hospital, Ohio, US) for their technical assistance. Frederic Renaud (GSK) performed the statistical analyses. Pascal Cadot (GSK) provided scientific writing services. Ulrike Krause (GSK) provided editorial advice and coordinated the manuscript development.

Funding

This work was sponsored and financially supported by GlaxoSmithKline Biologicals SA, which was involved in all stages of the study conduct and analysis.

Conflicts of interest statement

CY, FG, PD, PH and VW are employees of the GSK group of companies. FG, PD, PH report ownership of GSK shares and/or restricted GSK shares. CY, PH are listed as inventors on patents owned by the GSK group of companies. LB has received grants and consulting fees from GlaxoSmithKline Biologicals SA. LN reports no financial conflicts of interest.
Author contributions

FG, LB, LN and PH were involved in the conception and design of the study. CY, LN and PH acquired the data. CY, FG, LB, LN, PH, PD and VW analyzed and interpreted the results. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

References


34. Mokrzan EM, Novotny LA, Brockman KL and Bakaletz LO. 2018. Antibodies against the majority subunit (PilA) of the Type IV pilus of nontypeable *Haemophilus influenzae* disperse *Moraxella catarrhalis* from a dual-species biofilm. MBio 9:e02423-02418.


Legends to the figures

**Figure 1. Antibody levels after immunization.** Mice (n = 33/group) were immunized three times intramuscularly (IM) at a 2-week interval with 1 µg PilA, 1 µg PE, or 1 µg PE-PilA, adjuvanted with alum or AS01, or mice (n = 20/group) were immunized three times intranasally (IN) at a 2-week interval with 6 µg PE, 6 µg PilA or 6 µg PE-PilA adjuvanted with the heat-labile toxin of Escherichia coli (LT). Sera were collected 14 days after the second and the third IM injection (days 28 and 42, respectively) or 14 days after the third IN injection (Day 42). PE-specific and PilA-specific antibody levels were measured by ELISA.

**Figure 2. Inhibition of vitronectin binding.** Mice (n=33/group) were immunized intramuscularly on Days 0, 14 and 28 with 1 µg PE, 1 µg PilA or 1 µg PE-PilA formulated with alum or AS01. Sera were collected on Day 42 and a pool was made with all sera within each group. Twofold dilutions of these serum pools were used to inhibit the binding of vitronectin to PE in microtiter plates. Bound vitronectin was
detected by specific antibody. The results were expressed as the dilution of serum able
to inhibit 50% of vitronectin binding.

**Figure 3. Inhibition of biofilm formation by anti-rsPilA and anti-PE-PilA antibodies.** Adult chinchillas (n=6) were immunized with rsPilA or PE-PilA fusion protein and sera were collected 10 days after the third immunization and pooled. NTHi biofilms of three different strains (86-028NP, 1714, 1128) were allowed to form in the wells of 8-well chambered glass slides. Immunized or naive (control) serum pools were added to the culture medium during formation of the biofilms. After overnight incubation, biofilms were fixed then viewed by confocal scanning microscope (A-I) Images were rendered as orthogonal projections to show a top-down view (to depict relative spatial distribution of the biofilm within the x-y planes), as well as a side view (to depict relative biofilm height within the z-plane) and analyzed with COMSTAT software to evaluate biofilm thickness and total biomass (J, K). Scale bars: 20 µm; Immune sera were compared to control: **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.

**Figure 4. Dispersal of established biofilms by anti-rsPilA and anti-PE-PilA antibodies.** Adult chinchillas (n=6) were immunized with rsPilA or PE-PilA fusion protein and sera were collected 10 days after the third immunization and pooled. NTHi biofilms of three different strains (86-028NP, 1714, 1128) were allowed to form overnight in the wells of 8-well chambered glass slides. Immunized or naive (Control) serum pools were added to the culture medium after biofilms were established. After overnight incubation, biofilms were fixed then viewed by confocal scanning microscopy (A-I) Images were rendered as orthogonal projections to show a
top-down view (to depict relative spatial distribution of the biofilm within the x-y planes), as well as a side view (to depict relative biofilm height within the z-plane) and analyzed with COMSTAT software to evaluate biofilm thickness and total biomass (J, K). Scale bars: 20 µm; Immune sera were compared with Control: ****, p < 0.0001.

Figure 5. Vaccine efficacy in an NTHi nasopharyngeal colonization model. Mice were immunized intranasally with LT-adjuvanted PE, PilA, PE-PilA or adjuvant alone (control) before they were intranasally challenged with NTHi strain 3224A. Bacterial colonies in nasal washings were counted on day 1 and day 2 post-challenge and expressed as log10 mean cfu. Each symbol represents a mouse. Dashed lines indicate the limit of detection; Black horizontal bars are geometric means. Statistical analyses were carried out with ANOVA2. Groups were compared to each other over time (Tukey adjusted). Groups PE and PE-PilA versus control: p < 0.001. Group PilA versus control: p = 0.9937. Group PE versus group PE-PilA: p = 0.4239. Representative experiment out of 4 with either 3224A or 3219C NTHi strain.

Figure 6. Development of otitis media (OM) in juvenile chinchillas. A. Adult chinchillas were immunized with adjuvanted PE, PilA, PE-PilA or adjuvant alone. Sera were collected and used for passive transfer to juvenile chinchillas. Anti-PE and anti-PilA immune sera were combined to form the PE-PilA group. After passive transfer, the juvenile chinchillas were intranasally challenged with NTHi strain 86-028NP and the development of otitis media was monitored by video otoscopy and tympanometry for 35 days after bacterial challenge. B. Adult chinchillas were immunized with adjuvanted PE-PilA or adjuvant alone. Sera were transferred to
juvenile chinchillas and the animals were intranasally challenged with NTHi strain 86-028NP. The development of otitis media was blindly monitored for 35 days after bacterial challenge.
Table 1. NTHi strains used in the study and their characteristics.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Geographical origin</th>
<th>Source</th>
<th>Disease</th>
<th>Tissue</th>
<th>Identity% c</th>
</tr>
</thead>
<tbody>
<tr>
<td>86-028NP</td>
<td>US</td>
<td>Child</td>
<td>Otitis media</td>
<td>NP a</td>
<td>99.3</td>
</tr>
<tr>
<td>1714</td>
<td>US</td>
<td>Child</td>
<td>Otitis media</td>
<td>MEF b</td>
<td>99.3</td>
</tr>
<tr>
<td>1128</td>
<td>US</td>
<td>Child</td>
<td>Otitis media</td>
<td>MEF</td>
<td>99.3</td>
</tr>
<tr>
<td>3224A</td>
<td>US</td>
<td>Child</td>
<td>Otitis media</td>
<td>MEF</td>
<td>99.3</td>
</tr>
<tr>
<td>3219C</td>
<td>US</td>
<td>Child</td>
<td>Otitis media</td>
<td>MEF</td>
<td>98.6</td>
</tr>
</tbody>
</table>

a NP, nasopharynx  
b MEF, middle ear fluid  
c Versus the vaccine antigen sequence (using the Super Needle software)