Safety, Immunogenicity, and Antibody Persistence following an Investigational Streptococcus pneumoniae and Haemophilus influenzae Triple-Protein Vaccine in a Phase 1 Randomized Controlled Study in Healthy Adults

Johan Berglund, Peter Vink, Fernanda Tavares Da Silva, Pascal Lestrate and Dominique Boutriau

Safety, Immunogenicity, and Antibody Persistence following an Investigational *Streptococcus pneumoniae* and *Haemophilus influenzae* Triple-Protein Vaccine in a Phase 1 Randomized Controlled Study in Healthy Adults

Johan Berglund, a Peter Vink, b Fernanda Tavares Da Silva, c Pascal Lestrate, c Dominique Boutriaux c

Blekinge Institute of Technology, School of Health Science, Karlskrona, Sweden; GlaxoSmithKline Vaccines, King of Prussia, Pennsylvania, USA; GlaxoSmithKline Vaccines, Wavre, Belgium

We investigated a protein-based nontypeable *Haemophilus influenzae* (NTHi) and pneumococcal (HiP) vaccine containing pneumococcal histidine triad D (PhtD), detoxified pneumolysin (dPly), and NTHi protein D (PD) in adults. In a phase I study, 40 healthy 18- to 40-year-old subjects were randomized (2:2:1) to receive two HiP doses administered 60 days apart, with or without AS03 adjuvant (HiP-AS and HiP groups, respectively), or Engerix B (GlaxoSmithKline, Belgium) as a control. Safety, antibodies, and antigen-specific CD4+ T-cell immune responses were assessed before and until 480 days after vaccination. No serious adverse events were reported, and no subject withdrew due to an adverse event. Local and systemic symptoms were reported more frequently in the HiP-AS group than in the other two groups. The frequency and intensity of local and systemic symptoms appeared to increase after the second dose of HiP-AS or HiP but not Engerix B. Antibody geometric mean concentrations (GMCs) for PhtD, dPly, and PD increased after each dose of HiP-AS or HiP, with higher GMCs being observed in the HiP-AS group (statistically significant for anti-PD after dose 1 and anti-Ply after dose 2). GMCs remained higher at day 420 than prior to vaccination in both the HiP-AS and HiP groups. Antigen-specific CD4+ T cells increased after each dose but were unmeasurable by day 480. Two doses of an investigational PhtD-dPly-PD protein vaccine induced humoral immunity and antigen-specific CD4+ T-cell responses after each dose, with generally higher responses when the vaccine was administered with AS03. HiP combined with AS03 appeared to be more reactogenic than the antigens alone. (This study has been registered at ClinicalTrials.gov under registration no. NCT00814489.)

*Streptococcus pneumoniae* and *Haemophilus influenzae* are important pathogens in human disease. *S. pneumoniae* is the most commonly identified cause of community-acquired pneumonia in adults (1), whereas nontypeable *H. influenzae* (NTHi) is an opportunistic pathogen that frequently causes respiratory infections in individuals with underlying respiratory diseases such as chronic obstructive pulmonary disease (COPD) (2). Currently available pneumococcal vaccines for adults suffer from a number of shortcomings. The 23-valent pneumococcal polysaccharide vaccine licensed for use in adults, although providing greater serotype coverage for the adult population (3), shows limited efficacy in preventing pneumonia in elderly individuals and those with COPD (4, 5). Furthermore, repeated vaccination with pneumococcal polysaccharide has been linked to the development of immune hyporesponsiveness to some serotypes (6). Pneumococcal conjugate vaccines contain polysaccharides from a limited number of pneumococcal serotypes that are specific to childhood pneumococcal disease, rather than adult disease. For example, serotypes contained in the licensed 13-valent pneumococcal conjugate vaccine account for only around one-third of invasive pneumococcal disease cases in adults (3). Serotype replacement following pneumococcal conjugate vaccination and capsular switching drive changes in the prevalence of serotypes of pneumococci over time (7–9), further decreasing the coverage by currently licensed pneumococcal vaccines. Development of the next generation of pneumococcal vaccines is therefore directed toward identifying antigens common to all serotypes, in order to expand the efficacy of the vaccines to all pneumococcal strains. To date, there is no available vaccine targeting *H. influenzae* (including NTHi) infections in adults.

GlaxoSmithKline Vaccines has developed a candidate vaccine that combines pneumococcal and NTHi proteins as pneumococcal histidine triad D (PhtD), detoxified pneumolysin (or pneumolysoid) (dPly), and NTHi protein D (PD) (PhtD-dPly-PD), hereafter referred to as the *H. influenzae* and pneumococcal (HiP) vaccine. PhtD is a surface-exposed protein that is highly conserved among pneumococcal serotypes but whose biological function remains incompletely described (10). PhtD may be implicated in adhesion of pneumococci to the mucosal surface, may be involved in bacterial zinc metabolism, and may play a role in complement inhibition through an undefined mechanism (11–13). Antibodies to PhtD prevent nasopharyngeal colonization of mice by pneumococci and protect mice against lethal systemic pneumococcal...
Pneumolysin (Ply) is a cytoplasmic virulence factor that is present in all pneumococcal serotypes and is released after spontaneous autolysis (18). Ply has multiple biological properties, including induction of cholesterol-dependent lysis of host cells, complement activation, inhibition of ciliary action, and promotion of epithelial disruption (19). Mice immunized with genetically detoxified Ply (dPly) survived invasive pneumococcal challenge, with an additional survival advantage being demonstrated when dPly was delivered with adjuvant (20, 21). A combined adjuvanted PhdT-dPly vaccine protected rhesus macaques against lethal pneumococcal pneumonia (22).

PD is a lipoprotein that is highly conserved among encapsulated and nonencapsulated strains of *H. influenzae*. PD is used as the protein carrier in the licensed 10-valent pneumococcal conjugate vaccine SynFlorix (PHID-CV; GlaxoSmithKline, Belgium). Administration of PD to children induced robust antibody responses and, in one study (the Pneumococcal Otitis Efficacy Trial (POET)), efficacy against episodes of acute otitis media caused by nontypable *H. influenzae* was also shown (23); however, this was not consistently observed in subsequent studies (24).

In this phase I study, the candidate combined HiP vaccine, when administered with or without adjuvant system AS03 (composed of α-tocopherol and squalene in an oil-in-water emulsion) to healthy adults, was evaluated. AS03 has been shown to enhance the vaccine antigen-specific adaptive responses of several candidate vaccines and is included in licensed H1N1 and H5N1 pandemic influenza vaccines (25). The potential immune-enhancing effects of AS03 were assessed in view of the target population of elderly individuals, in whom immune responses to vaccination may be attenuated. Here we report on safety, reactogenicity, and immunogenicity results following two doses of HiP vaccine administered with or without the AS03 adjuvant, with assessment of antibodies and cell-mediated immune responses until 1 year post-vaccination.

**MATERIALS AND METHODS**

**Study design and study subjects.** This phase 1 study (registered at ClinicalTrials.gov under registration no. NCT00814489) was conducted at the Blekinge Kompetenscentrum (Karlskrona, Sweden) between 8 January 2009 and 10 June 2010. The study protocol and associated documents were reviewed and approved by the Medical Product Agency in Sweden and the regional ethics review board in Lund (applications LU 545/2008 and 269/2010). The study was conducted in accordance with good clinical practice guidelines, applicable regulatory requirements, and the Declaration of Helsinki. Written informed consent was obtained from each subject prior to the performance of any study-specific procedures.

Participants were healthy adults between 18 and 40 years of age. Subjects were not eligible to participate if they had suffered from pneumonia or invasive pneumococcal disease within 3 years prior to the study; if they had immunosuppression from any cause, including administration of immunosuppressants for more than 14 days prior to vaccination; or if they had previously received vaccination against hepatitis B. Subject could not participate if they had received influenza vaccine within 14 days or any other vaccine within 30 days of the first dose of study vaccines. Other exclusion criteria included receipt of immunoglobulins or blood products within 3 months prior to the study, any serious or uncontrolled disease, chronic infection, past or current malignancy, laboratory evidence of clinically significant hematological or biochemical abnormalities, and a history of chronic alcohol consumption and/or drug abuse. Pregnancy was excluded for female participants prior to each vaccination, and female subjects were required to avoid pregnancy for 30 days prior to vaccination and for 2 months after completion of the vaccination series.

Participants were randomized (2:2:1) to one of 3 study groups, as follows: the HiP-AS group received the candidate HiP vaccine administered with the AS03 adjuvant, the HiP group received the candidate HiP vaccine without adjuvant, and the control group received a licensed hepatitis B vaccine (Engerix B; GlaxoSmithKline Vaccines). Three vaccine doses were planned (days 0, 60, and 180). However, during the study a predefined safety holding rule was met. Thus, the GlaxoSmithKline Vaccines internal vaccine safety monitoring board halted administration of the third vaccine dose to the HiP-AS and HiP groups.

A randomization list was used to number the vaccines, with a blocking scheme to ensure balanced allocation between the groups. Randomization at the study site used a centralized randomization system on the Internet.

Because the volumes of the investigational and control vaccines were different, the study was observer blinded. That is, the observer blinding was maintained by dedicated unblinded site personnel, who performed vaccination activities only and were not involved in any other study activities. The investigators, subjects, and safety observers were unblinded at approximately day 180, when the third dose of Engerix B was administered. The laboratory staff remained blinded until the completion of laboratory testing.

**Vaccines.** PhdT and dPly were produced as described previously (26). In brief, the full mature sequence of the PhdT protein devoid of its signal peptide was expressed in the recombinant *Esherichia coli* AR58 strain and further purified. Production of detoxified Ply (dPly) was done by fermentation of *E. coli* AR58, followed by protein purification and detoxification by formaldehyde. Recombinant PD was expressed in *E. coli* AR58 following amplification of the gene from NTHi (strain 772, biotype 2) and cloning in the plasmid pMG-MDPPrD under the control of the heat-inducible ypl promoter. PD was extracted and purified using chromatography and ultrafiltration.

The HiP vaccine contained 60 μg each of PhdT, dPly, and PD presented as a freeze-dried pellet and reconstituted with either AS03 (HiP-AS group) or diluent (Tween-modified phosphate-buffered saline). The dose of AS03 was the same as that used in licensed influenza vaccines (27, 28). After reconstitution, both investigational vaccines had a final volume for injection of 0.5 ml. Engerix B contained 20 μg recombinant hepatitis B surface antigen adsorbed on 0.5 mg aluminum hydroxide, in a 1.0-ml injection volume. All of the vaccines were administered intramuscularly, into the nondominant deltoid muscle.

**Study objectives.** The primary objectives of the study were to describe the safety and reactogenicity profiles of the HiP and HiP-AS investigational vaccines. Safety endpoints included the occurrence of solicited adverse events within a 7-day period after each vaccination, the occurrence of unsolicited adverse events within a 30-day period after each vaccination, the occurrence of hematological or biochemical abnormalities within 7, 180, 300, and 420 days after vaccination, and the occurrence of serious adverse events during the entire study. The assessments of vaccine immunogenicity in terms of humoral and cell-mediated immune responses after each vaccination and the persistence of responses until 12 months postvaccination were secondary objectives.

**Safety monitoring.** The vaccination phase was staggered to allow for controlled evaluation of vaccine safety during the study. No more than 5 subjects were vaccinated per day. Before proceeding to the next vaccine dose, safety data collected within 7 days after each dose underwent unblinded review by an internal safety review committee composed of a biostatistician, a clinical physician, and a safety physician at GlaxoSmithKline Vaccines who were not involved in HiP vaccine development. Any potential safety signal was escalated to the vaccine safety monitoring...
boad at GlaxoSmithKline Vaccines. Holding rules to temporarily suspend vaccination were predefined and are provided in Table 1. Vaccination could be put on hold at any time in case of death, a life-threatening vaccine-related serious adverse event or ulceration at the injection site, or discontinuation from further vaccination by the investigator in response to any adverse event.

**Safety and reactogenicity assessments.** Diary cards were used to solicit the occurrence of local symptoms (pain, redness, and swelling) and general symptoms (fever, headache, fatigue, gastrointestinal symptoms, malaise, and myalgia) for 7 days after each dose (days 0 to 6). The occurrence of other (unsolicited) adverse events was collected with diary cards for 30 days after each dose. Serious adverse events were recorded throughout the study. The investigators assessed the relationship between investigational product and the occurrence of each adverse event. Solicited local adverse events were all considered to be related to the vaccine.

Hematological and biochemical parameters were measured to assess subject eligibility and then again prior to each vaccination (days 0 and 60), local adverse events were all considered to be related to the vaccine. Serology was done with all vaccinated subjects (the total vaccinated cohort). The analysis of immunogenicity and persistence was done with eligible subjects who complied with all study procedures and for whom immunogenicity results were available for at least one assay (the according-to-protocol [ATP] cohort). All unsolicited signs and symptoms were coded according to Medical Dictionary for Regulatory Activities (MedDRA)-preferred terms.

Seroconversion rates (with exact 95% confidence intervals [CIs]) and geometric mean concentrations (GMCS)/geometric mean titer (GMTs) were calculated for each vaccine antigen-specific antibody (GMC) and for Hem-Ply antibodies (GMT) at each time point. Calculation of GMCS/GMTs was performed by taking the antilog of the mean of the log10 concentration/titer transformations. Antibody titers/concentrations below the assay cutoff value were given an arbitrary value of one-half the assay cutoff value for the purpose of GMC/GMT calculations.

Anti-PtD, anti-Ply, and anti-PD antibody responses 30 days after doses 1 and 2 were compared between the investigational groups using a one-way analysis of covariance (ANCOVA) model with log-transformed concentrations. The ANCOVA model included the group as a fixed effect and the prevaccination log-transformed concentration as the regressor. Exploratory comparisons should be interpreted with caution, as they were not adjusted for the number of endpoints and statistically significant findings could have occurred by chance. Analyses were performed using SAS version 9.1 software and Proc StatXact-7.

**RESULTS**

**Study subjects.** Forty-six subjects underwent screening and 40 subjects were enrolled in the study, of whom 39 subjects were included in the ATP immunogenicity cohort. One subject (control group) received a protocol-forbidden vaccine (Twintrix hepatitis A and B vaccine; GlaxoSmithKline, Belgium), subsequently had no blood sampling after dose 2, and was withdrawn from the ATP immunogenicity cohort. Demographic characteristics of the study groups are shown in Table 2.

**Analysis of safety.** No serious adverse events were reported during the study, and no subject withdrew from the study due to an adverse event (Table 2).

**Local and general solicited adverse events.** The most frequent local adverse event in all groups after each dose was pain at the injection site, which was reported by 93.3% of HiP-AS recipients after dose 1 and by 100% after dose 2. Pain was reported by 64% of subjects after the first HiP dose and by 94.1% after dose 2. Pain was also the most reported grade 3 adverse event and was reported by 33.3% of subjects after the second HiP-AS dose and by 17.6% after the second HiP dose. Redness and swelling of >50 mm were also reported more frequently after the second HiP-AS dose than after the first dose (Fig. 1).

<table>
<thead>
<tr>
<th>Table 1: Study holding rules</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Event</strong></td>
</tr>
<tr>
<td>Grade 3 general solicited adverse event that persisted for &gt;48 h (rule a)</td>
</tr>
<tr>
<td>Grade 3 unsolicited adverse event (rule b)</td>
</tr>
<tr>
<td>Grade 3 laboratory abnormality within 7 days after vaccination (rule c)</td>
</tr>
<tr>
<td>Combination of rules a and b</td>
</tr>
<tr>
<td>Combination of rules a, b, and c</td>
</tr>
</tbody>
</table>

*Grading as defined by the FDA (see http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Vaccines/ucm074775.htm).
The most frequently reported general symptoms in all groups after each dose were myalgia, headache, and fatigue (up to 73.3% of subjects). In addition, fever was reported by 66.7% of subjects in the HiP-AS group after dose 2. Although one-third (HiP) to two-thirds (HiP-AS) of subjects reported fever, mostly after dose 2, no cases were grade 3 (>39.5°C). Other grade 3 solicited general symptoms were reported by up to 20% of subjects (myalgia after dose 1 and fatigue after dose 2) in the HiP-AS group, by up to 11.5% (myalgia after dose 2) in the HiP group, and by up to 14.3% (headache after dose 2) in the control group.

Both local and general symptoms appeared to increase in frequency and intensity after the second dose of HiP-AS or HiP in the treatment groups but not Engerix B in the control group. None of the solicited local or general adverse events required medical attention.

(ii) Other (unsolicited) symptoms recorded until 30 days after each dose. At least one unsolicited symptom of any grade was reported by 10 subjects (66.7%) in the HiP-AS group, 10 subjects (58.8%) in the HiP group, and 6 subjects (75.0%) in the control group during the 30-day follow-up period after each vaccine dose (Table 3). The most commonly reported event in the HiP-AS group was chills (4/15 [26.7%] subjects), and those in the HiP group were headache, nasopharyngitis, and oropharyngeal pain (each reported by 3/17 [17.6%] subjects). Investigators were questioned regarding the definition of chills; the term “chills” was used to describe a range of symptoms, from the sensation of being cold, the sensation of being unable to warm oneself, and the disagreeable sensation of being cold up to actual shivering or quaking with or as if cold.

The percentages of subjects who reported at least one grade 3 (unsolicited) symptom in the 30-day period after each dose were 46.7% (7/15 subjects) in the HiP-AS group, 17.6% (3/17 subjects) in the HiP group, and 12.5% (1/8 subjects) in the control group (Table 3). No clustering of events was noted. No individual preferred term was attributed more than twice in any study group.

Eleven subjects reported at least one grade 3 unsolicited event after vaccination. Of these, 6 subjects (5 in the HiP-AS group and one in the HiP group) had events within 48 h after vaccination that were considered by the investigator to be causally related to vaccination. After dose 1 in the HiP-AS group, one subject reported grade 3 chills (see the definition of chills in Table 3), one subject reported a grade 3 injection site reaction, and one subject reported grade 3 oropharyngeal pain and a bursting earache. After dose 2 in the HiP-AS group, one subject reported grade 3 chills and one subject reported grade 3 vertigo lasting 3 days postvaccination. In the HiP group, one subject reported grade 3 muscularkeletal pain for 2 days after dose 2. All subjects recovered without sequelae.

At the protocol-scheduled internal safety review committee meeting after dose 2, holding rule 1bb (at least 5 of 16 subjects or >30% had a grade 3 unsolicited event within 7 days after either vaccination) was met for the HiP-AS group, in which 6 of the 15 subjects reported grade 3 unsolicited events. Four of these adverse events occurred after the first dose and two occurred after the second dose. Together, these included chills in 2 subjects and vomiting, vertigo, ear/oropharyngeal pain, and injection site reaction in one subject each. During the study, four HiP-AS subjects reported 5 events of chills (including two grade 3 events, two grade 2 events, and one grade 1 event), with all cases occurring on the day after vaccination and 4 cases occurring after dose 2. One subject reported chills after both dose 1 (grade 3) and dose 2 (grade 2). The duration of chills for all subjects was 1 to 2 days. All events of chills were assessed as being related to the vaccine by the investigator, and all of the events resolved. There were no reports of chills in the other groups. In view of the generally high overall level of reactogenicity and the proportion of subjects experiencing chills in temporal association with vaccination observed within one treatment arm, the GlaxoSmithKline vaccine safety monitoring board decided not to administer the planned third dose of both investigational vaccines.

(iii) Safety laboratory assessments. No grade 3 hematological
or biochemical laboratory abnormalities were reported 7 days or 30 days after each vaccination dose. A single grade 2 laboratory abnormality, which was not considered to be vaccine related, was observed during the study, i.e., a female subject in the HiP-AS group had a hemoglobin level of 107 g/liter (grade 2 interval range, 95 to 109 g/liter) 60 days after dose 1.

**Humoral immunogenicity.** Prior to vaccination, all subjects had anti-PhtD and anti-Ply antibodies above the assay cutoff value, indicating prior pneumococcal exposure or infection. For *H. influenzae*, anti-PD antibodies above the assay cutoff value, as evidence of prior exposure or infection, were present in 66.7% of subjects in the HiP-AS group, 35.3% of subjects in the HiP group, and all subjects in the control group (see Table S1 in the supplemental material).

Antibody GMCs for each protein increased markedly after each dose of HiP-AS or HiP (see Table S1 in the supplemental material). For anti-PhtD, the antibody GMCs after dose 2 increased above prevaccination concentrations by 6.7-fold in the HiP-AS group and by 4.7-fold in the HiP group. For anti-Ply, the GMCs increased by 19.2-fold and 11.6-fold, respectively (Fig. 2). For anti-PD, the antibodies increased by 17-fold and 10-fold, respectively. Exploratory analyses indicated a statistically significant difference between the HiP-AS and HiP groups in terms of the anti-PD GMC 1 month after dose 1 and the anti-Ply GMC 1 month after dose 2.

A functional assessment of anti-Ply antibodies used an assay of the inhibition of red blood cell hemolysis. All subjects were above the assay cutoff value for Hem-Ply antibodies prior to and after each dose (see Table S1 in the supplemental material). However, the Hem-Ply antibody GMTs were higher at the postvaccination time points than prior to vaccination in groups that received the PhtD, Ply, and PD proteins and tended to be higher in the HiP-AS group than in the HiP group.

At day 420, the observed anti-PhtD, anti-Ply, and anti-PD GMCs remained higher in the HiP-AS group than in the control group. For the HiP group, the observed antibody GMCs remained...
higher than in the control group for anti-Ply and anti-PhtD but not anti-PD antibodies.

**CD4⁺ T-cell responses.** PhtD-specific CD4⁺ T-cell expression of IL-2 and IFN-γ (Th1 profile) was observed at day 14 in the HiP-AS group and at 14 days after dose 2 (day 74) in both the HiP-AS and HiP groups (Fig. 3). IL-2 and IFN-γ expression increased between dose 1 and dose 2 and was observed to be higher in the HiP-AS group than in the HiP group.

Ply-specific CD4⁺ T-cell expression of IL-2 and of IFN-γ (Th1 profile) was observed at day 74 in the HiP-AS group (Fig. 3). Ply-specific CD4⁺ T-cell expression of IL-17 (Th17 profile) was observed at day 14 and day 74 in both the HiP-AS and HiP groups. Compared to baseline, 8- to 13-fold increases in median PD-specific CD4⁺ T-cell expression of IL-2 and IFN-γ (Th1 profile) were observed at day 74 in the HiP-AS group, whereas there was no increase in T-cell expression of IL-17 (Th17 profile) from baseline at either day 14 or day 74 in any treatment group (Fig. 3). Intracellular staining showed coexpression of IFN-γ, IL-2, and/or TNF-α by CD4⁺ T cells, although at low frequencies (<0.15%) (see Fig. S1 in the supplemental material).

At each time point, no more than 60/million (median) antigen-specific CD4⁺ T cells showed expression of IL-13 and/or IL-5 (Th2 profile) in the HiP-AS and HiP groups. No more than 113/million (median) antigen-specific CD8⁺ T cells showed expression of any cytokine at any postvaccination time point in the HiP-AS and HiP groups. No antigen-specific CD4⁺/CD8⁺ T-cell expression of any cytokine analyzed was detected 14 months after dose 2 (day 480) in any of the groups (Fig. 3).

### DISCUSSION

We investigated the safety and immunogenicity of a novel 3-component protein vaccine targeting *S. pneumoniae* and *H. influenzae* infections in a population of healthy young adults. Prior to this study, clinical studies of humans evaluated free dPly and PhdT and combinations of free dPly and free PhdT with either 10 μg or 30 μg of antigen administered as free antigen or adjuvanted with aluminum or with the AS02v adjuvant (ClinicalTrials.gov registration no. NCT00707798, 18- to 40-year-old subjects [unpublished results]; registration no. NCT01767402, 18- to 45-year-old subjects; registration no. NCT00756067 and NCT00307528, 65- to 85-year-old subjects [30]). Together, these studies enrolled more than 600 subjects, and their results showed clinically acceptable safety profiles of investigational dPly and/or PhdT vaccine formulations in adults. Robust humoral immune responses to vaccine antigens were observed in all groups in all four studies; however, the humoral immune responses were observed to be dose dependent and higher in the AS02v-adjuvanted group. In terms of reactogenicity, no consistent correlation with antigen dose was observed; however, AS02v-adjuvanted formulations showed increased local reactogenicity versus other formulations. The occurrence of fever and chills was uncommon in all four studies (only 4 cases of chills/feeling cold), and there was no evidence that they occurred more frequently with consecutive doses. The results of our study were generally in line with these studies, with the exception of the unexpected number of subjects who reported chills. We observed no serious adverse events during the present study, no subject withdrew due to an adverse event, and no clinically

### TABLE 3 Numbers of subjects with unsolicited symptoms classified by MedDRA-preferred terms during the 30-day (days 0 to 29) postvaccination period (total vaccinated cohort)

<table>
<thead>
<tr>
<th>Eventa</th>
<th>HiP-AS group (n = 15)</th>
<th>HiP group (n = 17)</th>
<th>Control group (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Any adverse event</td>
<td>10 66.7 (38.4–88.2)</td>
<td>10 58.8 (32.9–81.6)</td>
<td>6 75.0 (34.9–96.8)</td>
</tr>
<tr>
<td>Any related adverse event</td>
<td>8 53.3 (26.6–78.7)</td>
<td>6 35.3 (14.2–61.7)</td>
<td>1 12.5 (0.3–52.7)</td>
</tr>
<tr>
<td>Any grade 3 adverse event</td>
<td>7 46.7 (21.3–73.4)</td>
<td>3 17.6 (3.8–43.4)</td>
<td>1 12.5 (0.3–52.7)</td>
</tr>
<tr>
<td>Any related grade 3 eventb</td>
<td>5 33.3 (11.8–61.6)</td>
<td>1 5.9 (0.1–28.7)</td>
<td>0 0.0 (0.0–36.9)</td>
</tr>
</tbody>
</table>

a Any indicates at least one symptom experienced (regardless of the MedDRA-preferred term). Numbers and percentages indicate subjects reporting the symptom at least once.

b CI, confidence interval.

c Each subject might have reported more than one preferred term.

d Chills/shivering indicated a range of symptoms, from the sensation of being cold, the sensation of being unable to warm oneself, or the disagreeable sensation of being cold up to actual shivering or quaking with or as if cold.

---

**Figure 3.** Intracellular cytokine analysis. Intracellular IFN-γ, IL-2, and TNF-α expression was assessed in mononuclear cells isolated 14 days after the initial dose of the vaccine that included Ply, PhtD, or both. **A** and **B**, Flow cytometry analysis of CD4⁺ T cells. **A**, gated on CD4⁺ T cells. **B**, gated on CD8⁺ T cells. **C** and **D**, flow cytometry analysis of CD8⁺ T cells. **C**, gated on CD4⁺ T cells. **D**, gated on CD8⁺ T cells. **E** and **F**, intracellular expression of IL-17 and IL-13, respectively. **E**, gated on CD4⁺ T cells. **F**, gated on CD8⁺ T cells. Each subject might have reported more than one preferred term.
relevant hematological or biochemical events were detected during the study period.

The HiP-AS vaccine was associated with higher incidences and intensities of solicited symptoms than the HiP vaccine and the control, but it had an overall reactogenicity profile that appeared to be generally similar to those of other vaccines commonly administered to adults. For example, of healthy adults given combined adult diphtheria-tetanus vaccine, 93% reported pain at the injection site, 19% reported pain of grade 3 intensity, and 70% reported systemic symptoms, including headache (44%) and fever (33%), after vaccination (31). Of adults 18 to 60 years of age given AS03-adjuvanted influenza vaccine, 96.2% reported pain, 75.2% reported fatigue, and 74.0% reported myalgia within 7 days after vaccination (32). The AS03 adjuvant system is specifically designed to increase innate immune responses by enhancing migration of monocytes and macrophages and by increasing localized production of a range of cytokines and chemokines intramuscularly at the site of injection and in the draining lymph nodes. Thus, the higher local reactogenicity observed in the HiP-AS group than in the HiP group may be secondary to these enhanced innate immune responses elicited at the site of injection and therefore is not unexpected (32,33). This was consistently observed in studies evaluating AS03-adjuvanted influenza vaccines, in which higher local reactogenicity was observed with adjuvanted versus nonadjuvanted vaccines (34,35).

In this study, the high rate of occurrence of chills observed within one treatment arm was an unexpected finding, but results may have been confounded by the broad range of symptoms encompassed by this term. Chills were a common and transient event (all lasted ≤2 days) that was not associated with recorded fever and occurred in temporal association with the HiP-AS vaccine only. Four of the 5 episodes of chills were grade 2 or 3. One of the subjects reported chills after both doses, although the chills were of lesser intensity after the second dose. A possible causal association between the symptom of chills and HiP-AS vaccine administration cannot be ruled out. In studies of AS03-adjuvanted influenza vaccines, the occurrence of “shivering” after vaccination was actively solicited. Shivering was reported as being a “very common” event after vaccination with H1N1-AS03 (Pandemrix; GlaxoSmithKline, Belgium), being reported by up to 20% of vaccinees in all age groups (36).

Local inflammatory effects are expected, as the role of the adjuvant is to increase the immunogenicity of the vaccine. A study of several adjuvant systems, including AS03, administered with hepatitis B vaccine to adults showed early, short-lived increases in systemic levels of IL-6 and IL-10 after vaccination (37). However, no firm evidence clearly correlates increased local cytokine release with increased numbers of systemic events. Nonetheless, we hypothesize that a slight increase in systemic reactogenicity could occur as a consequence of locally produced cellular mediators or signal molecules spilling into the systemic circulation. Such events could possibly be linked to the transient local inflammatory response resulting from adjuvanted vaccines (28), but further work is needed to confirm this supposition. Given the small numbers of subjects per treatment group (15 subjects in the HiP-AS group and 16 subjects in the HiP group), firm conclusions or interpretations cannot be made on the basis of this study alone. Considering the overall increased reactogenicity of the vaccine after administration of the second vaccine dose, the GlaxoSmithKline vaccine safety monitoring board elected to halt the administration of planned third doses of the treatment vaccines in this study.

In terms of immunogenicity, the majority of adult subjects had preexisting antibodies to all three vaccine proteins prior to vaccination, suggesting past exposure or infection. HiP proteins induced marked increases in anti-PD, anti-PhtD, and anti-Ply anti-
body GMCs after each dose, with a trend toward higher GMCs for all three proteins in the HiP-AS group than in the HiP group.

It is not known whether antibodies to PhtD or Ply are protective in humans. While anti-PD antibodies have been linked to clinical protection against otitis media in children (23), their ability to prevent respiratory infections in the elderly is not known. Thus, the significance of post-HiP vaccination antibody levels in terms of clinical protection and the relative advantage, in terms of immunogenicity, of administering HiP proteins with AS03 are as yet undefined. Recent work has highlighted the importance of anti-PhtD antibodies in preventing the adherence of pneumococci to epithelial cells (38).

We used red blood cell lysis by Ply as a convenient surrogate measure of the ability of Ply to lyse respiratory epithelium. Reduced hemolysis was observed after vaccination, suggesting that Hem-Ply antibody activity is indicative of functionality.

PhtD- and dPly-specific CD4\(^+\) T cells were detected after the first vaccination, with a further increase after the second dose, in HiP protein recipients, in contrast to the control group, in which no observable increase was detected. The highest responses were observed in HiP-AS recipients, consistent with the reported effects of AS03 on CD4\(^+\) cell responses (25). Th17 has been implicated in vaccine-induced immunity against a range of bacterial pathogens, including pneumococcal infections at mucosal sites (39). A favorable CD4\(^+\) T-cell profile was induced, with increases in measured Th1 and Th17 cells and without increases in CD4\(^+\) Th2 cells. As expected, the CD4\(^+\) Th1 and Th17 responses tended to be more marked in the adjuvanted HiP-AS group than in the HiP group. Our data are similar to those reported by Sharma et al. (40), who showed that adults administered streptococcal and NTHi proteins, including PhtD and Ply, responded with expression of mainly Th1 cytokines. In contrast, no expression of IL-17 was observed, whereas Th2 cytokines (IL-4, IL-10, and IL-13) were expressed at lower levels than the Th1 cytokines.

All study subjects had serological evidence of being exposed to, colonized with, or infected with H. influenzae and pneumococci earlier in life, yet antigen-specific cell-mediated immunity was absent or very low prior to vaccination. This may indicate that, rather than circulating in the bloodstream, inactive memory T cells were sequestered in lymphoid tissue prior to stimulation by vaccination. Indeed, evidence for the presence of specific T-cell homing receptors may mean that T cells boosted by vaccination may have exited the circulation prior to blood sampling (41). Some T-cell responses, including Th17 responses, are known to be short lived (42). Finally, others showed CD4\(^+\) T-cell responses using assays that employed longer stimulation periods (12 days for Staphylococcus aureus) than in our study and that measured different cytokines (such as IL-10 and IL-22) (43). Thus, our assay may not have been optimal for detection of specific T cells occurring at low frequencies in the circulation. Similarly, we observed that cell-mediated immune responses after vaccination did not appear to be long-lasting, with little or no detectable antigen-spe-

**FIG 3** Antigen-specific CD4\(^+\) T cells expressing Th1 (IL-2 and IFN-γ) and Th17 (IL-17) cytokines over time (frequency per million cells). Baseline, prior to vaccination; day 14, 14 days after dose 1; day 74, 14 days after dose 2; day 480, 480 days after the first vaccination. Vertical lines indicate the interquartile range.
pecific CD4+ T-cell responses being seen at day 480. The immune mechanisms and clinical implications of the kinetics of persistence of antigen-specific CD4+ T-cell responses to both natural infection and vaccination are not known and warrant further investigation. It is also important to note that our results cannot be extrapolated to children, who may be unprimed or partially primed for the vaccine antigens.

This study confirms that two doses of a tricomponent PhtD-dPly-PD investigational vaccine are immunogenic in adults, inducing increases in antibody concentrations and antigen-specific Th1- and Th17-directed cell-mediated immune responses after each dose, with improvements in the antibody responses when the vaccine is administered with AS03 adjuvant. The study indicates that HiP combined with AS03 appears to be more reactogenic that the antigens administered without adjuvant.

ACKNOWLEDGMENTS

We thank the volunteers who participated in this study as well as the study nurses and other staff members. We also thank Sonia Mesia-Velázquez for study coordination, Silvia Damaso for performing the statistical analysis, and Thomas Moens for clinical report writing (all employed by GlaxoSmithKline Vaccines), Joanne Wolter (independent) for writing the initial draft of the manuscript, and Wouter Houthof (XPE Pharma & Science) for publication coordination on behalf of GlaxoSmithKline Vaccines.

J.B. declares no conflict of interest. P.V., F.T.D.S., P.L., and D.B. are employees of the GlaxoSmithKline group of companies, and all declare ownership of stock/stock options of the GlaxoSmithKline group of companies. D.B. also is an inventor of certain GlaxoSmithKline patents. Synflorix, Engerix, Pandemrix, and Twinrix are trademarks of the GlaxoSmithKline group of companies.

GlaxoSmithKline Biologicals SA was the funding source and was involved in all stages of study conduct and analysis. GlaxoSmithKline Biologicals SA also funded all costs associated with the development and publishing of this article.

All authors had full access to the data, and the corresponding author was responsible for submission of the manuscript.

REFERENCES


