Controlled Human Infection and Rechallenge with *Streptococcus pneumoniae* Reveals the Protective Efficacy of Carriage in Healthy Adults

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Rationale: **The immunological and protective role of pneumococcal carriage in healthy adults is not known, but high rates of disease and death in the elderly are associated with low carriage prevalence.**
Objectives: **We employed an experimental human pneumococcal carriage model to investigate the immunizing effect of a single carriage episode.**
Methods: **Seventy healthy adults were challenged, and of those with carriage, 10 were rechallenged intranasally with live 6B *Streptococcus pneumoniae* up to 11 months after clearance of the first carriage episode. Serum and nasal wash antibody responses were measured before and after each challenge.**
Measurements and Main Results: **A total of 29 subjects were experimentally colonized. No subjects were colonized by experimental rechallenge, demonstrating the protective effect of initial carriage against subsequent infection. Carriage increased both mucosal and serum IgG levels to pneumococcal proteins and polysaccharide, resulting in a fourfold increase in opsonophagocytic activity. Importantly, passive transfer of postcarriage sera from colonized subjects conferred 70% protection against lethal challenge by a heterologous strain in a murine model of invasive pneumococcal pneumonia. These levels were significantly higher than the protection conferred by either precarriage sera (30%) or saline (10%).**
Conclusions: **Experimental human carriage resulted in mucosal and systemic immunological responses that conferred protection against recolonization and invasive pneumococcal disease. These data suggest that mucosal pneumococcal vaccination strategies may be important for vulnerable patient groups, particularly the elderly, who do not sustain carriage.**

Keywords: *Streptococcus pneumoniae*; human challenge models; colonization; immunity

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The immunological and protective role of pneumococcal carriage in healthy adults is not known, but high rates of disease and death in the elderly are associated with low carriage prevalence.

What This Study Adds to the Field

We postulate that carriage is beneficial and works as a natural boosting mechanism to sustain protective immunity against disease in adults. The data suggest that mucosal pneumococcal vaccination strategies may be important for vulnerable patient groups, particularly the elderly, who do not sustain carriage. This study has important implications for current and future vaccination strategies. Currently, the direct and herd protection effects against carriage promoted by pneumococcal conjugate vaccination in children could reduce natural boosting of existing immunity in both children and adults.

Pneumococcal carriage is both the primary means of transmission and a necessary prerequisite of invasive pneumococcal disease (1, 2). In the young, high carriage rates and density are associated with frequent disease (3, 4). Conversely, reduced rates of both carriage and disease are seen in adult life, implying an immunizing effect of exposure (5, 6). Paradoxically, the elderly have low rates of carriage, but high rates of disease (7). As pneumococcal disease is the most common cause of infectious death worldwide, and carriage is critical in this process, we studied the immunizing effect of a single experimental carriage episode and its role in sustaining protective immunity in healthy adults.

The development of both antibody and antigen-specific T cell responses after pneumococcal colonization are protective against subsequent colonization and invasive disease in mice (8–12). Pneumococcal carriage in human infants and adults results in an increase in anticapsular (serotype specific) and antiprotein (non–serotype specific) serum antibody levels (13–15), which is associated with a reduced incidence of natural carriage, but direct rechallenge has not previously been attempted. The relative contribution of anticapsular and antiprotein responses in protection against carriage has been modeled in humans (16), but direct observation of Ig function has not previously been possible.

Our aim was to develop an experimental human pneumococcal carriage (EHPC) model and employ this model to investigate the immunizing effect of a single carriage episode. Our central

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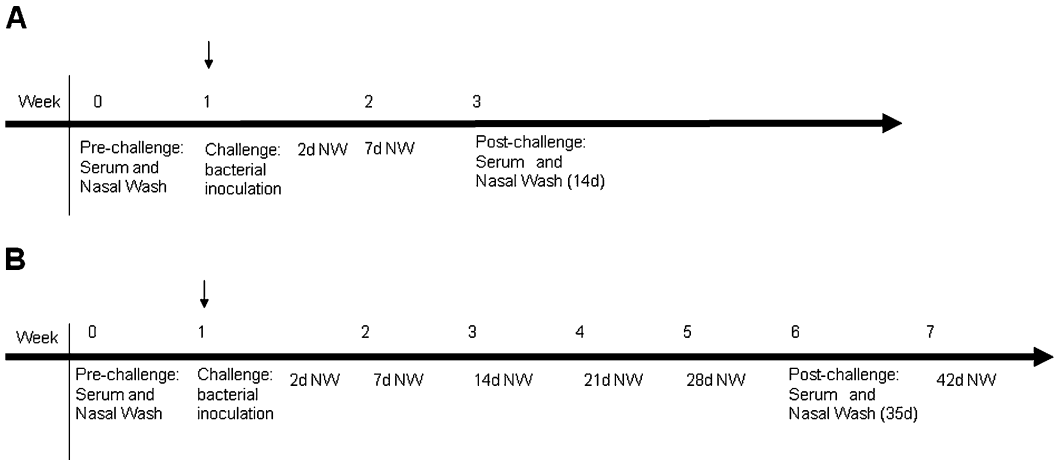


Figure 1. Experimental human pneumococcal carriage (EHPC) studies design. (*A*) In the dose-ranging study, carriage was monitored by nasal wash (NW) on Days 2, 7, and 14 after pneumococcal inoculation. (*B*) In the long-term carriage monitoring (ltcm) study, carriage was monitored by NW on Days 2, 7, 14, 21, 28, 35, and 42 after pneumococcal inoculation. Blood samples were obtained 1 week before challenge and 2 weeks (*A*) or 5 weeks (*B*) after challenge. Bacterial challenge is indicated by an *arrow*.

hypothesis was that carriage would boost the pre-exiting anti-pneumococcal antibody responses, and that these altered responses would be protective against reacquisition of carriage and disease. For this, we measured both antibody responses and function in serum before and after EHPC. We rechallenged carriers to determine the association of humoral responses with subsequent protection against reacquisition of carriage up to 1 year after the first experimental carriage episode. Using passive transfer experiments in a murine model of fatal invasive pneumonia, we distinguished between protection conferred by existing anti-protein antibodies in healthy adults and protective responses induced by a single carriage episode. Carriage induced increased opsonophagocytic activity of serum, cross-reactive antibodies against protein antigens, and, therefore, protection against invasive pneumonia by a nonhomologous capsular strain.

We postulate that carriage is the mechanism by which immunocompetent adults maintain elevated antibody function and, therefore, protection against pneumococcal disease. The failure of carriage in the elderly may be an important contributor to their relative immunodeficiency.

Some of the results of these studies have been reported previously in the form of an abstract (17).

METHODS

Detailed METHODS are presented in the online supplement.

Recruitment

We recruited healthy adults (18 and 60 yr) with no respiratory disease. Individuals naturally colonized with pneumococcus or in regular contact with at-risk individuals were excluded. Ethical approvals were obtained from the National Health Service Research Ethics Committee (08/H1001/52 and 11/NW/0592).

Study Design and Samples Collection

Samples of serum and nasal wash (NW) were obtained before and after bacterial inoculation, as described in detail in METHODS in the online supplement. Briefly, type 6B *Streptococcus pneumoniae* were instilled in 100 μl to each naris. Samples were obtained 1 week before and at 2, 7, and 14 days after pneumococcal inoculation for dose-ranging studies and 1 week before at 2, 7, 14, 21, 28, 35, and 42 days in a long-term carriage study (ltcm) (Figure 1). For rechallenge experiments, previously colonized volunteers were reinoculated with the 6B strain. NW samples were collected 1 week before and 2, 7, and 14 days after rechallenge.

Bacterial Stock Preparation and Inoculated Dose

Bacterial stock preparation was performed as previously described (18). On each day of inoculation, stored aliquots were thawed and diluted to reach the desired concentration. A total of 70 volunteers were inoculated, and no adverse event was reported. For dose-ranging studies, five cohorts of 10 subjects each (A–E) were inoculated with a dose of 10,000, 20,000, 40,000, 80,000, and 160,000 cfu/100 μl/naris, and a sixth cohort was inoculated with saline only (mock challenge group). For the ltcm study, 20 volunteers were inoculated with doses ranging between 20,000 and 40,000 cfu/100 μl/naris. For rechallenge experiments, 10 volunteers were inoculated with 40,000 cfu/100 μl/naris (19).

Nasal Washing and Determination of Carriage

NW samples were processed as previously described (20). Volunteers in whom 6B pneumococci were detected by classical microbiology from NW samples collected on any of the visits were defined as carriers.

Anti-Pneumococcal Capsular Polysaccharide and Protein IgG Determination

We measured anti–capsular polysaccharide (PS) IgG by the standard World Health Organization ELISA method (21). The Meso Scale Discovery (MSD) technology (21) was used to determine antibody levels for 27 pneumococcal proteins (Table E1) before and after carriage or

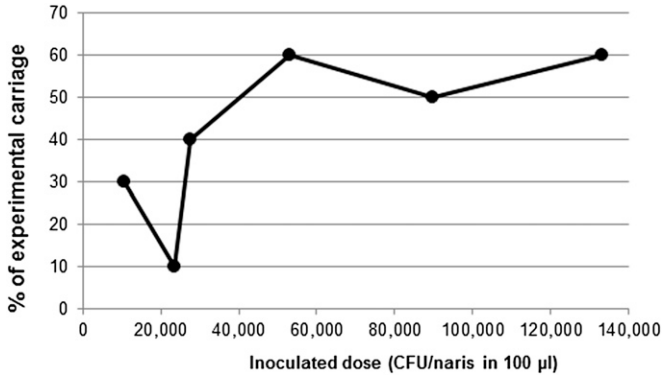


Figure 2. Dose–response curve of experimental carriage. Groups of 10 volunteers were inoculated with 100 μl of increasing doses of 6B *Streptococcus pneumoniae* (10,000, 20,000, 40,000, 80,000, and 160,000 cfu/naris). An extra cohort ($n = 20$) was inoculated with 40,000 cfu/naris. Inoculated doses were quantified by Miles and Misra dilution and plating. The average of inoculated doses and corresponding percentage of carriage achieved for each group are indicated.

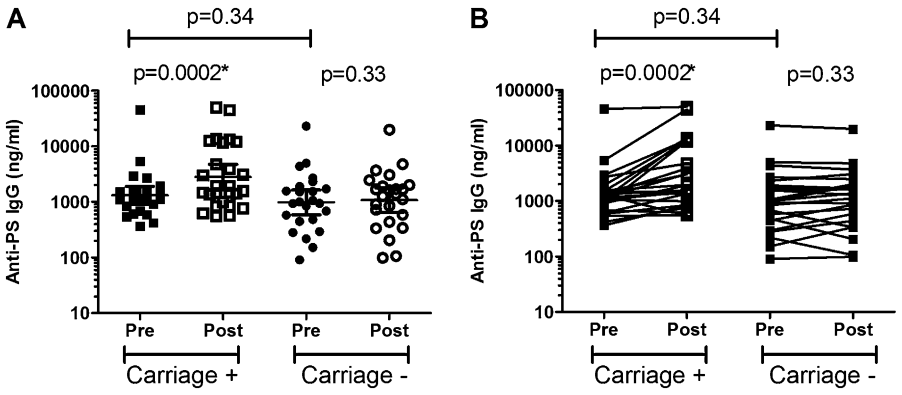


Figure 3. Serum anti–capsular polysaccharide (PS) IgG response to experimental human pneumococcal carriage. ELISAs were performed using 6B capsular PS as target to measure specific IgG levels (μg/ml) in serum from healthy human subjects in whom experimental pneumococcal carriage had been established (carriage$^+$, $n = 26$) and from the subjects who had been experimentally inoculated, but in whom carriage had not been established (carriage$^-$, $n = 24$). (*A*) Values are shown for each subject at pre- and postchallenge time points. Geometric mean and 95% confidence intervals are indicated by *bars*. (*B*) Pre- and postchallenge IgG levels to PS are linked for each subject. *Statistically significant difference between pre- and postchallenge antibody levels, using paired *t* test ($P \leq 0.05$).

challenge (no carriage after inoculation). We also used the N-terminal region of pneumococcal surface protein (Psp) A (clade1) and PspC (group 9) of the 6B pneumococcal strain used for inoculation in standard ELISA assays to determine IgG levels.

Opsonophagocytic Killing Assay Using HL60 Cells

A standard opsonophagocytic killing (OPK) assay using HL-60 cells was performed (22). Titer was determined by the last serum dilution in which the observed killing was 50% greater than the killing observed in the control wells containing bacteria, HL-60 cells, and complement.

Antibody Passive Transfer Experiments

Serum samples from six volunteers that were protected against reacquisition of carriage during rechallenge experiments were pooled for passive transfer experiments. Pooled sera were diluted 1:100 in saline and 100 μl was administered to mice by intraperitoneal injection 2 hours before challenge. Female MF1 mice (Charles River, Kent, UK) between 8 and 10 weeks of age were challenged with a lethal dose of D39 pneumococci in UK Home Office–approved experimental protocols (detail available in the online supplement). At 24 hours postinfection, tail vein blood was collected for assessment of bacteremia. At predetermined humane end points, mice were culled and

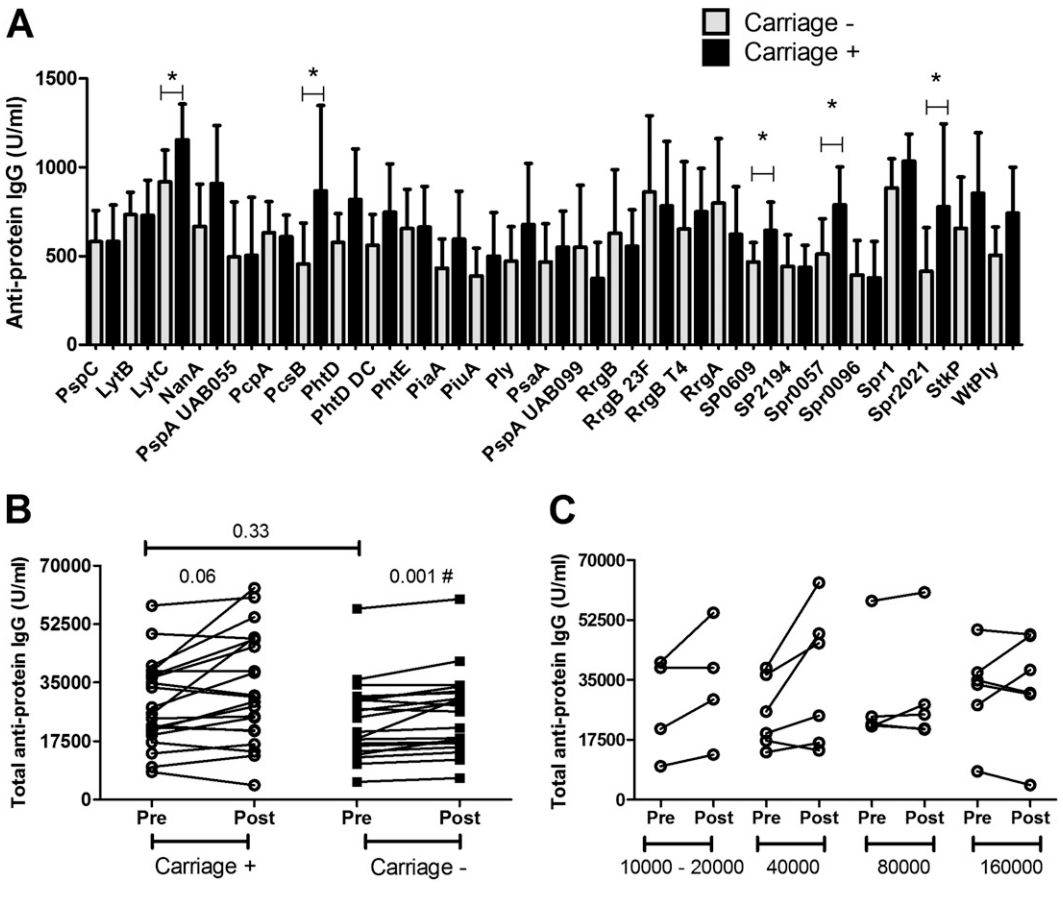


Figure 4. Serum IgG responses to 27 pneumococcal proteins. (*A*) IgG levels to 27 pneumococcal proteins (indicated on *x axis*) were measured by Meso Scale Discovery (MSD) in serum samples of 54 subjects obtained before pneumococcal challenge (baseline). IgG levels of subjects in whom experimental carriage had been established (carriage positive [carriage$^+$], *black bars*, $n = 27$) and of subjects who had been inoculated, but did not establish carriage (carriage negative [carriage$^-$], *gray bars*, $n = 27$). (*B*) Total anti–protein IgG values are the sum of IgG levels to the 27 pneumococcal proteins for each subject at pre- and postchallenge time points for carriage$^-$ and carriage$^+$ subjects. (*C*) Total anti–protein IgG levels in pre- and postchallenge serum samples of carriage$^+$ subjects divided by inoculated dose group (indicated on *x axis*). *Statistically significant difference between anti–protein IgG levels of carriage$^-$ and carriage$^+$ subjects, using unpaired *t* test ($P \leq 0.05$); $^{\#}$statistically significant difference between pre- and postchallenge IgG levels, using paired *t* test ($P \leq 0.05$).

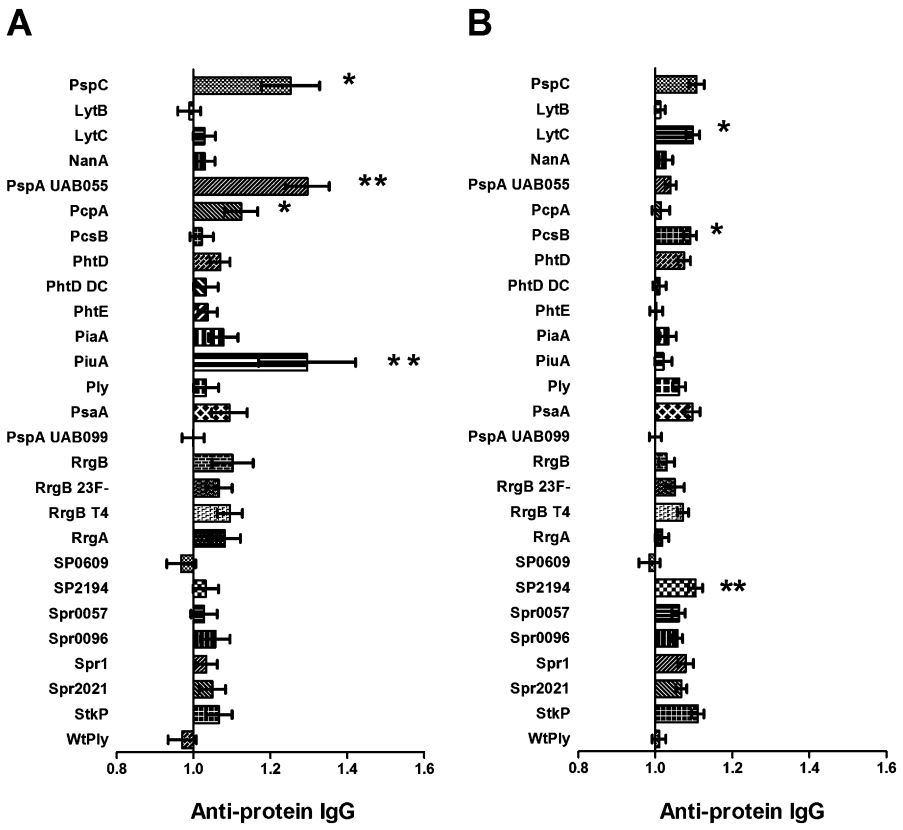


Figure 5. Anti–protein IgG response to experimental human pneumococcal carriage. Meso Scale Discovery was performed using 27 pneumococcal proteins as targets to measure specific IgG in pre- and postchallenge serum samples. Values are the antilog of differences of after levels minus before levels for each indicated antigen (mean ± SEM). Presamples were collected 1 week before, and postsamples were collected 2 weeks after pneumococcal inoculation. (*A*) Carriage-positive (carriage$^+$) subjects ($n = 21$) and (*B*) carriage-negative (carriage$^-$) subjects ($n = 20$) were determined by the presence of pneumococcus in nasal wash (NW) samples at 2, 7, or 14 days after inoculation. **Statistical significance ($P \leq 0.05$) and *borderline significance ($0.1 < P > 0.05$) comparing postchallenge levels of carriage$^+$ and carriage$^-$ subjects covariant adjusted by prechallenge levels, using multiple regression analysis.

blood and lung tissue taken for assessment of bacteremia and lung cfu levels.

Statistical Analysis

Log-transformed data were compared using appropriate parametric tests in GraphPad Prism v5 and Instat v3 (GraphPad Inc., San Diego, CA). Multiple regression analyses were used to compare differences between colonized and noncolonized postchallenge samples covariant adjusted for differences between prechallenge samples of both groups. Data from passive transfer experiments were analyzed by one-way ANOVA with Bonferroni's post test.

RESULTS

EHPC Studies

During prechallenge screening, we excluded 10% of the volunteers owing to natural carriage; 80 eligible volunteers were inoculated between November 2011 and February 2012 with minimal adverse events, including nonspecific nasal symptoms as previously described for this model (19). Details of all study cohorts, inoculated doses, and percentage of carriage achieved are given in Table1. The carriage rate achieved was dose dependent, ranging between 10 and 60%, as shown in Figure 2. Carriage density, recovered from NW at Days 2, 7, and 14 after challenge, was not different between cohorts inoculated with different doses (data not shown).

Responses Induced by Carriage Are Protective Against Reacquisition of Carriage

We reinoculated 10 volunteers with 35,473 (±5,164) cfu/naris of the same 6B strain used in the previous experimental carriage episode. Details of the volunteers, inoculated dose, and interval between first challenge and rechallenge are given in Table E2 (in the online supplement).

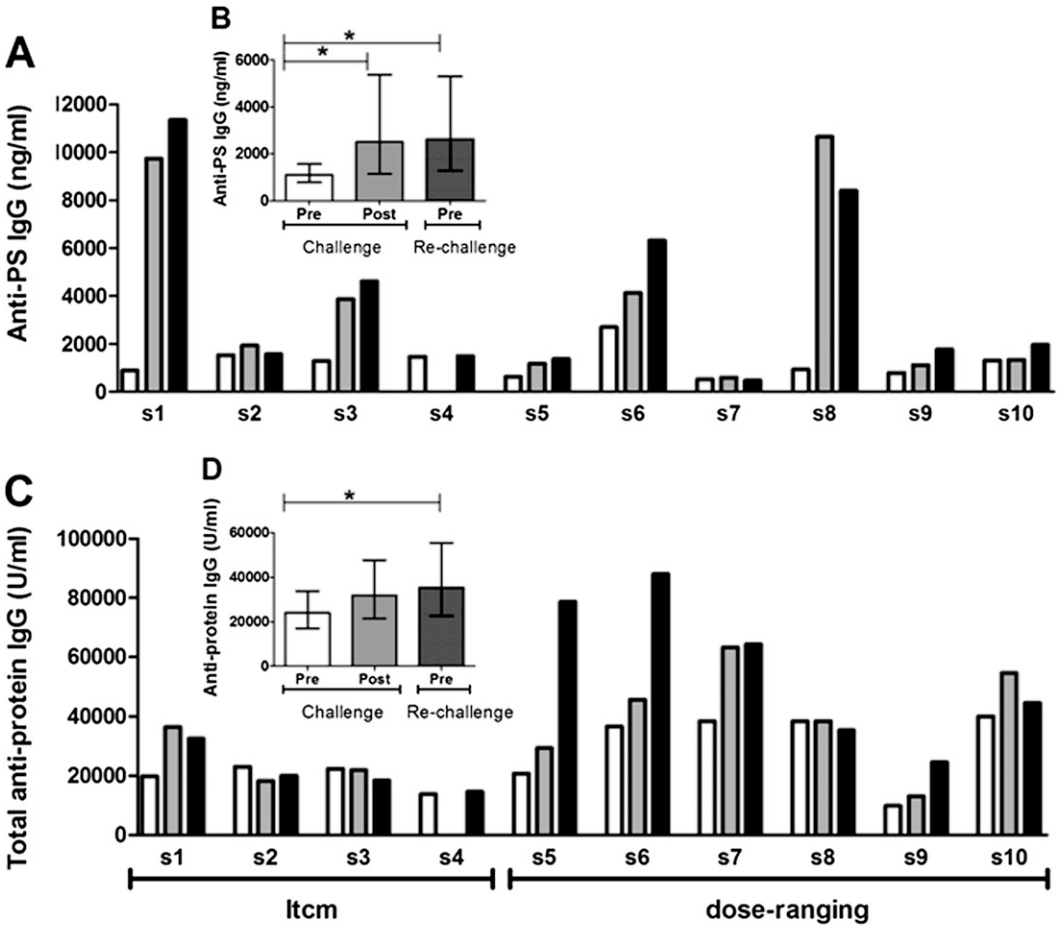
All volunteers examined were carriage negative by standard NW at Days 2, 7, and 14 after pneumococcal rechallenge. Our previous results showed 60% carriage rate at the inoculation dose chosen. In rechallenge experiments, there were no pneumococci detected by classical microbiology in any of the 30 samples (0% carriage rate). A previous carriage episode significantly protected against reacquisition of carriage by the same strain ($P = 0.01$, using Fisher's exact test).

Carriage Boosts IgG Levels to Specific Capsular PS in Serum

All prechallenge samples had measurable levels of IgG to PS 6B, and no difference at baseline was observed between volunteers who established carriage (carriage-positive individuals) and those who did not (carriage-negative individuals) ($P = 0.34$, unpaired t test; Figures 3A and 3B). Carriage-positive individuals showed a significant increase in serum anti-PS IgG levels after challenge compared with prechallenge levels (geometric mean [95% confidence interval (CI)]: before, 1,312 [898–1,917] versus after, 2,797 [1,637–4,780]; $P = 0.0002$, using paired t test). No significant increase was observed for carriage-negative individuals (before, 980 [586–1,639] versus after, 1,073 [644–1,787], $P = 0.33$). There was no difference in levels of IgG antibodies against PS 6B before and after mock challenge (data not shown).

No Association of Baseline IgG to Pneumococcal Proteins and Carriage

Full MSD data on 27 proteins before and after inoculation are presented in Tables E3 and E4. We rejected the hypothesis that baseline levels of IgG antibodies against individual pneumococcal protein would be lower in volunteers developing carriage (Figure 4A), and there was also no relation between carriage



E

Subjects	PS	Proteins	Neither
s1	++	+	
s2			=
s3	+		
s4			=
s5		++	
s6	+	++	
s7		+	
s8	++		
s9	+	+	
s10	+		

Figure 6. Serum anti–capsular polysaccharide (PS) and anti–protein IgG responses in subjects protected against reacquisition of carriage. A total of 10 subjects (*x axis*, s1–s10) were protected from reacquisition of carriage after rechallenge with the homologous pneumococcal strain. (*A*) IgG levels to 6B capsular PS for each of the 10 subjects at prechallenge (*white bars*), postchallenge (*gray bars*), and pre-rechallenge (*black bars*) time points. (*C*) Total anti–pneumococcal protein IgG levels for each subject were summed and expressed as total anti–protein IgG at each time point. For long-term carriage monitoring (ltcm; $n = 4$) and dose-ranging ($n = 6$) cohorts, the interval between pre- and postchallenge samples was 6 and 3 weeks, and between prechallenge and pre-rechallenge samples was 32–48 weeks and 16–19 weeks, respectively. Geometric mean and 95% confidence intervals (CIs) of all 10 subjects are given for IgG levels (*B*) to PS and (*D*) to proteins. Values from Subject 3 (s3) were excluded from analysis, and one-way ANOVA repeated measures test and Bonferroni's post test were employed. *Statistical significance compared with prechallenge samples ($P \leqslant 0.05$). (*E*) Increases in anti-PS and anti-protein responses by subject was tabulated (+ represents a modest increase, ++ represents more than onefold increase, and = represents no increase comparing levels at prechallenge and pre-rechallenge time points).

and the summed baseline antiprotein IgG levels ($P = 0.33$, unpaired t test; Figure 4B). Paradoxically, carriage-positive volunteers (expected to have lower levels) had significantly higher levels of IgG in prechallenge samples against the proteins LytC ($P = 0.05$, using unpaired t test), PcsB ($P = 0.03$), SP0609 ($P = 0.03$), Spr0057 ($P = 0.03$) and Spr2021 ($P = 0.05$) compared with carriage negative volunteers (Figure 4A).

A small increase in mean total protein IgG response postchallenge was observed for carriage-negative volunteers ($P = 0.001$) and for carriage-positive volunteers ($P = 0.06$) (Figure 4B). A small number of volunteers ($n = 7$) showed a drop in serum antiprotein IgG after carriage. These were stratified by the inoculated dose and showed that one was inoculated within the 40,000 cfu/naris group, two were inoculated in the 80,000 cfu/naris group, and four were inoculated within the highest dose group (160,000 cfu/naris) (Figure 4C).

Changes in Serum IgG to Pneumococcal Proteins after Intranasal Exposure

We compared serum samples collected before and 2 weeks after pneumococcal challenge from 20 carriage-negative and 21 carriage-positive volunteers. Geometric means and 95% CIs are shown in Table E3. Carriage induced a significant increase in the level of serum IgG to six antigens at 2 weeks after inoculation: PspC ($P = 0.0025$, using paired t test); PspAUAB0055 ($P < 0.0001$); PcpA ($P = 0.009$); PhtD ($P = 0.02$); PiuA ($P = 0.0046$); and RrgB T4 ($P = 0.01$). In the absence of carriage, pneumococcal challenge elicited increased levels of IgG antibodies against 14 antigens: PspC ($P < 0.0001$, using paired t test); LytC ($P < 0.0001$); PspAUAB0055 ($P = 0.01$); PcsB ($P < 0.0001$); PhtD ($P < 0.0001$); Ply ($P = 0.0014$); PsaA ($P < 0.0001$); RrgB T4 ($P < 0.0001$); SP2194 ($P < 0.0001$); SP0057 ($P = 0.0017$); SP0096 ($P = 0.0002$); Spr1 ($P = 0.0007$); Spr2021 ($P < 0.0001$); and Stkp ($P < 0.0001$).

TABLE 1. PARTICIPANTS OF DOSE-RANGING AND LONG-TERM CARRIAGE MONITORING STUDIES

Study	No. of Subjects	Age (*yr*)	Sex (*M:F*)	Study Period	Dose (*cfu/Naris*)	Experimental Carriage Rate
Cohort A	10	26.2 ± 5.8	5:5	Nov–Dec 2011	10,650 ± 704	30% (3/10)
Cohort B	10	22.6 ± 3.2	6:4	Nov–Dec 2011	23,433 ± 3,637	10% (1/10)
Cohort C	10	21.8 ± 1.4	5:5	Nov–Dec 2011	53,042 ± 3,266	60% (6/10)
Cohort D	10	25.4 ± 11.8	2:8	Jan–Feb 2012	89,833 ± 11,805	50% (5/10)
Cohort E	10	20.1 ± 1.85	4:6	Jan–Feb 2012	133,266 ± 3,492	60% (6/10)
ltcm (cohort F)	20	25.1 ± 7.3	9:11	Oct 2010–Sep 2011	27,424 ± 10,570	40% (8/20)
Mock challenge	10	23.1 ± 9.13	5:5	Apr–May 2012	Saline only	N/A

Definition of abbreviations: ltcm = long-term carriage monitoring study; N/A = not applicable.
Values are means ± SD.

We also calculated the ratios of postchallenge-to-prechallenge levels for each volunteer. Ratios analysis demonstrated variation by antigen and that carriage-positive volunteers had generally higher ratios than carriage-negative volunteers (Figure 5). High ratios were particularly noted for the antigens, PspAUAB055 ($P < 0.0001$, using multiple linear regression), PcpA ($P = 0.02$), PiuA ($P = 0.002$), and PspC ($P = 0.08$) (Figure 5A).

There were no changes observed in any of the analyzed antigens in mock-challenged volunteers (data not shown).

Persistently increased levels of IgG antibodies against 13 antigens was observed for carriage-positive but not for carriage-negative volunteers at 5 weeks after inoculation (Figure E1 and Table E4).

We also analyzed the humoral responses of the 10 previous carrier rechallenged subjects to test the hypothesis that increased IgG to either PS or pneumococcal protein would be associated with protection against carriage reacquisition. Data for anti-PS IgG are presented by volunteer in Figure 6A and pooled for the group in Figure 6B, showing an increased level postcarriage compared with precarriage. Pre-rechallenge levels were higher than prechallenge, but not different from postchallenge levels (Figure 6B). Antiprotein responses are presented in a similar manner in Figures 5D and 6C. Again, we observed generally higher antiprotein levels at the time points after the first inoculation (postchallenge and pre-rechallenge) than at baseline (prechallenge) (Figure 6D). The individual analysis of IgG levels to each protein confirmed that the levels in serum samples obtained 1 week pre-rechallenge were higher than prechallenge levels for 16 out of the 27 proteins analyzed (Figure E2 and Table E5). Response data are summarized in Figure 6E. We observed increased IgG levels to PS in three volunteers, increased IgG levels to proteins in two volunteers, and increased IgG responses to both PS and proteins in three volunteers. We also observed that two volunteers protected from carriage showed no significant increase in responses to either PS or proteins.

Carriage Increases Opsonophagocytic Activity of Antibodies in Serum

To determine the function of the anti-PS and protein responses to carriage described previously here, we compared paired serum samples in an OPK assay. Serum samples from most volunteers had measurable levels of killing in the OPK assay both pre- and postcarriage (Figure 7). No difference in the geometric mean antibody titer (GMT) was observed when prechallenge (baseline) samples of carriage-positive volunteers and carriage-negative volunteers were compared ($P = 0.20$, using paired *t* test). Carriage induced a fourfold increase in GMT (before, 139 [41–462] versus after, 1936 [1,069–3,508], $P = 0.0001$). In the absence of carriage, GMT remained similar (before, 212 [78–576] versus after, 209 [61–715], $P = 0.87$). There was no association of GMT in the OPK with carriage duration. There was no correlation between anti-PS IgG levels and OPK levels.

Carriage Increases Specific Mucosal Antibodies to PspA, but Not to PspC

There was no difference observed in baseline levels of IgG in NW between carriage-positive and carriage-negative volunteers for PspA (Figures 8A and 8B, $P = 0.84$ using unpaired *t* test) or for PspC (Figures 8C and 8D, $P = 0.43$).

Carriage elicited increased mucosal IgG to PspA from Day 7 after inoculation, reaching significantly higher levels than prechallenge levels at Day 28 ($P = 0.05$, using paired *t* test), Day 35 ($P = 0.02$), and Day 42 ($P = 0.0008$) (Figure 8A). Mucosal IgG levels to PspC did not significantly change from baseline in carriage-positive volunteers (Figure 8C). We did not observe significant changes in the level of IgG to either PspA or PspC in NW samples in the absence of carriage (Figures 8B and 8D). Bacterial rechallenge did not alter IgG levels to either protein (Figures 8A and 8C). IgG levels to both PspA and PspC were increased in serum after carriage.

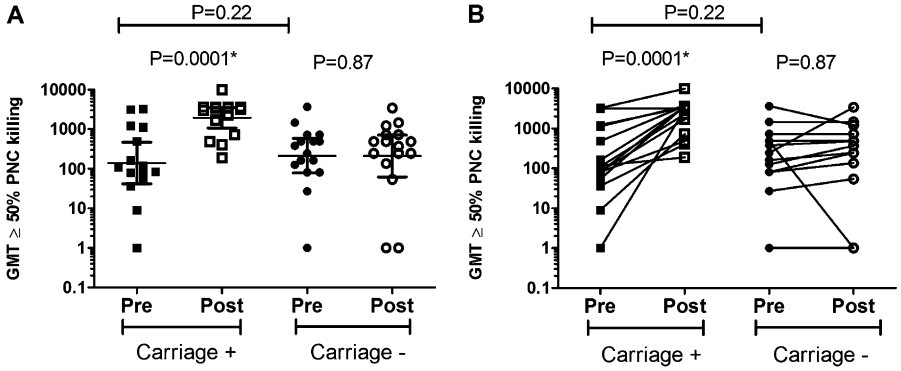


Figure 7. Opsonophagocytic activity of serum antibodies before and after pneumococcal carriage. Pre- and postserum samples of carriage-positive (carriage$^+$, $n = 15$) and carriage-negative (carriage$^-$, $n = 16$) subjects were heat inactivated and employed in an opsonophagocytic killing (OPK) assay using differentiated HL-60 cells, baby rabbit complement, and the 6B strain used for intranasal challenge. OPK was determined by the dilution (titer) of serum sample of each subject that killed at least 50% more bacteria than the control well in which no serum sample was added. We evaluated pre- and postchallenge samples of each subject in the same assay. (*A*) Geometric mean titer (GMT) values represent three independent experiments of duplicates of samples for each subject. Geometric mean of values and 95% confidence intervals are indicated by *bars*. (*B*) Pre- and postchallenge GMT levels are linked for each subject. *Statistically significant difference between pre- and postchallenge GMT levels, using paired *t* test ($P \leq 0.05$). PNC = pneumococcal.

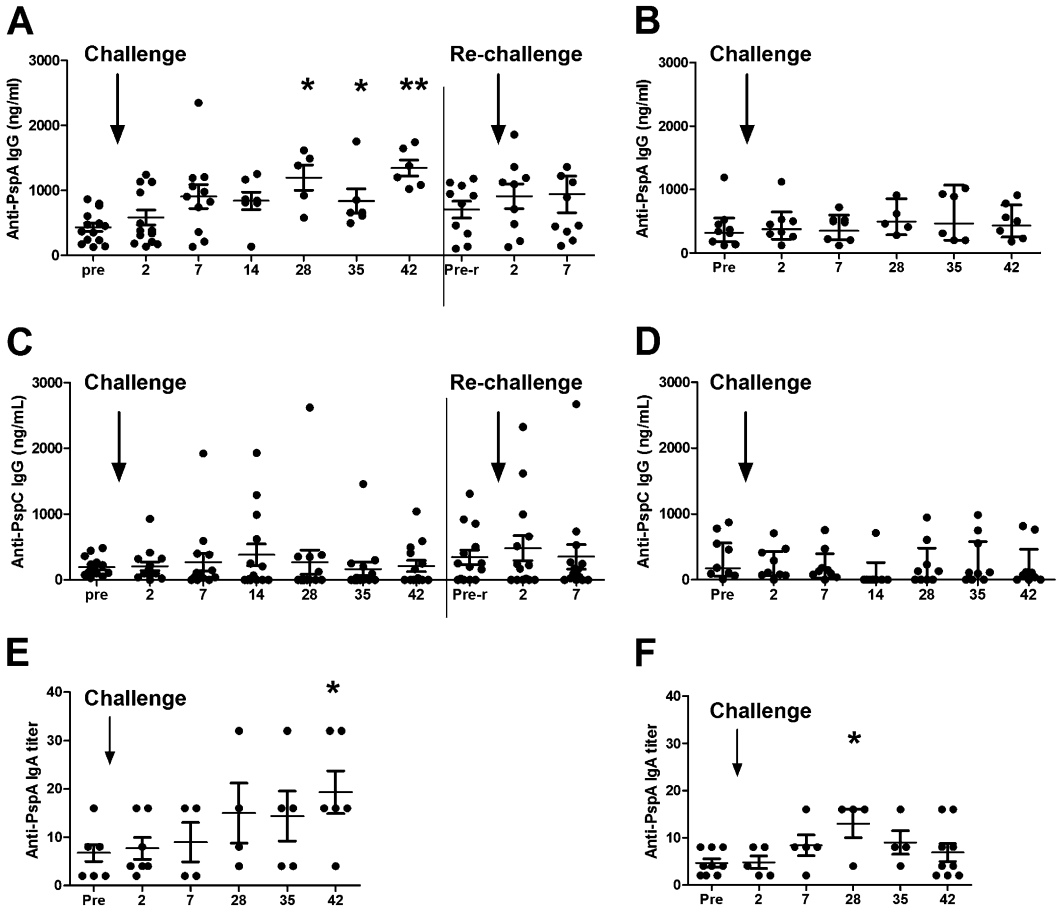


Figure 8. Kinetics of mucosal antibodies to pneumococcal surface protein (Psp) A and PspC after pneumococcal challenge. ELISAs were performed against the pneumococcal antigens, PspA (*A* and *B*, *E* and *F*) and PspC (*C* and *D*). IgG responses were determined using nasal wash (NW) samples from 14 carriage-positive (carriage$^+$) (*A* and *C*) and 9 carriage-negative (carriage$^-$) (*B* and *D*) subjects. IgA responses were determined using NW samples from eight carriage$^+$ (*E*) and nine carriage$^-$ (*F*) subjects. Samples were obtained before challenge (pre) and at the indicated days after challenge (*x axis*). Bacterial challenge and rechallenge are indicated by *arrows*. For the 10 rechallenge subjects, samples were also collected prerechallenge (Pre-r) and at Days 2 and 7 after rechallenge (*A* and *C*). Values shown are the mean (±SEM) antibody concentration. IgG antibodies to PspA and PspC are expressed in micrograms per milliliter and IgA antibodies to PspA are expressed in antibody titer. The assigned titer value was determined as the last dilution in which optical density was above 0.1. Asterisks indicate statistical significance when compared with prechallenge antibody levels using paired *t* test (*$P \leq 0.05$ and **$P \leq 0.001$).

We also measured IgA to PspA in NW samples prechallenge and at Days 2, 7, 28, 35, and 42 after challenge (Figures 8E and 8F). We observed increased IgA levels at Day 28 after challenge for carriage-positive and carriage-negative volunteers ($P = 0.01$, paired *t* test). For carriage-positive volunteers, the levels progressively increased and were significantly higher than prechallenge levels at Day 42 ($P = 0.01$), but for carriage-negative volunteers, although levels of IgA were significantly higher 28 days after challenge, levels declined and were not significantly different to prechallenge levels at Days 35 and 42.

Passive Transfer of Antibodies Elicited Cross-Protection in a Murine Model of Pulmonary Invasive Disease

Postcarriage sera diluted 1:100 in saline imparted significant protection against invasive pneumococcal disease in a lethal model of pneumococcal bacteremia (Figure 9). A total of 70% (7/10) of mice treated with 1:100 postcarriage sera survived, compared with just 20% (2/10) of mice given precarriage sera at the same dilution, and 10% of saline control mice (Figure 9A). Comparison of bacteremia levels at 24 hours postinfection revealed significantly reduced numbers of bacteria in the blood of mice treated with 1:100 postcarriage sera (Figure 9B) compared with those receiving 1:100 precarriage sera or saline. At the time of death, bacterial numbers in the blood (Figure E3A) and lungs (Figure E3B) showed a clear separation, with mice that survived the week of the experiment having completely eliminated the infection, whereas those that succumbed to the infection had high bacterial numbers in both blood and lungs.

DISCUSSION

We have shown here that the immunizing effects of pneumococcal carriage are of functional significance. Responses elicited by a single experimentally induced pneumococcal carriage episode protected humans against subsequent carriage and mice against invasive pneumococcal disease by passive transfer of sera from colonized individuals.

All subjects enrolled in our study had measurable baseline IgG levels to proteins and to the 6B capsular PS. We have found no relation of baseline serum IgG and carriage outcome after challenge. Intranasal exposure to bacteria boosted serum IgG levels to several pneumococcal proteins, with carriage-positive subjects showing the greatest magnitude of response. Increased IgG levels to the 6B PS and OPK mediated by postcarriage serum were found only in subjects that developed carriage.

Persistently increased IgG to proteins was observed in carriage-positive subjects at 5 weeks after inoculation and not in those without carriage. The boosting effect of exposure is temporary and therefore different than responses induced by persistent carriage. This increased and sustained response after carriage was reflected in protection against carriage acquisition after rechallenge up to 11 months after clearance of the first carriage episode. We showed that, in 8 out of 10 volunteers, protection against carriage reacquisition was associated with significantly altered levels of IgG to both proteins and PS. This does not exclude a role for mucosal CD4$^+$ T helper type 17 cells and, in particular, it is interesting to note that 2 out of the

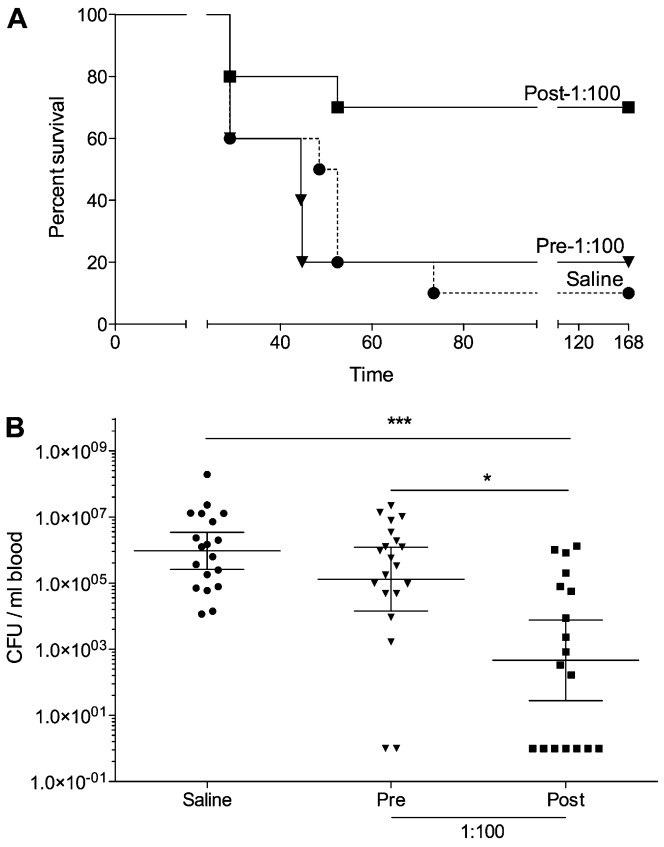


Figure 9. Passive transfer of postcarriage sera to mice confers significant protection against invasive disease. Mice were given intraperitoneal administration of 1:100 diluted pooled sera obtained from six subjects pre- or postcarriage with 6B strain. Sera were administered to mice 2 hours before an intranasal invasive challenge with 1×10^6 cfu of *Streptococcus pneumoniae* D39. (*A*) Survival of mice (indicated as percentage on *y axis*) after intranasal challenge (given in hours on *x axis*). (*B*) Colony-forming units per milliliter blood at 24 hours postinfection. Data presented are geometric means (±95% confidence interval). Asterisks indicate statistical significance using one-way ANOVA and Bonferroni's post test (*$P < 0.05$ and ***$P < 0.001$). Data in *A* are from a single experiment with 10 mice per group. Data in *B* are from two independent experiments with 10 mice per group.

10 volunteers did not have significantly increased levels of IgG. Results from some murine models suggest that T helper type 17–mediated immunity is involved in mucosal protection against pneumococcal colonization (23, 24). Our data are also consistent with the observation of Cobey and Lipsitch (16), in which a mathematical model was used to show that anticapsular immunity acquired during colonization is weak and confers only 30 to 60% reduction in susceptibility to future colonization with that type.

We demonstrated, for the first time, that the antibody response elicited by a single carriage episode in adults was protective against invasive pneumococcal disease in a serotype-independent manner in mice. We showed that a serotype 6B carriage episode induced antibodies that cross-reacted with a serotype 2 strain and were sufficient to confer cross-serotype protection against pulmonary invasive disease.

The EHPC model offered us the opportunity to associate the magnitude of antibody responses with known inoculated doses of pneumococcus. There was no significant association with inoculated dose, but, paradoxically, total pneumococcal protein responses dropped in serum for 7 out of the 20 carriage-positive volunteers, particularly in those inoculated with the highest doses of pneumococci. We speculate that nasopharyngeal colonization may result in circulating antibody sequestration. This is consistent with our findings that persistent antigen presentation during carriage elicited progressive increases in mucosal IgG and IgA to the PspA expressed by the inoculated strain.

The strengths of this study are the novel use of human challenge and rechallenge, multiple assessments of humoral responses in both serum and mucosal samples, and confirmation of effect in both OPK assays and a passive transfer model using a heterologous pneumococcal strain. We employed paired serum samples in our OPK, which allowed us to directly compare increased bacterial killing mediated by serum samples after a single carriage episode. The reference opsonophagocytic assay using HL-60 cells is used to determine quality of antibody after pneumococcal vaccination (22). We did not observe a significant correlation between levels of IgG to PS and OPK in the 61 samples analyzed, suggesting that simple anti-PS IgG levels are insufficient to explain this effect. Antibodies to certain proteins, such as PspA and PspC, could mediate complement deposition and facilitate phagocytosis (25, 26).

A further strength of our study was that the genome of the strain used for inoculation has been sequenced, allowing us to measure responses to specific antigens of interest. For instance, our MSD results showed increased IgG to the PspA UAB055 (family 1 clade 2), but not to the PspA UAB099 (family 2 clade 3). The PspA expressed by the 6B strain used for inoculation is of family 1 clade 1. Our data are therefore consistent with the literature showing that antibodies induced by immunization with a PspA clade 1 have high reactivity with strains expressing PspA clade 1 and clade 2, but very low reactivity with strains expressing PspA clade 3 (27, 28). The antibody response to carriage has been examined previously in healthy adults in an EHPC study (15, 29). That study showed that susceptibility to carriage did correlate with baseline serum IgG to PspA (15). In our study, however, we could not find a correlation between anti-PspA IgG antibodies and protection against carriage. This could be explained by the fact that PspA is a variable antigen, and the PspA expressed by our 6B strain (clade1) has 61% amino acid sequence identity (N-terminal region) with PspAUAB055 (clade 2) and 40% with PspAUAB099 (clade 3). A study using different fragment regions of the PspA from our strain might resolve this inconsistency.

We postulate that carriage is beneficial and works as a natural boosting mechanism to sustain protective immunity against disease in adults. The reason why this effective immunity observed in adults wanes with aging, and pneumococcal disease incidence increases despite decreased carriage, is not yet fully understood (7). It is unlikely to be an artifact of sampling methods, but more likely to be due to "failure" to establish carriage due an "inappropriate" response by the immune system in aged adults. Increased production of inflammatory mediators has been described in the elderly, a condition termed "inflammaging" (30, 31). The presence of a balanced response between T cell–derived IFN-γ and IL-10, as well as regulatory T cells, in nasal-associated lymphoid tissue has been associated with carriage persistence in children (32, 33), and provides a clue as to how the elderly might fail to take advantage of carriage as a means of autoinoculation. A lack of "natural boosting" provided by carriage episodes could explain the observed decline in anti-pneumococcal antibody levels (34, 35) and antibody function with aging (36) while total IgG and IgM remain unchanged (37). The benefit of low-level pneumococcal carriage at the time of vaccination has also been reported in mice (38).

This study has important implications for current and future vaccination strategies. Currently, the direct and herd protection effects against carriage promoted by pneumococcal conjugate vaccination in children could reduce natural boosting of existing immunity in both children and adults. Antibody responses after

vaccination last between 5 and 10 years and, in the absence of intermittent carriage episodes, it may be necessary to administer additional doses of pneumococcal vaccine to all ages to maintain protection—as is the recommendation for tetanus, diphtheria, yellow fever, and typhoid fever vaccination.

This study supports the development of future mucosal vaccination strategies. We have previously reported that intranasal challenge without subsequent carriage was sufficient to elicit increased IgG to whole-cell pneumococcus in the lung of healthy subjects after a double inoculation protocol using a 23F pneumococcal strain (18). We have now shown that mucosal antigen presentation and persistent carriage induced both systemic and local production of antibodies to several pneumococcal proteins. Together, these results support the use of a protein-based mucosal vaccine to induce mucosal (in the upper and lower airways) as well as systemic responses. By enhancing immunity in both sites, mucosally active vaccines could increase protection against invasive disease as well as pneumonia (39) and carriage.

Author disclosures are available with the text of this article at www.atsjournals.org.

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