Antibodies to the Iron Uptake ABC Transporter Lipoproteins PiaA and PiuA Promote Opsonophagocytosis of Streptococcus pneumoniae

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Received 1 February 2005/Returned for modification 13 April 2005/Accepted 24 May 2005

PiaA and PiuA are the lipoprotein components of the Pia and Piu Streptococcus pneumoniae iron uptake ABC transporters and are required for full virulence in mouse models of infection. Active or passive vaccination with recombinant PiaA and PiuA protects mice against invasive S. pneumoniae disease. In this study we have analyzed the antibody responses and mechanism of protection induced by PiaA and PiuA in more detail. For both proteins, two booster vaccinations induced stronger antibody responses in mice than a single or no booster vaccinations, and 5 μg of protein induced similar levels of antibody responses as 20 μg. Immunoglobulin G (IgG) subclass-specific enzyme-linked immunosorbent assays demonstrated that the antibody response to PiaA and PiuA was predominantly IgG1, with induction of only low levels of IgG2a. Anti-PiaA and anti-PiuA polyclonal rabbit antibodies bound to the surface of live S. pneumoniae when assessed by flow cytometry but did not inhibit growth of S. pneumoniae in cation-depleted medium or bacterial susceptibility to the iron-dependent antibiotic streptonigrin. However, anti-PiaA and anti-PiuA did increase complement-independent and -dependent opsonophagocytosis of different serotypes of S. pneumoniae by the human neutrophil cell line HL60. Hence, vaccination with PiaA and PiuA protects against S. pneumoniae infection by inducing antibodies that promote bacterial opsonophagocytosis rather than inhibiting iron transport.

In both developing and industrialized countries, the gram-positive pathogen Streptococcus pneumoniae is responsible for a significant burden of potentially preventable disease (2, 16). The currently available S. pneumoniae vaccines are based on capsular polysaccharide antigens, but as S. pneumoniae is capable of synthesizing at least 90 antigenically different capsular polysaccharides, a polyvalent vaccine containing many capsular antigens is needed for protection against the common serotypes. The 23-valent polysaccharide vaccine protects against 80% of clinically significant serotypes but is ineffective in children under the age of 2 and has reduced efficacy in the elderly, the two main risk groups for severe disease (15). Conjugated vaccines composed of 7 to 11 selected polysaccharides bound to nonneumococcal protein carriers have been developed and protect children aged less than 2 years from invasive S. pneumoniae infections (3, 14). However, the conjugated vaccines protect against only a limited number of capsular serotypes, and their use results in nonvaccine serotypes becoming more prevalent (11). In addition, conjugated vaccines are expensive, limiting their availability in the developing world. Because of these drawbacks with capsular antigen vaccines, there has been much interest in developing a protein-based S. pneumoniae vaccine, using surface-expressed proteins which are well conserved across all serotypes (1, 8, 21, 22, 25, 28).

PiaA and PiuA are recently identified lipoprotein components of two separate S. pneumoniae iron uptake ABC transporters that are required for full virulence in mouse models of infection (4). Both PiaA and PiuA are likely to be expressed on the bacterial cell surface (24, 26), and active or passive vaccination of mice with recombinant PiuA and PiaA protects against invasive S. pneumoniae disease, especially when mice are vaccinated with both antigens (8). Mouse antibodies to recombinant PiuA and PiaA derived from a capsular serotype 2 S. pneumoniae strain react with identical proteins from nine other capsular serotypes (8), and human sera recovered from patients with recent episodes of S. pneumoniae sepsicemia due to different capsular serotypes contain anti-PiuA and anti-PiaA which cross-react with recombinant PiuA and PiaA from a single capsular serotype (27). Hence, PiuA and PiaA are potential candidates for an S. pneumoniae vaccine that may induce protection against many different S. pneumoniae capsular serotypes and overcome some of the limitations of the existing capsular antigen vaccines. However, ABC transporter lipoproteins are probably attached to the outer surface of the cell membrane (24, 26) and therefore separated by both the capsule and cell wall from the external environment, and recent data have shown that the surface accessibility to antibodies of another lipoprotein vaccine candidate, PsaA, was poor (12). It
is therefore unclear whether antibodies to PiuA and PiaA can bind to the lipoprotein in live S. pneumoniae cells and assist opsonophagocytosis of the bacteria by host phagocytes. Potentially, antibodies to PiuA and PiaA could aid immunity by other mechanisms, such as inhibiting the function of these proteins and therefore reducing iron uptake by S. pneumoniae. As iron uptake is required for in vivo growth (4–6), this would result in delayed replication of S. pneumoniae and protection of the host against infection.

In the present study, we have characterized the antibody responses to different schedules of vaccination with these antigens in more detail. In addition, we have investigated the mechanism of protection provided by anti-PiuA and anti-PiaA, using growth in cation-depleted medium and streptolysin sensitivity assays to assess their effect on iron transport and a flow cytometry assay to assess their effect on opsonophagocytosis of S. pneumoniae.

MATERIALS AND METHODS

Bacterial strains and media. The capsular serotype 2 strain D39 and its derivatives were used for all experiments. D39 ΔpiuB and ΔpiaA strains were constructed by standard transformation techniques using DNA isolated from the previously described strain 0100993 capsular serotype 3 ΔpiuB and ΔpiaA strains and selection for mutant strains with appropriate antibiotics (4). Representative strains of common strain types (according to multilocus sequence typing) for the S. pneumoniae and selection for mutant strains with appropriate antibiotics (4). Representative strains of common strain types (according to multilocus sequence typing) for the S. pneumoniae were expressed and purified using the QIAexpress (QIAGEN) bacterial strains and media.

Cloning, expression, and purification of recombinant PiuA and PiaA. Recombinant PiuA and PiaA proteins (excluding the N-terminal lipoprotein peptidase signal sequence) were expressed and purified using the QIAexpress (QIAGEN) expression system. Fragments of piuA and piaA were amplified using high-fidelity PCR and S. pneumoniae D39 chromosomal DNA as the template with the oligonucleotide primer pairs PiaA forward (5′-CGGATCCCTCTATAATCTCGTTAATAGTGA-3′) and PiuA reverse (5′-GAGGCTTGGCAGATTTGAAC-3′). Bacterial strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract; when necessary, cations were depleted using Chelex 100 as previously described (4). Single-use aliquots of D39 for use in the experiments were prepared from cultures grown in Todd-Hewitt-yeast (THY) broth (optical density at 540 nm [OD540], 0.2 to 0.4) and stored at −70°C.

Cloning, expression, and purification of recombinant PiuA and PiaA. Recombinant PiuA and PiaA proteins (excluding the N-terminal lipoprotein peptidase signal sequence) were expressed and purified using the QIAexpress (QIAGEN) expression system. Fragments of piuA and piaA were amplified using high-fidelity PCR and S. pneumoniae D39 chromosomal DNA as the template with the oligonucleotide primer pairs PiaA forward (5′-CGGATCCCTCTATAATCTCGTTAATAGTGA-3′) and PiuA reverse (5′-GAGGCTTGGCAGATTTGAAC-3′). Bacterial strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract; when necessary, cations were depleted using Chelex 100 as previously described (4). Single-use aliquots of D39 for use in the experiments were prepared from cultures grown in Todd-Hewitt-yeast (THY) broth (optical density at 540 nm [OD540], 0.2 to 0.4) and stored at −70°C.

To investigate the optimum immunization protocol for anti-PiuA and anti-PiaA sera in a modified ELISA, antibody titers were measured as the lowest dilution giving an OD492 equal to or greater than 0.30.

IgG binding assays. Flow cytometry assays of IgG deposition on the surface of S. pneumoniae were performed using a previously described protocol (7). Briefly, bacterial stocks were thawed, washed by centrifugation at 20,000 × g for 6 min in PBS, incubated in 10 μl of mouse serum for 20 min, washed twice with 500 μl of PBS-0.1% Tween 20, and resuspended in 50 μl of PBS-0.1% Tween 20 containing a 1:100 dilution of phycoerythrin-conjugated goat anti-rabbit IgG (Sigma). After incubation on ice for 30 min, the bacteria were washed with 500 μl of PBS-0.1% Tween 20 and resuspended in 400 μl of PBS for flow cytometry analysis.

Opsonophagocytosis assays. To assess the effect of anti-PiuA and anti-PiaA antibodies on the interaction of S. pneumoniae with the human monocyte/macrophage cell line U937, S. pneumoniae strains were fluorescently labeled by incubation with 5.6-carboxyfluorescein-succinimidyl ester (FAM-SE; Molecular Probes, Eugene, Ore.) solution (10 mg/mL in dimethyl sulfoxide; Sigma) in 0.1 M sodium bicarbonate buffer for 1 h at 37°C and then washed six times with Hanks balanced salt solution (HBSS) in 0.2% bovine serum albumin and stored in aliquots at −70°C in 10% glycerol (approximately 10^7 CFU/mL). The human cell line HL-60 (promyelocytic leukemia cells; CCL240, American Type Culture Collection, Manassas, Va.) was used to provide the effector cells after differentiation into granulocytes by using previously described protocols (17). Differentiated HL60 cells were harvested by centrifugation (160 × g, 8 min, 4°C) and washed twice with HBSS and once with HBSS in the presence of Ca^2+ and Mg^2+. FAM-SE-labeled bacteria (10^6 CFU) were opsonized with 1:100, 1:40, and 1:10 dilutions of serum in a 96-well plate for 20 min at 37°C with horizontal shaking (170 rpm). Negative controls were included, using the same volume of HBSS. HL60 cells (100 μl) were added to the opsonized bacteria in the microplate plate and incubated for 30 min at 37°C with shaking, after which the bacteria and cells were fixed using 3% paraformaldehyde and analyzed using flow cytometry. A minimum of 8,000 cells per sample were analyzed.

Rabbit polyclonal antibodies to PiaA or PiuA. Rabbit polyclonal anti-PiaA and anti-PiuA were prepared by CovalAb (United Kingdom) by immunization of 6- to 8-week-old rabbits three times at three weekly intervals i.p. with either PiaA or PiuA and collecting sera by tail bleeds. A proportion of the antisera was used for affinity purification of anti-PiaA and anti-PiuA by using a protein A bead column, providing approximately 2 ml of affinity-purified antibody at 4 mg/ml.

Streptolysin sensitivity assays. Streptolysin susceptibility assays were performed using bacteria grown in cation-depleted medium in the presence or absence of rabbit affinity-purified anti-PiuA or anti-PiaA (4, 5). One hundred microliters of the culture was removed, to which was added 2.5 μg/mL of streptolysin (Streptolysin, Sigma), followed by incubation at 37°C for up to 80 min. Aliquots of the reaction cultures were diluted and plated at different time points and the CFU milliliter^-1 for each time point calculated. Each experiment used triplicate samples and was repeated three times. Results were expressed as a proportion of the starting CFU milliliter^-1 to allow comparison between experimental groups.

Statistical analysis. Data presented are representative of data from experiments performed two or three times. Data from the survival experiments were analyzed using log rank tests, and the results for all other comparisons were analyzed using two-tailed t tests.

RESULTS

Effects of booster immunization with PiuA and PiaA and IgG subclass responses. To investigate the optimum immunization protocol for anti-PiuA and anti-PiaA sera in a modified ELISA, antibody titers were measured as the lowest dilution giving an OD492 equal to or greater than 0.30.
zation regimen, the effects of one or two booster vaccinations on the strength of mouse antibody responses to PiaA and PiaA were assessed. Groups of mice were given one, two, or three 10^{-6} g doses of PiaA or PiaA by i.p. injection on days 0, 7, and 14, and the titers of total IgG to the antigens in sera obtained on days 7, 14, 21, and 28 immediately before booster vaccinations were measured using ELISAs. For both PiaA and PiaA, antibody titers after one dose of antigen were relatively weak, but they were substantially increased when mice were given one or two booster vaccinations (Table 1). Antibody titers were higher after two than after one booster dose. As previously reported, PiaA appears to be more immunogenic than PiaA, with maximum antibody titers of 1/12,800 compared to 1/3,200 (8).

The combination of immunization with PiaA and PiaA improves protection against systemic S. pneumoniae infection (8); therefore, additional experiments investigated the effect of vaccination with varying and combined doses of PiaA and PiaA on the antibody titers. Different groups of animals were vaccinated with three doses of 10 or 20 µg of individual antigen or 5 or 10 µg each of PiaA and PiaA in combination, and the antibody responses were measured 14 days after the last vaccination. There were only small differences in the antibody titers to PiaA and PiaA in mice given 5, 10, or 20 µg of each antigen singularly or in combination (Fig. 1A and B), suggesting that vaccination with 5 µg of each antigen should be as effective as higher doses. The predominant IgG subclass responses after vaccination with PiaA and PiaA were assessed using ELISAs specific for mouse IgG1 and IgG2a. Both PiaA and PiaA elicited strong IgG1 subclass responses, with only weak IgG2a responses (Fig. 1C and D).

### Table 1. Specific IgG titers in mouse sera over time in response to one, two, or three i.p. vaccinations with PiaA and PiaA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>No. of vaccinations</th>
<th>Titer after day since first vaccination:</th>
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<tr>
<td></td>
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<td>7</td>
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<tr>
<td>PiaA</td>
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<td>2</td>
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<td>3</td>
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<tr>
<td>PiaA</td>
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* Mice were vaccinated on day 0 and after sera were obtained on days 7 and 14.
* ND, not done.
* —, no detectable response.

**FIG. 1.** (A and B) Antibody titers to PiaA (A) and PiaA (B) in mouse sera after i.p. vaccination with 10 µg (diagonally hatched bars) or 20 µg (vertically hatched bars) of single antigen or with 5 µg plus 5 µg (horizontally hatched bars) or 10 µg plus 10 µg (open bars) of PiaA and PiaA in combination. (C and D) Serum IgG1 (C) and IgG2a (D) subclass antibody titers to PiaA and PiaA on day 7 (open bars, 7 days after the initial i.p. vaccination), 14 (diagonally hatched bars, 7 days after first booster vaccination), or 21 (vertically hatched bars, 7 days after the second booster i.p. vaccination). Solid bars represent results for sera from mice vaccinated with alum alone, and error bars represent standard deviations. Titers are presented as log_{10} values of the reciprocal dilutions giving an OD_{405} equal to or greater than 0.30.
Rabbit anti-PiuA and anti-PiaA protect mice against systemic S. pneumoniae infection. In order to analyze the mechanism of protection induced by anti-PiuA and anti-PiaA antibodies, high-titer polyclonal rabbit anti-PiuA and anti-PiaA sera were obtained (titers of 1/8,000 and 1/32,000, respectively). Before assessment of the mechanisms by which anti-PiuA and anti-PiaA can prevent S. pneumoniae infection, whether polyclonal rabbit anti-PiuA and anti-PiaA can protect mice against S. pneumoniae infection was confirmed using passive immunization experiments. Groups of mice were given 100 μl of rabbit anti-PiuA (diluted in PBS to a titer of 1/8,000) and anti-PiaA sera by i.p. injection, followed 12 h later by i.p. challenge with 10^6 CFU of S. pneumoniae D39 and a second i.p. vaccination with rabbit antisera at 24 h. In keeping with previous data using mouse antisera, passive immunization with rabbit anti-PiuA and anti-PiaA in combination significantly enhanced survival of mice (55%, compared to 10% for mice given rabbit preimmune sera; P < 0.01) (Fig. 2) (8). Passive immunization with the individual antisera resulted in smaller, nonsignificant improvements in survival (30% for PiuA and 20% for PiaA). These results demonstrate that rabbit anti-PiuA and anti-PiaA in combination can protect against S. pneumoniae infection.

IgG binding to S. pneumoniae. Whether polyclonal rabbit anti-PiuA and anti-PiaA bound to the surface of live S. pneumoniae was measured using viable D39 cells and a flow cytometry assay. IgG was deposited on a high proportion of S. pneumoniae cells after incubation in either undiluted anti-PiuA or anti-PiaA serum, and to a lesser degree in diluted anti-PiuA or anti-PiaA serum (Fig. 3A). The level of IgG binding to PiuA serum was similar to that to PiaA despite the higher titer of antibody to PiuA, consistent with reverse transcription-PCR data which show that the piaA operon is more highly expressed than the piuA operon in laboratory medium and during infection in mice (5; J. S. Brown, unpublished data). To confirm that IgG binding to S. pneumoniae in rabbit anti-PiuA and anti-PiaA sera was antigen specific, IgG binding experiments were repeated using the ΔpiaA and ΔpiuB strains, in which, respectively, the expression of PiaA and PiuA is disrupted (4). Although there was some nonspecific IgG binding, the proportion of bacteria positive for IgG increased substantially when the ΔpiuB strain was incubated with anti-PiaA antibodies and when the ΔpiaA strain was incubated with anti-PiuA antibodies (Fig. 3B). These experiments demonstrate that anti-PiuA and anti-PiaA bind to the surface of live S. pneumoniae and that this binding is mainly antigen specific.

Effects of anti-PiuA and anti-PiaA on iron transport. Whether anti-PiuA and anti-PiaA can inhibit iron uptake by the Piu and Pia ABC transporters was investigated using growth of S. pneumoniae in cation-depleted medium and bacterial susceptibility to streptonigrin. Growth of the wild-type D39 S. pneumoniae strain in cation-depleted medium with or without addition of anti-PiuA and anti-PiaA was assessed by measuring the ODs of broth cultures over time. To avoid competitive binding of IgG by polyclonal serum, experiments were performed using purified and biotinylated rabbit polyclonal anti-PiuA and anti-PiaA sera. These sera were incubated with S. pneumoniae D39, followed by streptavidin-phycoerythrin conjugate and flow cytometry. The proportion of bacteria positive for IgG increased substantially when the ΔpiuB strain was incubated with anti-PiaA antibodies and when the ΔpiaA strain was incubated with anti-PiuA antibodies (Fig. 3B). These experiments demonstrate that anti-PiuA and anti-PiaA bind to the surface of live S. pneumoniae and that this binding is mainly antigen specific.

FIG. 2. Survival curves for groups of 20 mice vaccinated passively with rabbit polyclonal anti-PiuA and anti-PiaA sera at a titer of 1/8,000. Circles, preimmune serum; diamonds, anti-PiaA serum; squares, anti-PiuA serum; triangles, combination of anti-PiuA and anti-PiaA serum. For passive vaccination with anti-PiuA and anti-PiaA in combination versus preimmune serum, P < 0.01. The differences in survival between mice given either anti-PiuA or anti-PiaA and mice given preimmune serum or the combination of anti-PiuA and anti-PiaA were not significant.

FIG. 3. Anti-PiuA and anti-PiaA binding to S. pneumoniae D39 assessed by identifying bacteria coated with antibody by using phycoerythrin-conjugated goat anti-rabbit IgG and flow cytometry. (A) Proportion of wild-type bacteria positive for IgG after incubation in rabbit polyclonal anti-PiuA and anti-PiaA. Solid bars, PBS negative control; open bars, 1/20 dilution of antisera; diagonally hatched bars, 1/5 dilution of antisera; vertically hatched bars, undiluted antisera. For all antisera dilutions versus PBS, P < 0.015. (B) Proportions of ΔpiuB, ΔpiaA, and ΔpiuB ΔpiaA bacteria positive for IgG after incubation in a 1/10 dilution of preimmune sera (solid bars), rabbit polyclonal anti-PiuA (open bars), and anti-PiaA (diagonally hatched bars). For the ΔpiaA strain, results for anti-PiuA compared to the results for anti-PiaA, P < 0.0001. For the ΔpiuB strain, results for anti-PiaA compared to the results for anti-PiuA, P < 0.0001. Error bars represent standard deviations.
contamination with iron-containing compounds present in serum, affinity-purified anti-PiuA and anti-PiaA were used for these experiments. As previously demonstrated for a capsular serotype 3 S. pneumoniae strain, growth of a ΔpiuB ΔpiaA double mutant strain derived from D39 was substantially impaired in cation-depleted medium compared to that of the wild-type parental strain (Fig. 4). However, addition of the combination of anti-PiaA and anti-PiuA antibodies did not delay growth of the wild-type strain, indicating that these antibodies do not substantially inhibit the function of the Pia and Piu iron transporters. In addition, the presence of anti-PiaA and anti-PiuA did not inhibit growth of the ΔpiuB and ΔpiaA strains, respectively, in cation-depleted medium (data not shown).

To further investigate whether anti-PiaA and anti-PiuA may affect iron transport, the susceptibility of D39 strains to streptongrigin in the presence or absence of the antibodies was assessed. Streptongrigin is an antibiotic whose efficacy is dependent upon intracellular iron content, and it is a sensitive indirect indicator of low iron levels in S. pneumoniae (4). However, addition of anti-PiuA in combination with anti-PiaA antibody to D39, of anti-PiuA to the ΔpiaA strain, or of anti-PiaA to the ΔpiuB strain grown in cation-depleted medium did not affect S. pneumoniae susceptibility to streptongrigin (data not shown). Hence, data for both growth in cation-depleted medium and susceptibility to streptongrigin suggest that anti-PiuA and anti-PiaA do not inhibit Piu and Pia function, and these antibodies probably protect against S. pneumoniae infection by other mechanisms.

Anti-PiuA and anti-PiaA increase opsonophagocytosis of S. pneumoniae. Anti-PiuA and anti-PiaA may assist immunity to S. pneumoniae by opsonizing bacteria and improving phagocytosis. To investigate this possibility, we used an in vitro flow cytometry assay to measure the association of S. pneumoniae with the human neutrophil cell line HL60 after incubation with 1/100, 1/40, and 1/10 dilutions of affinity-purified anti-PiuA and anti-PiaA. The role of complement was investigated by repeating the experiments with and without exogenous supplementation with rabbit complement. In the absence of complement there was a small increase in the association of D39 with HL60 cells in the presence of either anti-PiuA or anti-PiaA (Fig. 5A). Overall, the level of association of D39 with HL60 cells was considerably higher when rabbit complement was added to the assay, and this increase was more marked in the presence of anti-PiuA and anti-Pia (Fig. 5B). Further opsonophagocytosis assays with 1/10 dilutions of affinity-purified anti-PiuA and anti-PiaA were performed using representative strains of the common S. pneumoniae serotypes 6A, 6B, 9V, 14, and 23F. For all the serotypes investigated, in the presence of complement both anti-PiuA and anti-PiaA increased the association of bacteria with HL60 cells compared to HBSS alone (Fig. 5C). These results demonstrate that anti-PiuA and anti-PiaA assist opsonophagocytosis of D39 and a range of other S. pneumoniae capsular serotypes.

Discussion

Several surface-expressed S. pneumoniae proteins are under current investigation as alternative vaccine candidates that may overcome the limitations of vaccines based on capsular polysaccharide (25). The work presented in this paper further characterizes the antibody responses to two of these candidate proteins, the iron uptake ABC transporter lipoproteins PiuA and PiaA. Previously we have shown that active vaccination with either PiuA or PiaA improves protection against systemic S. pneumoniae infection and that vaccination with both antigens is highly protective (8). In addition, passive vaccination with polyclonal mouse anti-PiuA and anti-PiaA was protective against S. pneumoniae (8), and the results presented here confirm that this was also true for passive vaccination with rabbit anti-PiaA and anti-PiuA. To help optimize vaccination schedules for future studies, we have investigated the effects of one or two booster vaccination doses and different quantities of antigen on the strength of mouse antibody responses to PiaA and PiuA. The results indicate that three doses of vaccine induce the strongest response and that using 5 μg of each protein should be adequate to stimulate good antibody titers. As has previously been shown for other S. pneumoniae protein vaccine candidates (29), i.p. vaccination of BALB/c mice with PiaA and PiuA induces mainly IgG1 subclass antibodies, with only a small rise in IgG2a. The predominance of IgG1 suggests that in this model of vaccination there is mainly a Th2 immune response, contrasting with the Th1 response seen after DNA vaccination (19, 20).

The mechanism of protection afforded by many vaccine candidates remains ill-defined. Passive vaccination with antibodies to various S. pneumoniae protein antigens prevents infection, indicating that the protection provided by active vaccination is likely to be at least partially dependent on antibodies (8, 18, 21, 28). Antibodies to PiaA and PiuA are detectable in convalescent-phase sera from patients recovering from S. pneumoniae infections (27), suggesting that these antigens are immunogenic during natural infection. However, these antibodies could be generated by bacterial breakdown products rather than by responses to cell membrane antigens in live bacteria, and whether S. pneumoniae lipoprotein vaccine candidates such as PiuA and PiaA are accessible to antibodies in extracellular fluid or are shielded by the capsule and the cell wall was unclear. Using a flow cytometry assay, we have now demonstrated that live bacteria incubated in either anti-PiuA or
anti-PiaA are coated in IgG. Moreover, using bacterial strains deficient in either antigen, we have shown that the IgG binding to \textit{S. pneumoniae} was dependent on the presence of specific antigen, providing good evidence that these antigens are accessible to antibodies even in live bacteria. There was also a degree of nonspecific IgG binding, especially when the \textit{H9004 piaA} strain was incubated in anti-PiaA serum. This may reflect nonspecific binding of IgG to the bacterial surface or, as previously has been demonstrated, cross-reactivity between anti-PiaA and PiuA (8). Higher levels of anti-PiaA and anti-PiuA binding to \textit{H9004 piaA} and \textit{H9004 piuA} strains than in wild-type bacteria could be due to increased expression of the remaining iron uptake systems in the mutant strains.

These results contrast with those of a recently published study in which anti-PsaA failed to detect significant levels of expression of the lipoprotein vaccine candidate PsaA on the surface of \textit{S. pneumoniae} (12). Why there are differences between the accessibilities of PiuA, PiaA, and PsaA to antibody binding is unclear, but they could reflect differences in the capsular structure and relative expression levels of each protein. In addition, although lipoproteins are considered to be bound mainly to membrane, potentially a proportion of some lipoproteins may also be found within the cell wall or capsule, where there would be more likely to bind to antibody and aid immunity.

Iron is an essential cofactor for many pathogenic bacteria, but within the host, iron availability is greatly restricted. In common with many pathogens, \textit{S. pneumoniae} has specialized mechanisms for iron uptake that are necessary for full virulence, including the Pia and Piu ABC transporters (4, 6). Binding of specific antibody to PiuA and PiaA could protect against infection by inhibiting Piu and Pia function, and this would readily explain the synergistic effect of dual vaccination with these antigens. However, using growth in cation-depleted medium and the highly sensitive streptonigrin susceptibility assays, we have been unable to demonstrate any inhibitory effect of anti-PiuA or anti-PiaA on iron uptake, even in mutant strains already deficient in either PiuA or PiaA. It is therefore unlikely that anti-PiuA or anti-PiaA protects against \textit{S. pneumoniae} infection by preventing iron uptake by Piu or Pia.

\begin{figure}
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\caption{(A and B) Opsonophagocytosis measured by association of \textit{S. pneumoniae} D39 with HL60 cells in different dilutions of affinity-purified rabbit anti-PiuA or anti-PiaA in the absence (A) or presence (B) of purified rabbit complement. Solid bars, HBSS; open bars, 1/100 dilution of serum; diagonally hatched bars, 1/40 dilution of serum; vertically hatched bars, 1/10 dilution of serum. For the 1/10 dilution of anti-PiuA compared to HBSS, \(P < 0.01\) in either the presence or absence of complement; for the 1/10 dilution of anti-PiaA compared to HBSS, \(P < 0.005\) in either the presence or absence of complement. For the 1/20 dilutions, \(P < 0.03\) for both antisera in the presence or absence of complement. Error bars represent standard deviations. (C) Opsonophagocytosis measured by association with HL60 cells of different serotypes of \textit{S. pneumoniae} in the presence of complement after incubation in HBSS (open bars), a 1/10 dilution of anti-PiuA (diagonally hatched bars), or a 1/10 dilution of anti-PiaA (vertically hatched bars). Anti-PiuA and anti-PiaA results that are significantly different from the results for HBSS for that strain are marked by asterisks.}
\end{figure}
Alternatively anti-PiuA and anti-PiaA may prevent infection by assisting host immunity. IgG serotype-specific antibodies to capsular polysaccharides are known to assist opsonophagocytosis of S. pneumoniae, as do antibodies to the cell surface-associated proteins PspA and PmpA (1, 17, 20, 23). We have adapted a flow cytometry assay of opsonophagocytosis widely associated with serotypes PspA and PmpA (1, 17, 20, 23). We have adapted a flow cytometry assay of opsonophagocytosis of the S. pneumoniae serotype 2 strain in either the presence or absence of complement. In addition, anti-PiaA and anti-PiuA improved opsonophagocytosis of representative strains of the common capsular serotypes 6A, 6B, 9V, 14, and 23, demonstrating that these antibodies are likely to provide protection against many of the clinically important S. pneumoniae serotypes. In general, anti-PiaA stimulated opsonophagocytosis to a greater degree than anti-PiuA, possibly because PiaA is expressed to a higher degree than PiuA (5). Hence, antibodies to membrane-anchored proteins such as PiuA and PiaA and potentially other vaccine candidates located in the same cellular compartment (9, 19, 21) can be effective opsonins and promote humoral immunity, and this is likely to be one mechanism by which anti-PiuA and anti-PiaA protect against S. pneumoniae infection. Recent data suggest that cellular immune responses are also important for immunity to S. pneumoniae (13). Whether immunization with PiuA and PiaA, in addition to stimulating humoral immunity, can also improve cellular immune responses to S. pneumoniae infection requires further investigation.

This study provides further data on the antibody response induced by vaccination with PiuA and PiaA and the mechanism by which these proteins induce protection against i.p. infection with S. pneumoniae. Future research is required to determine whether vaccination with PiaA and PiuA can also protect against S. pneumoniae after intranasal inoculation, an infection model that leads to pneumonia and represents the natural route of infection in humans. In addition, combinations of PiaA and/or PiuA and other existing protein vaccine candidates need to be investigated to identify the most effective combination vaccine for preventing S. pneumoniae infections (21).

ACKNOWLEDGMENT
This work was funded by Microscience.

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