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Citation

Published Version
doi:10.1128/IAI.00141-08

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Protection against Nasopharyngeal Colonization by *Streptococcus pneumoniae* Is Mediated by Antigen-Specific CD4⁺ T Cells

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Received 1 February 2008/Returned for modification 11 March 2008/Accepted 26 March 2008

CD4⁺ T-cell-dependent acquired immunity confers antibody-independent protection against pneumococcal colonization. Since this mechanism is poorly understood for extracellular bacteria, we assessed the antigen specificity of the induction and recall of this immune response by using BALB/c DO11.10Rag⁻/− mice, which lack mature B and T cells except for CD4⁺ T cells specific for the OVA₃₂₃–₃₃₉ peptide derived from ovalbumin. Serotype 6B *Streptococcus pneumoniae* strain 603S and unencapsulated strain Rx1ΔlytA were modified to express OVA₃₂₃–₃₃₉ as a fusion protein with surface protein A (PspA) (strains 603OVA¹ and Rx1Δlyt4OVA³) or with PspA, neuraminidase A, and pneumolysin (Rx1Δlyt4OVA³). Whole-cell vaccines (WCV) were made of ethanol-killed cells of Rx1ΔlytA plus cholera toxin (CT) adjuvant, of Rx1Δlyt4OVA³ + CT (WCV-OVA³), and of Rx1Δlyt4OVA³ + CT (WCV-OVA³). Mice intranasally immunized with WCV-OVA¹, but not with WCV or CT alone, were protected against intranasal challenge with 603OVA¹. There was no protection against strain 603S in mice immunized with WCV-OVA¹. These results indicate antigen specificity of both immune induction and the recall response. Effector action was not restricted to antigen-bearing bacteria since colonization by 603S was reduced in animals immunized with vaccines made of OVA-expressing strains when ovalbumin or killed Rx1Δlyt4OVA³ antigen was administered around the time of challenge. CD4⁺ T-cell-mediated protection against pneumococcal colonization can be induced in an antigen-specific fashion and requires specific antigen for effective bacterial clearance, but this activity may extend beyond antigen-expressing bacteria. These results are consistent with the recruitment and/or activation of phagocytic or other nonspecific effectors by antigen-specific CD4⁺ T cells.

Colonization of the upper respiratory tract (URT) is a step prior to *Streptococcus pneumoniae* infection (1, 4). Most carriage episodes are asymptomatic and last on the order of weeks to a few months (1, 8). In principle, colonization may be prevented or terminated by the innate and/or adaptive immune system or by competing microbial flora (15, 33), yet the particular host and pathogen factors affecting resistance to pneumococcal colonization are still poorly understood. The successes of antipneumococcal therapy using passive transfer of serotype-specific antibodies (14) and of vaccinations that depend on anticapsular antibodies (5) showed the importance of humoral immunity as one mechanism of protection against colonization and disease from *S. pneumoniae*. For some, but not all serotypes, such immunity appears to play a role in naturally acquired protection (34). However, several lines of evidence indicate that factors other than acquisition of anticapsular antibodies play a crucial role in the development of natural protection against pneumococci. The reduction in pneumococcal disease incidence after the first birthday in the general population occurs simultaneously for many rare and common serotypes, suggesting the acquisition of one rather than many individual immune responses; in particular, it seems to precede by several years the age-related rise in anticapsular antibody (12). Similar patterns have been suggested for nasopharyngeal carriage (8). Experimental (20, 21) and observational (7, 16) studies in adults have found little or no evidence that higher anticapsular antibody concentrations are associated with greater protection from colonization.

Mouse studies have similarly shown that immunity to pneumococcal colonization acquired from prior exposure to live bacteria (31) or a killed, whole-cell vaccine (WCV) (19) is antibody independent, while other studies have shown a similar mechanism for clearance of longstanding carriage in previously unexposed animals (33). Acquired immunity was shown to be dependent on the presence of CD4⁺ T cells at the time of challenge (19, 31).

Apart from their role in providing help for the production of antibodies, the role of CD4⁺ T cells in acquired immunity to extracellular bacteria remains poorly understood. A basic question is whether such responses depend on classical antigen presentation to the T-cell receptor. Two types of observations particularly raised this concern. First, while wild-type mice inoculated intranasally with a strain of pneumococcus rapidly cleared bacteria from the lungs and blood within 2 days, major histocompatibility complex class II-knockout mice in the same experiment showed persistent infection in both lungs and blood over 3 days, suggesting a nonspecific role for CD4⁺ T cells in early host defense (10). Further, the pneumococcal toxin pneumolysin caused apparently nonspecific activation and migration of CD4⁺ T cells in vitro in the absence of antigen presentation (10). Second, we observed (data pre-
sent below) that immunization of mice with very small quantities of killed whole pneumococci could protect them against subsequent intranasal challenge. These data are in accord with the results of a recent report by Roche et al. (26) showing that, in mice, a single exposure to a live attenuated pneumococcal strain conferred resistance against colonization and invasive disease and raise concern about the antigenic specificity of the observed protection.

We therefore sought to test the hypothesis that exposure to killed pneumococci with choler toxin adjuvant induces CD4+ T-cell-dependent immunity in an antigen-specific fashion. We approached this question by using BALB/c DO11.10 Rag−/− mice, which lack mature B and T cells except for CD4+ T cells specific for the OVA 323–339 peptide derived from ovalbumin (22). By varying the presence of this peptide in the immunizing and challenge doses, we assessed (i) whether the induction of CD4+ T-cell-dependent immunity was antigen specific; (ii) whether the memory immune response required antigen-specific stimulation of CD4+ T cells; and (iii) whether the effector function, once stimulated by cognate antigen, was limited to cells bearing that antigen. We measured the effectiveness of this immunity by evaluating the impact of vaccination with killed bacterial cells on subsequent S. pneumoniae colonization.

**TABLE 1. Streptococcus pneumoniae strains used in the study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP1296</td>
<td>Derived of laboratory strain R6; chp3kan-rpsL*. Km' Sm*</td>
<td>29</td>
</tr>
<tr>
<td>TIGR4</td>
<td>Derivative of clinical strain TIGR4 but Sm' by selection of mutants; Km*</td>
<td>30</td>
</tr>
<tr>
<td>TIGR4ΔpspA</td>
<td>TIGR4ΔpspA but pspA:kan-rpsL* by transformation with ligation product of PCR fragments amplified with primer pairs TTM053-TTM066 in TIGR4, DAM406-DAM351 in CP1296, and TTM067-TTM069 in TIGR4, and prior to ligation digested with BamHI, BamHI, and ApaI and ApaI restrictionases, respectively; Km' Sm*</td>
<td>This study</td>
</tr>
<tr>
<td>TIGR4OVA1</td>
<td>TIGR4OVA1 derivative, kan-rpsL*:pspA OVA by transformation with PspA-OVA fusion protein cassette constructed using TIGR4 genomic DNA; Km' Sm*</td>
<td>This study</td>
</tr>
<tr>
<td>R6</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Rx1</td>
<td></td>
<td>25</td>
</tr>
<tr>
<td>Rx1ΔlytA</td>
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<td>25</td>
</tr>
<tr>
<td>Rx1ΔlytAΔpspA</td>
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</tr>
<tr>
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<td></td>
<td>This study</td>
</tr>
<tr>
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<tr>
<td>Rx1ΔlytAΔnanA</td>
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<tr>
<td>Rx1ΔlytAΔnanA ΔΔnanA</td>
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<tr>
<td>603</td>
<td>Serotype 6B clinical isolate; Km' Sm*</td>
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<td>603 derivative, Sm' by selection of mutants</td>
<td>This study</td>
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<td>603ΔpspA derivative, kan-rpsL*:pspA OVA by transformation with primer pair TTM053-TTM069 PCR product of TIGR4ΔpspA; Km' Sm*</td>
<td>This study</td>
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<tr>
<td>603OVA1</td>
<td>603ΔpspA derivative, kan-rpsL*:pspA OVA by transformation with primer pair TTM053-TTM069 PCR product of TIGR4OVA1; Km' Sm*</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Km, kanamycin; Sm, streptomycin.

**MATERIALS AND METHODS**

**Streptococcus pneumoniae strains.** Pneumococcal isolates were maintained as previously described (32). Strains were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (Becton Dickinson, Sparks, MD) or on blood agar base no. 2 medium (Acumedia Manufacturing, Lansing, MI) supplemented with 5% defibrinated sheep blood (Colorado Serum Company, Denver, CO) (SBA). An autolysin-negative mutant of unencapsulated strain Rx1 (Rx1ΔlytA) (25, 26), serotype 4 strain TIGR4 (30), and serotype 6B strain 603 (17) were modified to express the OVA 323–339 peptide on the bacterial surface as a fusion protein with pneumococcal surface protein A (PspA). To construct these, the pspA locus in TIGR4 was replaced with a Janus-type cassette (29) by using the transformation protocol described by Pozzi et al. (24). The cassette was then replaced with a DNA fragment coding for PspA with OVA 323–339 peptide on the bacterial surface as a fusion protein with pneumococcal surface protein A (PsP). To construct these, the pspA locus in TIGR4 was replaced with a Janus-type cassette (29) by using the transformation protocol described by Pozzi et al. (24). The cassette was then replaced with a DNA fragment coding for PspA with OVA 323–339, generated in a two-step PCR as described by Park et al. (23), to create strain TIGR4OVA1. Strains Rx1ΔlytAΔOVA1 and 603OVA1 were created by transforming parental strains with PCR products generated in TIGR4 mutants. Rx1ΔlytAΔOVA1 was further modified to express fusion proteins of OVA 323–339 with pneumolysin and pneumolysin and neuraminidase A (Rx1ΔlytAΔOVA1) by using cassettes constructed in strain R6 (9). See Table 1 for a detailed description of strains and Table 2 for primers used in the course of the study. Fusion protein integrity was confirmed by sequencing open reading frames. PspA-OVA expression was confirmed by Western blotting using antiserum to both chicken egg albumin (Sigma) and PspA (gift of Susan
Four weeks after the last immunization, all animals were challenged intranasally with a single dose of 10^8 CFU of strain 603. One week after the challenge, animals were euthanized and URT washes were collected and counted following serial dilution and culturing on SBA supplemented with gentamicin (2.5 mg/liter) to evaluate the presence of the challenge strain in the URT. The same protocol was followed in other experiments unless stated otherwise.

### Specificity of immune induction.
To test the antigen specificity of immune induction, three groups of DO11.10 RAG−/− mice were immunized twice with WCV derived from strain Rx1ΔlytA, WCV-OVA1 derived from Rx1ΔlytA-OVA1, or CT alone and challenged with 603OVA2. The presence of the challenge strain in the URT was evaluated by using SBA plates supplemented with streptomycin (150 mg/liter). Two groups of BALB/c female mice were immunized with either WCV-OVA2 or CT alone and challenged with a streptomycin-resistant mutant of strain 603 (603S) to test if WCV-OVA1 elicits protection in normal mice similar to that previously observed for unmodified WCV.

### Specificity of recall responses.
To assess the recall immune responses, wild-type C57BL/6J mice were ordered from Jackson Laboratory, BALB/c mice from Jackson Laboratory or Taconic Farms, and BALB/c DO11.10 RAG−/− mice were immunized with either WCV-OVA1 or CT alone and challenged with 603OVA2. The specificity of the recall responses was determined by using rabbits raised against OVA3 and challenging them with OVA3-derived WCV-OVA3 and with strain 603S to test if WCV-OVA1 elicits protection in normal mice similar to that previously observed for unmodified WCV.

### Specificity of effector action.
To assess the specificity of effector action, wild-type C57BL/6J mice were immunized twice with WCV derived from strain Rx1ΔlytA, WCV-OVA1 derived from Rx1ΔlytA-OVA1, or CT alone and challenged with 603OVA2. The specificity of the recall responses was determined by using rabbits raised against OVA3 and challenging them with OVA3-derived WCV-OVA3 and with strain 603S to test if WCV-OVA1 elicits protection in normal mice similar to that previously observed for unmodified WCV.

### Immunization and challenge.
In all experiments, a single dose of the WCV was composed of ethanol-killed cells equivalent to 10^9 CFU and 1 μg of cholera toxin (CT) mucosal adjuvant (List Biological Laboratories, Campbell, CA) in a 10-μl dose volume unless stated otherwise (17). The exception was the preparation of WCV dose responses, in which four groups of C57BL/6J female mice were immunized as previously described (17) twice, one week apart, with the regular dose of Rx1ΔlytA WCV (group 1), a dose composed of 1/100 or 1/10,000 dilution of this CFU dose (groups 2 and 3, respectively), or CT alone (group 4). Four weeks after the last immunization, all animals were challenged intranasally with 2 × 10^6 CFU of strain 603. One week after the challenge, animals were euthanized and URT washes were collected and counted following serial dilution and culturing on SBA supplemented with gentamicin (2.5 mg/liter) to evaluate the presence of the challenge strain in the URT. The same protocol was followed in other experiments unless stated otherwise.
the question of the antigen specificity of the protection observed. Before proceeding to assess this question in DO11.10 RAG−/− mice, we verified that *S. pneumoniae* strains expressing OVA323–339 were still protective in normal mice. WCV was made from strain Rx1ΔlytA (WCV-OVA1), a variant of Rx1ΔlytA expressing the OVA323–339 peptide as a fusion protein with pneumococcal surface protein A (PspA-OVA). WCV-OVA1 proved to be protective in immunocompetent BALB/c mice (Fig. 1B).

**Induction of CD4+ T-cell-mediated immunity is antigen specific.** Immunization with WCV-OVA1 protected DO11.10 RAG−/− mice against subsequent colonization with the unrelated *S. pneumoniae* strain 603OVA1 expressing PspA-OVA (Fig. 2). The level of colonization in the group of 11 animals immunized with WCV-OVA1 (median log10 of CFU recovered per nasal wash, 2.67; interquartile range, 2.47 to 3.48) was significantly lower than the levels in groups of 12 animals either vaccinated with WCV made of the OVA-negative variant of Rx1ΔlytA (3.84; 3.53 to 4.14 [P = 0.0006]) or immunized with CT alone (3.67; 3.51 to 3.96 [P = 0.0035]). These results show that the induction of CD4+ T-cell-mediated immunity to pneumococcal colonization was antigen specific.

**Recall responses to WCV-OVA1 are antigen specific.** Immunization of DO11.10 RAG−/− mice with WCV-OVA1 had no detectable effect on subsequent colonization with the non-OVA-expressing strain 603S (Fig. 2). The number of 603S bacteria recovered from the URT of animals immunized with WCV-OVA1 was nearly identical to the number of cells recovered from mice immunized with CT alone (3.76; 3.42 to 4.26 versus 3.88; 3.18 to 4.25 [P = 0.88]). This result confirmed that recall of the response observed in the previous experiment also requires antigen-specific CD4+ T-cell stimulation. Altogether, the expression of vaccine-induced T-cell immunity in these mice which can only express T-cell immunity to OVA323–339 peptide depends upon the presence of OVA in both the vaccine and the colonizing bacteria.

**RESULTS**

**Protection by low doses of WCV and by WCV-OVA1 in normal mice.** Previously we showed that immunization of immunocompetent mice with intranasally applied vaccine made of ethanol-killed whole cells of Rx1ΔlytA, an autolysin (LytA)-negative mutant of Rx1 (100 µg of the vaccine, dry weight, equivalent to 10^8 CFU per dose), and CT mucosal adjuvant protected animals against subsequent colonization by heterologous pneumococcal strains (17, 19). In the present study, we observed similar protection after immunization with a dose of WCV 100× lower than that previously used (Fig. 1A), indicating that a vaccine containing a small amount of antigen is sufficient to stimulate efficient mucosal immunity. This raised the question of the antigen specificity of the protection observed. Before proceeding to assess this question in DO11.10 RAG−/− mice, we verified that *S. pneumoniae* strains expressing OVA323–339 were still protective in normal mice. WCV was made from strain Rx1ΔlytA (WCV-OVA1), a variant of Rx1ΔlytA expressing the OVA323–339 peptide as a fusion protein with pneumococcal surface protein A (PspA-OVA). WCV-OVA1 proved to be protective in immunocompetent BALB/c mice (Fig. 1B).

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**FIG. 2.** Comparison of the density of intranasal colonization by *S. pneumoniae* strains 603OVA1 and 603S in DO11.10 RAG−/− mice after immunization with CT alone (CT), WCV made of the Rx1ΔlytA strain (WCV), or WCV made of Rx1ΔlytA-OVA1 (WCV-OVA1) as indicated below the x axis. The number of animals in each group is depicted at the bottom. Solid lines indicate group medians. P values refer to results of the Mann-Whitney test for differences in the distribution of CFU per nasal wash between groups.
Next, we evaluated whether the effector action was limited to antigen-bearing bacteria. We found that the protective response can be seen in trans by exposure to the challenge strain without OVA applied at the time of challenge, prior to the induction of Th17 cells in response to the conserved cell wall polysaccharide antigen of pneumococcus. Thus, it is tempting to speculate that the in trans effect observed here reflects the recruitment and activation of neutrophils by Th17 cells following antigen-specific stimulation and that these neutrophils kill pneumococci regardless of whether they bear the relevant antigen.

Our experiments show that S. pneumoniae is highly susceptible to this nonspecific effector response. It remains to be seen whether these same mechanisms of immunity could also impact colonization by other respiratory pathogens. In this regard, Lysenko et al. (15) described a key role of complement-dependent phagocytic killing of pneumococci by neutrophils in a mouse carriage model in which neutrophil-like cells were recruited and activated, not by pneumococcal proteins but by surface-covalently applied OVA (WCA). These two additional OVA fusions (with NanA and Ply) were introduced to increase the opportunity for peptide presentation at the time of immunization and to induce stronger responses at the time of challenge. After immunization with WCV made of Rx1ΔlytA-OVA3 cells (WCV-OVA3), the colonization of DO11.10 Rag-/- mice by strain 603S was significantly lower (P = 0.0002) in the group stimulated at the time of challenge with dead cells of strain Rx1ΔlytA-OVA3 (2.90; 2.29 to 3.56) than in the group stimulated with killed cells of the OVA-negative variant of the same strain (4.04; 3.88 to 4.12), a result similar to that observed with soluble ovalbumin as a stimulating factor (Fig. 3).

**DISCUSSION**

The existing pediatric vaccine against S. pneumoniae—the 7-valent pneumococcal conjugate vaccine—has been remarkably successful in preventing disease in vaccinated children and also has reduced colonization in these children and thereby protected unvaccinated individuals against pneumococcal disease (5). Indeed, it is estimated that these indirect (herd immunity) effects have prevented more than twice as many cases as have been directly prevented in vaccinated persons (5). As second- and later-generation vaccines are developed, with the goals of increasing the number of pneumococcal serotypes covered and reducing vaccine cost, a better understanding of mechanisms of immunity to colonization could lead to the development of novel strategies against this pathogen and, potentially, against other nasopharyngeal pathogens. Previous work by our group and others (19, 31, 33) demonstrated the critical role of CD4+ T cells in resistance to pneumococcal carriage. Our initial observation (Fig. 1) that very low doses of a killed WCV could confer immune protection, combined with observations by others of nonspecific roles for CD4+ T cells recruited by pneumococci (10), raised the possibility that protection by this strategy may be nonspecific. Using DO11.10/Rag-/- mice, we found that both the induction and recall of the protective response require specific antigen seen by the Th17 subset of CD4+ T cells but that clearance of pneumococci, once set in motion by specific antigen, could work in trans against pneumococci not expressing the antigen. Elsewhere, we have shown that the Th17 subset of CD4+ T-helper cells is critical to this protective activity and that neutrophil-like cells are also required (Y. J. Lu, J. Gross, D. Bogaert, A. Finn, L. Bagrade, Z. Qibo, J. Kolls, A. Srivastava, A. Lundgren, S. Forte, C. M. Thompson, K. F. Harney, P. W. Anderson, M. Lipsitch, and R. Malley, submitted for publication), consistent with our previous findings on the importance of Th17 cells in response to the conserved cell wall polysaccharide antigen of pneumococcus (18) and to pneumococcal proteins (2). Thus, it is tempting to speculate that the in trans effect observed here reflects the recruitment and activation of neutrophils by Th17 cells following antigen-specific stimulation and that these neutrophils kill pneumococci regardless of whether they bear the relevant antigen.
possible in principle that the type of immunity elicited by immunization with WCV expressing OVA is particularly effective at clearing pneumococci from the respiratory tree but may not be as potent against other organisms. If there are other organisms in the respiratory tree that are similarly susceptible to the action of these clearance mechanisms, then the action observed in trans against non-antigen-bearing pneumococci might also serve as a mechanism of interspecific bacterial antagonism, mediated by antigen-specific responses.

The approach described here, in which a whole organism is used in transgenic mice to stimulate an immune response to a defined antigen, may provide a useful basis for future work to understand the mechanisms by which whole organisms can be more immunogenic than individual, soluble moieties (6). By separating the antigenic function from possible adjuvant or other effects of the whole organism on the immune system, it should be possible to elucidate the identity and effect of other, immunogenicity-affecting components of the whole organism.

There is growing evidence that various naturally acquired mechanisms of immunity to pneumococcal carriage and infection are antibody independent. Rosseau et al. (27), in a comparative study on transcriptional profiling of the mouse lung during pneumococcal pneumonia, revealed a simultaneous down-regulation of B-cell-mediated responses and increase in the expression of T-cell-specific genes during inflammation caused by S. pneumoniae. The inflammatory responses were dominated by interleukin-1 (IL-1)-family cytokines, IL-6, and tumor necrosis factor alpha. Interestingly, there was also significant up-regulation of IL-17 expression (27). In a study of elderly adults with chronic obstructive pulmonary disease, we have shown that systemic antipneumococcal antibodies did not predict resistance to the acquisition of a new pneumococcal strain, suggesting that other mechanisms, possibly including T cells, may be responsible (16). In a recent study, significantly lower proliferative and cytokine peripheral blood T-cell responses to pneumolysin were observed in children who were colonized with S. pneumoniae than in noncolonized children, raising the intriguing hypothesis that T-cell responses to this antigen may be associated with increased resistance to pneumococcal colonization (35).

In conclusion, we have shown that CD4+ T-cell-mediated WCV-induced protection against pneumococcal colonization is antigen specific and can be triggered even in the absence of antigen-bearing pneumococci, provided that the target antigen is present at the time of challenge. While many aspects of the immune response to this antigen remain to be analyzed, these data provide additional support for a strategy based on mucosal immunization with a killed pneumococcal antigen.

ACKNOWLEDGMENTS

We thank Jyothi Rengarajan, Barry Bloom, and Porter W. Anderson for helpful discussions during the course of this work, Susan Hollinghead for providing anti-PspA serum, and Donald A. Morrison for supporting the project. We thank Jyothi Rengarajan, Barry Bloom, and Porter W. Anderson for helpful discussions during the course of this work, Susan Hollinghead for providing anti-PspA serum, and Donald A. Morrison for supporting the project.

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