Streptococcus pneumoniae Pilus Subunits Protect Mice against Lethal Challenge

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Streptococcus pneumoniae is a major public health threat worldwide. The recent discovery that this pathogen possesses pili led us to investigate their protective abilities in a mouse model of intraperitoneal infection. Both active and passive immunization with recombinant pilus subunits afforded protection against lethal challenge with the S. pneumoniae serotype 4 strain TIGR4.

Streptococcus pneumoniae causes severe diseases, including pneumonia, meningitis, and acute otitis media. The World Health Organization estimates that each year, 1.6 million people die from pneumococcal diseases; most of these deaths occur in young children in developing countries (8). Due to the increasing resistance to antibiotics (6), vaccination represents the most effective strategy for preventing S. pneumoniae infection. The current 23-valent polysaccharide vaccine is not effective in children under 2 years of age, while the 7-valent conjugate vaccine is effective, but its limited strain coverage can favor serotype replacement (3, 4, 13). Current research focuses on protein antigens as potential vaccine candidates able to elicit serotype-independent protection (2, 11).

The recent discovery that gram-positive pathogens possess pili (12, 14) has opened a new area of research into their function in pathogenesis and their role as protective antigens. More recently, pili were also discovered in S. pneumoniae (1, 7). Pneumococcal pili, which are present in some but not all clinical isolates (10), are encoded by the rrlrA islet, which includes the genes for the three pilus subunits (RrgA, RrgB, and RrgC) (1, 5). The recent finding that pneumococcal pilin contributes to adherence and virulence and elicits host inflammatory responses (1), together with the preliminary observation that serum antibodies against pilus antigens are detectable in patients diagnosed with pneumococcal diseases (unpublished data), led us to investigate their potential use as vaccine candidates.

Pilus subunits are immunogenic in mice. His-tagged, recombinant pilus subunits RrgA, RrgB, and RrgC (molecular masses: 93, 66, and 40 kDa, respectively), corresponding to the sequence of the S. pneumoniae serotype 4 TIGR4 strain, were expressed in Escherichia coli and purified in soluble form by affinity chromatography on His-Trap high-performance columns (GE Healthcare).

Animal experiments were done in compliance with the current law. Six-week-old, specific-pathogen-free female BALB/c mice (Charles River) were immunized intraperitoneally on days 0, 14, and 28 with heat-inactivated TIGR4 (10⁸ CFU); with recombinant RrgA, RrgB, and RrgC (20 μg); or with the combination RrgA-RrgB-RrgC (10 μg each), along with Freund’s adjuvant. Controls received identical courses of saline plus adjuvant.

Immunoglobulin G (IgG) antibodies were quantified by an enzyme-linked immunosorbent assay on sera obtained after the third immunization. Serial dilutions of sera were dispensed in Maxisorp 96-well plates (Nalge Nunc International) coated with recombinant RrgA, RrgB, or RrgC (0.2 μg/well). Antibody binding was detected by alkaline phosphatase-conjugated anti-human (Sigma) or anti-mouse (Southern Biotechnology Association) IgG, followed by the substrate p-nitrophenyl-phosphate (Sigma). Absorbance was measured at 405 nm. Sera were titrated by comparison with

FIG. 1. Immunogenicity of pilus subunits in mice. Enzyme-linked immunosorbent assay quantification of specific IgG titers against recombinant RrgA, RrgB, or RrgC in sera of immunized mice is indicated. Eight mice were used for each group, with the exception of the control (ctrl) group, in which 16 mice were used. P was <0.001 (one-tailed Mann-Whitney U test) for each immunized group for comparison to the corresponding control. Columns represent the means for the groups. A+B+C, the combination RrgA-RrgB-RrgC; bars, standard deviations.

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the curves obtained with reference sera, using a reference line calculation program. Reference sera consisted of pooled mouse anti-RrgA, -RrgB, or -RrgC sera, to which the titer of 50,000 was assigned.

Mice vaccinated with heat-inactivated TIGR4, containing native pilus structures, generated serum antibodies able to detect the recombinant pilus antigens. The most evident response was against RrgB (Fig. 1), possibly due to the fact that RrgB is the most abundant subunit in the native pilus, constituting its backbone (1). Serum IgG response was also quantified in mice immunized with recombinant pilus subunits. Individual recombinant pilus antigens (20 μg each) elicited high IgG responses (Fig. 2), with sera becoming titrable at a 1:50,000 dilution. Immunization with the combination RrgA-RrgB-RrgC also elicited high IgG levels against each of the three antigens, with titers slightly reduced, consistently with the lower antigen dose used (10 μg each) (Fig. 1).

Immunization with recombinant pilus antigens is protective in mice. Mice were immunized intraperitoneally as described above and then challenged intraperitoneally with 10^6 CFU of

FIG. 2. Protective efficacies of pilus subunits in mice. The protective efficacies against TIGR4 challenge of either active vaccination with the indicated immunogens (active immunization) or passive transfer of the indicated antisera (passive immunization) are shown. Eight mice were used for each group, with the exception of the control groups in the “Freund’s adjuvant” and “passive immunization” panels, in which 16 mice were used, and the RrgA-RrgB-RrgC (A+B+C) group in the “high-dose challenge” panel, in which 5 mice were used. (A) Bacteremia at 24 h postchallenge. Circles indicate numbers of CFU per ml of blood for single animals; horizontal bars indicate the geometric mean for each group; the dashed line indicates the detection limit (i.e., no CFU were detected in blood samples at levels below the dashed line). (B) Mortality course. Diamonds indicate survival times in days for single animals; horizontal bars indicate the median survival time for each group; the dashed line indicates the endpoint of observation (i.e., animals with survival times above the dashed line survived at the endpoint); ctrl indicates mice receiving only the corresponding adjuvant plus saline; * and ** indicate P values of <0.05 and <0.01, respectively (one-tailed Mann-Whitney U test), for comparison with the corresponding control groups.
TIGR4 per mouse, a dose previously observed to correspond to a 100% lethal dose in naïve or Al(OH)$_3$-treated mice and to a 50% lethal dose in Freund’s adjuvant-treated mice. As shown in Fig. 2, control animals had a geometric mean of $>10^5$ CFU/ml, including 7 mice with $>10^4$ CFU/ml and 5 mice with undetectable bacteremia (Fig. 2A); 9/16 mice did not survive at 10 days (Fig. 2B). In marked contrast, bacteremia was undetectable in mice vaccinated with heat-inactivated TIGR4 (Fig. 2A), and all mice of this group were alive at 10 days (Fig. 2B). All groups vaccinated with recombinant pilus antigens showed lower bacteremia levels and higher survival rates than controls. Immunization with RrgB resulted in only one of eight mice becoming bacteremic and all mice surviving at the endpoint. RrgA or the combination RrgA-RrgB-RrgC also afforded protection, with only one of eight mice becoming bacteremic and seven of eight mice surviving challenge in each group. In the group immunized with RrgC, three of eight mice were bacteremic and did not survive. The bacteremia levels and survival rates for the groups immunized with pilus antigens were not statistically different ($P > 0.1$) from those for the group vaccinated with heat-inactivated TIGR4. The combination RrgA-RrgB-RrgC showed a similar protective efficacy when Freund’s adjuvant was replaced by Al(OH)$_3$ and the amount of each antigen was reduced to 1 μg each [Fig. 2, “Al(OH)$_3$” panels]. Moreover, immunization with the combination RrgA-RrgB-RrgC (10 μg each) along with Freund’s adjuvant was also found to be protective against challenge with 3,800 CFU of TIGR4 (Fig. 2, “high-dose challenge” panels): eight of eight control mice were bacteremic and died within 3 days postchallenge, while three of five immunized mice were not bacteremic and survived.

Passive transfer of sera to recombinant pilus antigens is protective in mice. In order to further investigate whether the protective efficacies of pilus subunits are antibody dependent, we tested mouse antisera raised against recombinant pilus antigens for their protective abilities by passive serum transfer. For this purpose, 10-week-old mice received 50 μl of immune serum intraperitoneally 15 min before challenge with $10^6$ CFU of TIGR4. As shown in Fig. 2A, at 24 h postchallenge, controls presented a geometric mean of $>10^3$ bacteria per ml of blood, with 10/16 mice having $>10^5$ CFU/ml, one mouse having $<10^3$ CFU/ml, and 5 mice having undetectable bacteremia. At 10 days postchallenge, 8/16 control mice were still alive (Fig. 2B). All eight mice receiving anti-TIGR4 serum were not bacteremic and survived at 10 days (Fig. 2B). The results were similar to those obtained with active immunization: all groups receiving antisera against recombinant pilus antigens showed reduced bacteremia levels and increased survival rates compared to the control group. The passive transfer of anti-RrgA-RrgB-RrgC serum resulted in undetectable bacteremia at 24 h (Fig. 2A) and survival at the endpoint (Fig. 2B) for all eight mice. Moreover, after passive transfer of either anti-RrgA or anti-RrgB serum, only one or two mice, respectively, were found bacteremic at 24 h postchallenge (Fig. 2A), and eight of eight mice in each group survived lethal challenge (Fig. 2B). Passive transfer of anti-RrgC serum resulted in five of eight mice having no detectable bacteremia (Fig. 2A) and seven of eight mice surviving (Fig. 2B). Bacteremia levels and survival rates in groups receiving antisera to pilus antigens were not statistically different ($P > 0.1$) from those in the group that received anti-TIGR4 antiserum. These results indicate that antibodies play a relevant role in the protection elicited by pilus subunits.

We aimed at expanding the results obtained with TIGR4 pilus antigens to a different S. pneumoniae serotype. Thus, the recombinant pilus antigens RrgA$_{6B}$, RrgB$_{6B}$, and RrgC$_{6B}$ specific for the 6B serotype were expressed and purified. The 6B pilus subunits share 83% (RrgA), 47% (RrgB), and 99% (RrgC) amino acid identity with the corresponding TIGR4 pilus subunits (unpublished data). Passive transfer of mouse immune serum raised against the combination RrgA$_{6B}$-RrgB$_{6B}$-RrgC$_{6B}$ was able to protect mice against heterologous challenge with $10^2$ CFU of TIGR4. None of the eight mice receiving anti-RrgA$_{6B}$-RrgB$_{6B}$-RrgC$_{6B}$ antiserum were bacteremic at 24 h postchallenge, and all were still alive at 10 days (Fig. 2). This preliminary result suggests the possible cross-protective abilities of pilus subunits against different S. pneumoniae serotypes.

In conclusion, this report provides evidence that the recombinant subunits of the recently discovered S. pneumoniae pilus are novel protective antigens. Pilolated S. pneumoniae strains constitute a subset of pneumococcal strains (10); however, studies are ongoing to better define the percentage of pilolated pneumococci among the currently circulating strains, their geographic distribution, their role in infection and pathogenesis, and their possible association with severe pneumococcal diseases. Even though pilus antigens could afford protection against a particular group of S. pneumoniae strains, a combination of one or more pilus subunits with other protective antigens could lead to an effective multiprotein vaccine against S. pneumoniae, following a strategy similar to that successfully exploited for group B Streptococcus (9).

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