Immune Response to Capsular Polysaccharide and Surface Proteins of *Streptococcus pneumoniae* in Patients with Invasive Pneumococcal Disease

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The immune response to pneumococcal capsular polysaccharides (CPSs) and to the pneumococcal surface proteins cell wall–associated serine proteinase A (PrtA), pneumococcal surface protein A (PspA), and *Streptococcus pneumoniae* pullulanase A was evaluated in 45 patients with invasive pneumococcal disease compared with healthy adults. In serum from patients with meningitis and pneumonia, CPS antibody levels were low, compared with healthy adults; antibody levels did not differ between groups and did not change between phases. Levels of immunoglobulin G directed against the pneumococcal surface proteins in patients with invasive pneumococcal disease were in the same range as in healthy adults. However, median PrtA and PspA antibody levels tended to increase during early convalescent phase. Low levels of CPS antibody, rather than of antibodies directed against the pneumococcal surface proteins, may predispose to invasive pneumococcal infection.

*Streptococcus pneumoniae* is one of the most important bacterial pathogens responsible for respiratory and invasive infections. Invasive pneumococcal disease is common in young children, the elderly, and immunocompromised patients. To prevent life-threatening pneumococcal infections, vaccination of patients at risk has been recommended. In the past, a vaccine composed of the 23 most frequent serotypes of pneumococcal capsular polysaccharides (CPSs) was used. However, this vaccine was poorly immunogenic in children aged <2 years and immunocompromised patients [1]. Conjugation of pneumococcal polysaccharides to carrier proteins improved immune response and has been successfully introduced into clinical practice [2]. However, after intense use of these efficient vaccines, a shift to infections with pneumococci encapsulated with polysaccharides other than those included into the vaccine might occur. Pneumococcal proteins, especially surface proteins, have been considered for the development of a protein-based pneumococcal vaccine.

Antibodies not only directed against the cell-wall polysaccharides of pneumococci but also with specificity to pneumococcal surface proteins are produced naturally. During the first 4 years of life, children develop anti-protein antibodies in response to pneumococcal colonization and infection [3]. Therefore, efforts have been made to identify immunogenic pneumococcal proteins that could serve as the basis of a protein-based vaccine. In animal models, pneumococcal proteins elicited a protective immune response [4]. Preliminary studies have been conducted using recombinant pneumococcal surface protein A (PspA) for the immunization of humans. PspA has been demonstrated to be immunogenic, and human immune serum protected mice from pneumococcal infection [5].

Recently, we described pneumococcal surface proteins that were immunogenic in an adult patient with bacteremic pneumococcal meningitis [6]. Of the newly discovered proteins, we found cell wall–associated serine proteinase A (PrtA) and *S. pneumoniae* pullulanase A (SpuA) to be present in the pneumococcal population with limited sequence variability and expressed continuously at the bacterial surface in vitro; we also found that PrtA contributes to pneumococcal virulence [7, 8]. In the present study, we investigated the immune response to these pneumococcal surface proteins to the well-characterized surface protein PspA and to pneumococcal CPSs in patients with invasive pneumococcal disease.

**Methods.** The study population consisted of 45 patients. Invasive pneumococcal infections were confirmed by microbiological methods in the case of bacteremic pneumococcal pneumonia (*n* = 20) and meningitis (*n* = 25). Serum samples were obtained from the patients 0–6 (acute phase, *n* = 21), 7–50 (early convalescent phase, *n* = 27), and >100 (late convalescent phase, *n* = 23) days after first isolation of the pneumococcal strain.
mococcus. For comparison, 62 serum samples from healthy adults (blood donors) were used.

The levels of IgG antibodies to Pneumovax 23 (Aventis Pasteur) in serum were determined by an ELISA. The content of each of 5 vials of Pneumovax 23 (0.5 mL containing 23 25-μg portions of the CPS corresponding to the 23 pneumococcal types) was added to 1.5 mL 0.01 M NaOH and mixed immediately. The solution was transferred to a tube containing 6.5 mg of cyanuric chloride (Sigma-Aldrich) and mixed gently, with continuous monitoring of the pH. After 5 min at pH 8.3, the solution was transferred to a tube containing a solution of 0.3 mg poly-L-lysine in 0.3 mL of 0.05 M Tris buffer (pH 8.1). The solution was dialyzed against PBS (pH 7.4), diluted to 100 mL with PBS (pH 7.4), dispensed in 100-μL samples onto microtiter plates (Maxisorp; Nunc), and incubated for 24 h at room temperature. The plates were washed 3 times with distilled water and incubated for 10 min with PBS (pH 7.4; containing 0.05% Tween 20 and 0.5% NaCl). The washing buffer was removed, and the plates were dried at room temperature and stored at 4°C. Serum samples were stored at −20°C until use. Serial 5-fold dilutions ranging from 1:25 to 1:3125 were prepared in PBS. The dilutions were dispensed into the wells and incubated for 2 h at 37°C. After washing, 0.1 mL of a 1:4000 dilution of alkaline phosphatase–conjugated goat anti–human IgG (Sigma-Aldrich) was added to each well, and the plates were incubated at 4°C overnight. After washing, 100 μL of the substrate solution (p-nitrophenylphosphate [1 mg/mL] in 0.05 M sodium carbonate buffer [pH 9.6] that contained 0.001 M magnesium chloride) was added to each well, and the plates were incubated for 1 h at 37°C. The reaction was stopped by the addition of 50 μL of 1 M NaOH, and the absorbance of the substrate was measured at 414 nm in a multichannel photometer. The antibody titer was calculated according to the method of Caulfield and Shaffer [9]. For each run, a reference serum was additionally measured, to avoid interassay variation. If the measured value for the reference serum was outside the consensus value, the results for the tested serum samples were discarded, and the ELISA was repeated.

The aminoterminal part of PrtA (SP-15; accession no. AF127143, nt 330–1041), the central part of PspA (SP-52, highest sequence homology to accession no. AF071807, nt 306–864), and the aminoterminal part of SpuA (SP-21; accession no. AF217414, nt 354–1629), which had been identified as immunogenic in the aminoterminal part of SpuA (SP-21; accession no. AF217414, nt 354–1629), which had been identified as immunogenic in human invasive pneumococcal disease [6], were purified and chromatographically using Ni-NTA agarose. Wells of microtiter plates were coated with 50 μL purified protein (0.01 μg/mL) overnight at 4°C. The solution was replaced by 200 μL PBS containing 5% skim milk and 10% goat serum for 1 h at room temperature. After washing, human serum samples were added to the wells. Serial dilutions of 1:100 to 1:900 were used. A reference serum was placed on each microtiter plate at dilutions of 1:100, 1:300, and 1:2700 in triplicate. Diluted serum samples were added to the wells in 50-μL volumes. The plates were incubated at room temperature for 1 h. After washing, 50 μL of a 1:5000 dilution of alkaline phosphatase-conjugated goat anti–human IgG was added to the wells and incubated for 1 h at room temperature. As substrate, 200 μL p-nitrophenylphosphate (1 mg/mL) in 100 mM Tris-HCl (pH 9.5, 100 mM NaCl, and 5 mM MgCl2) was added. The development of the color reaction was stopped by adding 50 μL of 10 mM EDTA (pH 8.0). The optical density was measured at 405 nm. The background was subtracted, and the means of triplicates were given in relation to the reference values. If the measured value for the reference serum was outside the consensus value, the results for the tested serum samples were discarded, and the ELISA was repeated.

Data were compared by Kruskal-Wallis nonparametric analysis of variance and Dunn’s multiple comparisons test for P value adjustment because of repeated testing.

Results. Serum samples from 45 patients with invasive pneumococcal infections were included in the study. The patients were grouped by clinical syndrome: bacteremic pneumococcal meningitis ( ) or pneumococcal pneumonia ( ). Serum samples were lower than those from healthy control subjects (HCs; *P<.001; **P<.05). M, pneumococcal meningitis; P, bacteremic pneumococcal pneumonia.
female ratio, 12:13). Twelve patients with meningitis and 8 patients with bacteremic pneumonia had concomitant diseases (diabetes mellitus, hematologic disease, splenectomy, AIDS, IgG deficiency, alcoholism, and premature infancy). As a control, 62 serum samples from healthy adults were used. The mean age of the control subjects was 35 years (range, 20–58 years).

An ELISA measuring IgG directed against the 23-valent pneumococcal polysaccharide vaccine antigen preparation (Pneumovax 23) was used. The median of the CPS antibody titers in healthy control subjects were 276.5; the concentrations varied slightly between age groups without reaching statistical significance. CPS antibody concentrations of healthy control subjects was significantly higher than those of patients with invasive pneumococcal disease. In most serum samples from patients with meningitis, the antibody titer was below the detection limit of 25. The CPS antibody titers in serum samples from patients with pneumonia did not differ significantly from those in samples from patients with meningitis. No distinct differences were measured between serum samples drawn during the acute, early, or late convalescent phases (figure 1). Between patients with and without known concomitant diseases, no difference in antibody titers was observed.

Recombinant polypeptides of the aminoterminal part of PrtA [7], the central part of PspA [10], and the aminoterminal part of SpuA [8] were used as antigens for ELISA. Antibodies directed against these 3 surface proteins were measured from a series of acute, early, and late convalescent-phase serum samples obtained from patients with invasive pneumococcal disease and from healthy adults. PrtA antibody titers in patients with meningitis and pneumonia were in the same range as titers measured in healthy adults (figure 2A). No significant changes during the acute and convalescent phases were observed. The tendency of higher anti-PrtA levels in patients with pneumonia than in those with meningitis was apparent, as was a trend toward higher antibody levels during the early convalescent phase. The medians of the SpuA antibody titers were below the detection limit in serum samples from patients with both meningitis and pneumonia. In 30% of the serum samples from healthy adults, no anti-SpuA IgG was detectable (figure 2B). Therefore, no significant differences between serum samples of patients and healthy adults were detected. PspA antibody titers in patients did not change significantly after invasive pneumococcal infection. The titers were all within the same range as those in healthy adults (figure 2C). The median values of PrtA and PspA antibody titers tended to increase during the early convalescent phase; during the late convalescent phase, the median values decreased (not statistically significantly different). Patients with known concomitant diseases did not have lower antibody titers than those without known immunosuppression.

**Discussion.** The immune response to pneumococcal protein antigens develops during early childhood. Antibodies to pneumococcal CPS increased with age in young children. The development of anti-pneumococcal antibodies was associated with pneumococcal colonization and local pneumococcal infection: Therefore, in healthy adults, high antibody concentrations were measured [3, 11, 12]. However, the presence of antibodies does not necessarily mean that these antibodies can protect an individual from infection.
In our study, we also found high antibody titers to CPS in healthy adults. High antibody concentrations to the pneumococcal surface proteins PrtA and PspA were present in most of these serum samples, whereas, in nearly half of the serum samples, no detectable amount of anti-SpuA antibodies were measured. Because the concentration of anti-PspA antibodies seemed to correlate with invasive pneumococcal disease in children [12], and seroconversion to a 22-kD protein similar to the N-terminal region of PspA after pneumococcal colonization in healthy adults was described by McCool et al. [13], the presence of anti-PspA antibodies seemed to protect against invasive pneumococcal infection. The protein fragments of PrtA and SpuA that were used for ELISA are highly conserved, as demonstrated elsewhere [7, 8], whereas PspA epitopes were highly variable [5]. Because we measured the PspA antibody response to only one PspA type, the antibody levels to different PspA clades might differ from the demonstrated results. However, our results do not confirm the hypothesis that significant levels of antibody to the pneumococcal proteins PsPA, PrtA, and SpuA develop during invasive pneumococcal disease or that patients with invasive pneumococcal disease were at risk because of low titers of antibody to one of these pneumococcal proteins. During the early convalescent phase, a tendency toward elevated anti-protein IgG titers was demonstrated, which might reflect a transient immunologic activation. Elevated titers did not persist during the late convalescent phase. The presence of anti-pneumococcal antibodies in serum samples from healthy adults demonstrated that the immune system had been stimulated by these antigens in the past.

However, anti-CPS levels were significantly lower among patients with invasive pneumococcal pneumonia and meningitis than in the healthy population. Titers of anti-CPS antibodies did not change during convalescent phase, even though the polysaccharide serotypes of >80% of the strains causing these infections were included in the CPS antigen preparation. Patients predisposed to develop invasive pneumococcal infection appear to lack a immune response to CPS antigens, as has been described elsewhere [14]: in bacteremic pneumococcal pneumonia, the opsonic indices did not rise during the convalescent phase, whereas, in patients with nonbacteremic pneumococcal pneumonia, opsonic indices increased. The major risk factor for development of invasive pneumococcal infection seemed to be the lack of functional anti-CPS antibodies [15]. In summary, independent of the presence of antibodies to pneumococcal proteins, the absence of CPS antibodies is the major risk factor for development of invasive pneumococcal infection.

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References