Quantitative Relationship between Anticapsular Antibody Measured by Enzyme-Linked Immunosorbent Assay or Radioimmunooassay and Protection of Mice against Challenge with Streptococcus pneumoniae Serotype 4

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We have recently shown that a substantial proportion of antibody to pneumococcal polysaccharide as measured by enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay is removed by adsorption with pneumococcal cell wall polysaccharide (CWPS). The present study was undertaken to validate the hypothesis that only serotype-specific antibody that remains after adsorption with CWPS provides protection against pneumococcal infection. Serum samples were obtained from human subjects before and after they had been vaccinated with pneumococcal polysaccharide vaccine. Antibody to Streptococcus pneumoniae serotype 4 was measured by ELISA without adsorption or after adsorption of serum with CWPS. Groups of mice were injected with graded doses of serum and then challenged intraperitoneally with 10, 100, or 1,000 50% lethal doses (LD₅₀) of S. pneumoniae serotype 4. Without adsorption, prevaccination sera from five healthy adults appeared to contain up to 33 µg of antibody to S. pneumoniae serotype 4 antigen per ml; adsorption with CWPS removed all detectable antibody, and pretreating mice with up to 0.1 ml of these sera (<3.3 µg of antibody) failed to protect them against challenge with 100 LD₅₀. In contrast, postvaccination sera contained 2.9 to 30 µg of antibody per ml that was not removed by adsorption. Diluting sera to administer desired amounts of serotype-specific immunoglobulin G showed a significant relationship between protection and antibody remaining after adsorption (P < 0.05 by linear regression analysis); 150 ng was uniformly protective against 1,000 LD₅₀ and 50 ng was protective against 100 LD₅₀. These studies have, for the first time, quantitated the amount of serotype-specific antibody that protects mice against challenge with S. pneumoniae type 4. In light of these observations, it is necessary to reassess current concepts regarding the presence of antipneumococcal antibody in the unvaccinated population, responses to pneumococcal vaccination, and protective levels of immunoglobulin G.

There is little disagreement that humoral factors, specifically immunoglobulin G (IgG) antibody reactive with capsular polysaccharide, provide the strongest degree of immunity to infection with Streptococcus pneumoniae (1, 9, 11). Radioimmunoassay (RIA) has been used widely in epidemiological and clinical studies (22) and has been thought to accurately measure levels of anticapsular antibody (23). In the course of earlier studies, however, we (19) found poor correlation between antibody levels as measured by RIA and the opsonizing capacity of serum for nine of the serotypes of S. pneumoniae that are included in the currently available 23-valent pneumococcal vaccines. We also showed, as had earlier investigators (26), that antigenic preparations used to vaccinate human subjects or to measure antibody responses contain various amounts of noncapsular bacterial constituents. More recently, we (20) and others (17, 24) have demonstrated that a substantial proportion of antibody measured by enzyme-linked immunosorbent assay (ELISA) or RIA and regarded as being directed against the capsule can be removed by adsorption of serum with unencapsulated mutants of S. pneumoniae or isolated pneumococcal cell wall polysaccharide (CWPS). Our findings have suggested that much of the antipneumococcal antibody that has been regarded as serotype specific is, in fact, reactive with CWPS and may provide little or no protection.

To support the relevance of these findings, it is necessary to show that the protective effect of serum is directly proportional to levels of antibody that we measure by ELISA. In this study, we examined the capacity of sera obtained from healthy adults before and after vaccination to protect mice against challenge with S. pneumoniae serotype 4 and related the degree of protection to the level of serotype-specific IgG. Our results show that (i) sera from normal, unvaccinated young adults generally does not contain IgG antibody to capsular constituents of S. pneumoniae serotype 4, (ii) antibody in normal serum that reacts in RIA or ELISA with serotype 4 antigen but is removed by adsorption with CWPS is not protective, and (iii) vaccination induces IgG antibody that is not removed by adsorption with CWPS and protects mice against pneumococcal challenge.

MATERIALS AND METHODS

Bacteria. S. pneumoniae serotype 4 was obtained from the American Type Culture Collection (Rockville, Md.). After passage in mice, the number of organisms that killed 50% of mice (LD₅₀) was found to be 2 to 5 CFU. This serotype was selected because it is lethal for mice and because large differences in IgG reactive with type 4 antigen were observed before versus after adsorption in our earlier study (20). In one recent survey (14), this was also the most common serotype causing invasive disease in adults. Unencapsulated mutants Rx and R6, originally derived from S.
pneumoniae serotype 2 (30), were provided by Donald Morrison, University of Illinois, Chicago. Strain CSR SCS-2 (CSR), an unencapsulated mutant of S. pneumoniae serotype 2 that produces large amounts of cell wall (21), was provided by Uffe Sorensen, National Institutes of Health, Bethesda, Md. S. pneumoniae DW3.8 is an unencapsulated strain produced in our laboratory as a result of a single transposon insertion into the genome of S. pneumoniae serotype 3 (32). All pneumococci were cultured in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.5% yeast extract (Becton Dickinson, Cockeysville, Md.).

**Bacterial antigens.** CWPS purified from strain CSR was provided by Praxis Biologics, Rochester, N.Y. This material does not remove antibody to unrelated antigens, including pneumococcal capsular polysaccharide (20, 24; Donna Phipps, Praxis Laboratories, personal communication). S. pneumoniae serotype 4 capsule polysaccharide antigen (lot 75668) was obtained from the American Type Culture Collection. We previously reported that CWPS comprises about 0.5% of this type 4 capsule preparation (19); this concentration of CWPS antigen yields ELISA readings of 0.3 to 0.4 optical density units with sera from most healthy young adults, thus explaining how normal, prevaccination serum might appear to contain substantial levels of anticapsular antibody (20).

**Sera.** Sera were obtained before or 4 to 6 weeks after vaccination of healthy young adults (age 24 to 34) with 23-valent pneumococcal vaccine (Pneumovax [Merck, Sharp & Dohme, West Point, Pa.] or Pnu-Immune 23 [Lederle Laboratories, Pearl River, N.Y.]). Serum was separated from clotted blood by centrifugation and stored in 0.5- to 1-ml samples at −70°C. Serum from a patient with acquired immunodeficiency syndrome (AIDS) who had no antibody to S. pneumoniae serotype 4 capsule and <1 µg of antibody to CWPS per ml was used as a control.

**Adsorption.** Sera were adsorbed either with washed, unencapsulated bacteria or with CWPS. For bacterial adsorption, organisms were grown overnight at 37°C, collected by centrifugation, and washed three times in phosphate-buffered saline. Samples containing 10⁶ CFU were distributed to tubes, and bacteria were collected by centrifugation at 15,000 × g for 3 min. The bacterial pellets were suspended in 1 ml of each serum to be adsorbed, and this suspension was kept at 8°C on a rocking platform for 30 min. The serum was then collected by centrifugation, and this procedure was repeated twice again. After the third adsorption, sera were filtered (0.2-µm pore size; Acrodisc; Gelman Sciences, Ann Arbor, Mich.). Adsorption with CWPS was carried out by addition of 10 µg of CWPS per ml of serum and incubation at room temperature on a rocking platform for 30 min.

**ELISA and RIA.** ELISA was carried out as described previously (21), using Immulon II microtiter plates (Dynatech, Alexandria, Va.), with modifications. Capsular polysaccharide from S. pneumoniae serotype 4 or CWPS (12.5 µg/ml) was suspended in phosphate-buffered saline (PBS; pH 7.4) and used directly to coat wells by incubation at 37°C for 5 h followed by incubation at 4°C overnight. Blocking was done with PBS that contained 1% bovine serum albumin (Sigma). Triplicate samples of sera were studied in 1:100, 1:300, and 1:900 dilutions, and a laboratory reference standard that contained known amounts of IgG reactive with capsular polysaccharide or CWPS was included on every plate. Alkaline phosphatase-conjugated goat antibody to human IgG (Sigma) at a 1:1,000 dilution was used to detect IgG, and the reaction was developed by addition of p-nitrophenol phosphate in diethanolamine buffer. All washings were done with phosphate-buffered saline containing 0.02% Tween. Optical density was read in an ELISA reader (Dynatech) at 495 nm, with subtraction of optical density of the appropriate blank. Antibody concentrations were calculated from optical density as described previously (20). Antipneumococcal antibody was measured by RIA (23) in the laboratory of Gerald Schiffman, University of New York Downstate Medical Center, Brooklyn; that laboratory reports levels of ≥300 ng of antibody nitrogen per ml to be consistent with immunity.

**Animal infectivity.** Swiss outbred mice (Harlan Sprague Dawley, Inc., Indianapolis, Ind.), 25 to 30 g, were distributed randomly so that each cage contained no more than six mice. Food (chow) and water were provided ad libitum. The room containing these cages had fluorescent lighting for 10 to 12 daytime hours each day. Mice were injected intraperitoneally with serum that had been diluted to the desired antibody concentration or with saline for controls. This was followed 30 to 45 min later by intraperitoneal injection of S. pneumoniae serotype 4 from a log-phase broth culture diluted to yield the approximate desired dose. Bacterial suspensions were kept on ice after dilutions were made, and injections were completed within 40 min. In various experiments, mice received an estimated 10, 100, or 1,000 LD₅₀, the actual number of CFU of injected bacteria was determined by serial dilution and culture immediately at the end of the injection period. All injections were made by using tuberculin syringes and 28-gauge needles in a volume of 0.1 ml. Although earlier investigators (7) comixed bacteria and antibody before injection, we preferred two separate injections 30 to 45 min apart because of the agglutinating effect of antibody.

**RESULTS**

**Pilot studies.** Results of pilot studies suggested that protection of mice was largely or even entirely related to antibody that remained after adsorption with S. pneumoniae mutant Rx, R36a, or DW3-8 or purified, soluble CWPS. Serum samples from six unvaccinated young adults were found by ELISA to contain a 4- to 33-µg/ml concentration of IgG reactive with S. pneumoniae serotype 4 polysaccharide antigen. After adsorption with CWPS, five of these six serum samples contained no detectable antibody and were selected for further study. Two of these five serum samples were found by RIA to contain 995 and 548 ng, respectively, of antibody nitrogen to S. pneumoniae serotype 4 before adsorption; no antibody was detectable by RIA after adsorption with CWPS.

Results from a representative study are shown in Table 1. This prevaccination serum was shown by ELISA to contain 14.8 µg of IgG per ml and by RIA to contain 548 ng of antibody nitrogen reactive with type 4 antigen; all reactive IgG measured in either assay was removed by adsorption with CWPS. Passive immunization with 0.1 ml of this serum unadsorbed or adsorbed with CWPS or bacterial mutant DW3.8 did not protect mice against the lethal effect of 2,000 LD₅₀; however, when the serum that had been adsorbed contained a 30-µg/ml concentration of IgG reactive with type 4 antigen by ELISA; after adsorption with CWPS, 6.9 µg of IgG per ml remained. Pretreatment of mice with 0.1 ml of undiluted serum or 0.1 ml of a 1:5 dilution of this serum was uniformly protective against 2,000 LD₅₀. These data showed that 138 ng of IgG specifically reactive with capsular polysaccharide protected mice against challenge with 2,000 prevaccination serum but was removed by adsorption with
TABLE 1. Representative pilot study of pre- and postvaccination serum antibody levels and protective effect

<table>
<thead>
<tr>
<th>Serum</th>
<th>Prevaccination (µg/ml) ELISA</th>
<th>RIA (ng of antibody N)</th>
<th>Postvaccination ELISA (µg/ml)</th>
<th>Mouse protection*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadsorbed</td>
<td>14.8</td>
<td>548</td>
<td>30</td>
<td>0/5</td>
</tr>
<tr>
<td>Adsorbed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CWPS</td>
<td>&lt;1</td>
<td>0</td>
<td>6.9</td>
<td>0/5</td>
</tr>
<tr>
<td>DW3.8</td>
<td>&lt;1</td>
<td>ND</td>
<td>ND</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* Number of animals remaining alive at the end of the experiment (usually 120 h) per number initially infected. Mice were injected with the indicated amount of unadsorbed serum from subject 1 in a total volume of 0.1 ml and challenged 45 min later with 2,000 CFU of S. pneumoniae type 4. Prevaccination serum from subject 2 that contained 214 mg/ml by ELISA and 995 ng of antibody nitrogen by RIA before adsorption and no detectable antibody after adsorption yielded essentially identical results.

* ND, Not determined.

CWPS did not. Similar results were obtained for all five sera studied in this fashion.

Protection by nonimmune serum. Even though injection of prevaccination serum did not alter the lethality of 2,000 LD₅₀, this treatment appeared to prolong the survival time. Furthermore, this prolongation appeared to be abrogated by adsorbing the serum with unencapsulated mutant strains of S. pneumoniae, DW3.8 or CSR. To examine these phenomena more carefully, experiments were designed in which 1,000, 100, or 10 LD₅₀ was used to challenge mice after passive immunization with 0.1 or 0.02 ml of prevaccination serum that lacked antibody specifically reactive with capsular polysaccharide; serum was used either unadsorbed or after adsorption with DW3.8 or CSR.

Injection of prevaccination serum delayed the time of death after challenge with 1,000 LD₅₀ and actually reduced lethality after challenge with smaller inocula (Fig. 1). This protective effect was removed by adsorbing serum with DW3.8 or CSR (Fig. 2). To determine whether antibody or other serum factors were responsible for the protection, serum from a patient with AIDS that contained normal levels of complement but lacked detectable antibody to capsular polysaccharide or CWPS was used to pretreat mice; this serum protected mice to the same degree as did normal human serum (Fig. 2). In three separate experiments, adsorbing AIDS serum with DW3.8 or CSR or heating any of these sera to 56°C for 20 min abolished the protective effect (data not shown).

Protection by immune serum. Postvaccination sera that contained various concentrations of IgG specifically reactive with capsular polysaccharide were adsorbed with DW3.8. These sera were diluted (dilutions ranged from 1:1.9 to 1:20, depending on the IgG level) to yield 1,500 ng of antibody per ml, after which three further threefold dilutions were made. Groups of mice received 0.1 ml from each of these dilutions, thus being given 150, 50, 17, or 6 ng of IgG, after which they were challenged with 1,000, 100, or 10 LD₅₀ of S. pneumoniae serotype 4. Protection was directly related to the amount of serotype-specific IgG injected. In two representative experiments (Table 2), 50 ng of anti-type 4 IgG was uniformly protective against 100 LD₅₀, 17 ng reduced lethality, and 6 ng had no effect, although this amount of antibody protected some mice against 10 LD₅₀. Doses of IgG ≤2 ng were not protective. Similar results were observed in sepa-

FIG. 1. Survival of mice (six per group) passively immunized with 0.1 ml of prevaccination serum (from subject 2) that contained 33 µg of IgG to CWPS per ml and no detectable (<1 µg/ml) IgG to S. pneumoniae serotype 4 capsular polysaccharide. Treated mice and saline-treated controls were then challenged with 1,000, 100, or 10 LD₅₀ of S. pneumoniae serotype 4. Curves from left to right indicate saline pretreatment plus 1,000 (●), 100 (■), or 10 (□) LD₅₀ and serum pretreatment plus 1,000 (●), 100 (■), or 10 (□) LD₅₀.

FIG. 2. Survival of mice (six per group) passively immunized with 0.1 ml of prevaccination serum (from subject 2) that was unadsorbed or had been adsorbed with unencapsulated mutant DW3.8 or CSR. Control mice received saline or serum from a patient with AIDS who had no detectable IgG to CWPS or capsule. All mice were then challenged with 100 LD₅₀ of S. pneumoniae serotype 4. Curves from left to right indicate pretreatment with saline (●), normal serum adsorbed with mutant DW3.8 (■), normal serum adsorbed with mutant CSR (●), unadsorbed normal serum (□), and AIDS serum (●).
TABLE 2. Effect of IgG specifically reactive with capsular polysaccharide on the outcome of pneumococcal challenge

<table>
<thead>
<tr>
<th>Serum no.</th>
<th>IgG reactive with cap polysaccharide PS (ng)</th>
<th>Survival after LDF_50 of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1,000</td>
</tr>
<tr>
<td>3</td>
<td>150</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>5</td>
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<td>6</td>
<td>0</td>
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* Amount specifically reactive with S. pneumoniae serotype 4 capsular polysaccharide (PS) after adsorption of serum with CWPS.

rate experiments using four different postvaccination sera. When results from these experiments were averaged, curves could be constructed (Fig. 3) showing the relationship between nanograms of serotype-specific IgG injected and survival for each bacterial inoculum. Linear regression showed that survival after challenge increased significantly as a function of increasing antibody titer (P < 0.05).

DISCUSSION

The experiments described above show a close relationship between antibody that is specifically reactive with S. pneumoniae serotype 4 capsular polysaccharide and protection against challenge with this organism. These results provide direct support for three important conclusions drawn from earlier work done in our laboratory (20, 21): (i) normal adults usually do not have anticapsular antibody to most pneumococcal serotypes; (ii) a substantial proportion of the antibody that is normally present before vaccination or that appears after pneumococcal vaccination and has been thought, on the basis of RIA, to be specifically reactive with capsular polysaccharide is, in fact, reactive with pneumococcal CWPS; and (iii) antibody to CWPS does not provide an appreciable degree of protection against pneumococcal challenge.

Medical literature on the efficacy of pneumococcal vaccines is equivocal, and in the case of elderly adults who have underlying chronic diseases and for whom vaccination is recommended, it is not entirely clear that protection results despite the appearance of antibody as measured by RIA (reviewed in references 22 and 27). Our initial studies (22) demonstrated increases in antibody levels and opsonic capacity of serum for pneumococci after vaccination, but there was a surprising lack of relationship between the two. Subsequent experiments (20), together with the work of other investigators (17), demonstrated that a varying proportion of antibody measured by ELISA or RIA and thought to be specific for capsular polysaccharide could be removed by adsorption with unencapsulated mutants or with isolated CWPS. The presence of contaminating CWPS in antigen preparations (19, 24, 26) appears to be responsible for these observations.

Despite numerous reasons to accept the validity of these results, it might still have been argued that antibody levels as determined heretofore by RIA are reliable and that the loss of antibody after adsorption in our ELISA somehow represents an artifact of the assay. Accordingly, we designed experiments to relate our in vitro findings to protection in vivo, using the technique of passive immunization that was developed nearly a century ago to demonstrate immunity after natural infection or vaccination. Our results show that mice were protected against pneumococcal challenge in direct proportion to the amount of specific anticapsular IgG that they received (i.e., that measured after adsorption with CWPS) but unrelated to levels of antibody as measured before adsorption. In fact, when postvaccination sera from different subjects were appropriately diluted to provide equivalent amounts of anticapsular IgG, the degrees of protection conferred by the various sera were nearly identical. A direct relation between levels of anticapsular antibody and protection was recently shown by Linuma and Okinaga (13), whose ELISA was based on the use of monoclonal antibody to S. pneumoniae serotype 6 as a capture antibody.

Our studies also shed light on the question of whether young adults normally have antibody that might provide specific protection against pneumococcal infections. Widespread use of RIA has suggested that antibody to many pneumococcal polysaccharides is present in the sera of normal infants (10) or adults (16). We (20; this report) have found that adsorption of normal serum with CWPS removes much of this low-level activity, thereby showing that these antibodies are not directed against pneumococcal capsular polysaccharide.

Pretreatment of mice with 0.1 ml of normal human serum that lacked anticapsular antibody still reduced lethality of 100 or 10 LD_50 of S. pneumoniae serotype 4 and prolonged the time to death after 1,000 LD_50. The hypothesis that this result was due to anti-CWPS antibody initially appeared to
be supported by experiments in which the protective effect was removed by prior adsorption of sera with unencapsulated mutant strains of *S. pneumoniae*. Mice were, however, protected by pretreatment with serum that had no detectable anti-CWPS antibody, and protection by any serum that lacked anticapsular IgG was eliminated by heating to 56°C for 20 min. These data suggest that human complement was responsible for the modest protective effect of normal human serum. Mice are said to be complement poor (6), and an infusion of human complement may confer some protection against pneumococcal challenge. Removal of complement components might explain the loss of protection after extensive adsorption with unencapsulated bacteria.

Several studies have suggested that anti-CWPS antibody may play a role in protection. Immunization with whole, killed pneumococci (15, 29), isolated cell walls (2, 8), or conjugated phosphocholine (31) has been shown to be protective, as has infusion of monoclonal IgG or IgM directed against phosphocholine determinants of CWPS (3, 18, 28, 31). Antibody to CWPS does not, however, facilitate phagocytosis of pneumococci in vitro (4, 12, 19) or in vivo (5), presumably because it localizes at a site that is inaccessible to phagocytic cells (25). Furthermore, we have recently shown (21) in a seroepidemiologic study that there was no association between preexisting levels of anti-CWPS antibody and the degree of severity of pneumococcal infection. Although it remains possible that some extraordinarily high dose of anti-CWPS antibody might be protective, we found no effect from as much as 3,300 ng of anti-CWPS IgG against challenge with 100 L.D.50 of *S. pneumoniae* serotype 4, even though 50 ng of serotype-specific anticapsular antibody was uniformly protective. It is improbable that other serum constituents contributed to the protection observed with postvaccine sera, since dilutions up to 20-fold of high-titered sera were made before injection of the initial 150-ng IgG dose.

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LITERATURE CITED