Protection of Mice from Infection with *Streptococcus pneumoniae* by Anti-Phosphocholine Antibody

JANET YOTHER,* COLYNN FORMAN, BARRY M. GRAY, AND DAVID E. BRILES

The Cellular Immunobiology Unit of the Tumor Institute, Departments of Microbiology and Pediatrics, and the Comprehensive Cancer Center, University of Alabama in Birmingham, Birmingham, Alabama 35294

Received 3 August 1981/Accepted 24 November 1981

Anti-phosphocholine (PC) antibody mediated protection against many strains of *Streptococcus pneumoniae*, and hybridoma anti-PC antibodies protected mice from fatal infections with types 1 and 3 *S. pneumoniae*. Live types 1, 3, 5, 6A, and 19F *S. pneumoniae* had similar amounts of surface PC accessible to antibody. Furthermore, mice expressing the X-linked immunodeficiency (*xid*) of the CBA/N strain were found to be more susceptible to infection with *S. pneumoniae* of types 3, 6A, and 19F than were immunologically normal mice. The only exception to these results was the type 5 strain, which was highly virulent for both *xid* and normal mice. In addition, we were unable to protect mice against infection with the type 5 strain by using anti-PC antibody.

Several studies indicate that there is an as yet undefined species-specific pneumococcal antigen(s) that can elicit antibody protective against multiple *Streptococcus pneumoniae* serotypes (1, 9, 15, 16). One candidate for such an antigen is the phosphocholine (PC) determinant of pneumococcal C carbohydrate, which is believed to be a cell wall component of pneumococcal strains of all capsular types (7). Previously, we have demonstrated that antibody specific for PC can be protective against infections with type 3 *S. pneumoniae* in mice hemizygous for the X-linked immunodeficiency (*xid*) allele (4). These mice have very low antibody responses to most carbohydrate antigens, including the PC determinant of C carbohydrate, and are extremely susceptible to infection with type 3 pneumococci. However, they can be protected with naturally occurring anti-PC antibody found in the normal serum of other mouse strains (4).

The protective effects of anti-PC antibody have been unanticipated (12) since it has been expected that much or all of the C carbohydrate antigenic determinants are masked by the thick type 3 capsule (17). To determine whether anti-PC antibody would protect mice from other *S. pneumoniae* strains, we examined the binding of radiolabeled anti-PC antibody to strains of several pneumococcal types, including the type 3 strain WU2 used in earlier experiments. We also studied the relative ability of these pneumococci to infect *xid* and normal mice and the ability of hybridoma anti-PC antibody to protect against pneumococcal infection in normal mice. Our results indicate that type 3 strain WU2 is representative of other pneumococci and that anti-PC antibody can protect immunologically normal mice against infection with several pneumococcal strains.

**MATERIALS AND METHODS**

*Mice.* (CBA/N × DBA/2)F, (C × D) males and females and (DBA/2 × CBA/N)F, (D × C) males were obtained from the Rodent and Rabbit Production Unit of the National Institutes of Health, Bethesda, Md. CBA/I mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, and CFW Swiss-Webster mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

*Bacterial strains and growth conditions.* *Streptococcus pneumoniae* strains included type 3 strains WU2 (4) and A66 (2), clinical isolates of types 19F, 6A, and 1 which were kept virulent by mouse passage, and the unencapsulated strain R36A originally derived from a type 2 strain (2). A type 5 strain was obtained from S. Szu (Bureau of Biologics, Bethesda, Md.). The *Listeria monocytogenes* strain LM-22 was obtained from D. McGregor (Baker Institute for Animal Health, Ithaca, N.Y.). Stock cultures of streptococci were stored at -70°C in medium (Todd-Hewitt broth supplemented with 0.5% yeast extract) plus 10% glycerol. Broth cultures were grown from frozen stock in 5.0 ml of medium and 0.2% heparinized human blood at 37°C. After 4 to 6 h, 1.0 ml of this culture was passed to a second tube containing medium but no blood and was grown for 2 to 3 h (log phase). Bacteria for experimental infection were harvested by centrifugation (4,000 × g, 10 min, 4°C) and resuspended in Ringer lactate. Bacterial concentration was determined by optical density at 420 nm (optical density of 1 = 3 × 10⁸ colony forming units [CFU]/ml). Dilutions were made in 1% heat-inactivated (50°C, 45 min) fetal calf serum in Ringer (FCS-Ringer) and kept at 0°C until the time of infection (less than 1 h later). Immediately after injection the inoculum was plated onto blood agar to determine the number of CFU. The *Listeria* strains were grown in Brain Heart Broth (BBL, Cockeysville, Md.) supplemented with 0.5% yeast extract, 0.5% glutamate, and 1% glucose (BBL). Cultures were grown at 37°C for 24 h.
were stored at -70°C in Todd-Hewitt broth plus 0.5% yeast extract and grown in this same medium for 6 h (log phase) at 37°C.

**LD₉₀ determinations.** The number of pneumococci required to kill 50% of the infected mice (50% lethal dose, LD₉₀) was determined by infecting mice intravenously with doses of 10¹, 10², 10³, 10⁴, or 10⁵ pneumococci and recording the number of mice living for 10 days or more postinfection. The LD₉₀ value was calculated by the method of Reed and Muench (13).

**Hybridoma antibodies.** Isolated anti-PC antibodies (immunoglobulin M [IgM], 22.1A4; IgG₃, 59.6C3) obtained from L. Claffin (University of Michigan, Ann Arbor) were isolated by affinity chromatography on PC Sepharose and quantitated by absorbance at 280 nm (8). All hybridoma antibodies were made by fusion with the nonsecreting fusion line SP2/0-Ag14. Thus, the hybridoma antibodies produced would be expected to be pure clonal products and not to include any mixed molecules containing heavy or light chains from the fusion line. Both anti-PC hybridoma antibodies bear the TEPC-15 idiotype (8). Anti-type 3 antibodies (IgG₃, CA3-1; IgG₃, 16.3) (14) obtained from K. Schroer (National Cancer Institute, Bethesda, Md.) and anti-Salmonella antibody (IgM, ST-1) (3a) were used as diluted ascites fluid and quantitated by isotype-specific radioimmunoassays (5).

**Assay for anti-PC-binding capacity.** Radioactive antibody solution containing 20,000 cpm of ¹²⁵I-labeled (11) IgM antibody to PC (22.1A4) and 20,000 cpm of ⁵⁷CoCl₂ (as a volume marker) per 0.2 ml in 1% bovine serum albumin and 0.1 M diethylenetriamine pentaacetic acid (DTPA) in phosphate-buffered saline (0.15 M NaCl, 0.0092 M K₂HPO₄, 0.0022 M KH₂PO₄, pH 7.2). This solution was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) immediately before use to remove aggregates. ⁵⁷CoCl₂ and DTPA were mixed in phosphate-buffered saline before the addition of other components. Serial 10-fold dilutions of log-phase cultures of pneumococci were made in 1% bovine serum albumin in phosphate-buffered saline. A 0.1-ml amount of each dilution was added to 0.2 ml of the radioactive antibody solution in 1.5-ml Eppendorf tubes (in triplicate). These mixtures were incubated for 30 min at 37°C and then centrifuged for 10 min at 12,000 × g. Approximately 90% of the supernatant was removed and the remainder plus the pellet were counted in a two-channel γ-counter. The percentage of antibody bound was calculated by the following formula (10):

\[
\% \text{ bound} = \frac{\text{Final } ¹²⁵I \text{ cpm} - \text{Initial } ¹²⁵I \text{ cpm}}{\text{Initial } ¹²⁵I \text{ cpm}} \times 100
\]

A set of controls containing only medium or dilutions of medium (in 1% bovine serum albumin in phosphate-buffered saline) was used to determine background binding (always less than 1.5%), which was subtracted from experimental binding. The actual number of organisms in each dilution was determined by colony count of blood agar plates.

**Mouse protection tests.** Infecting doses of bacteria (about 10 × LD₉₀) were incubated with antibody diluted in 1% FCS-Ringer. The solution of antibody and FCS-Ringer was sterilized by filtration (0.45-μm membrane filter) before the addition of the bacteria. Control mice were infected with bacteria that had been incubated either with FCS-Ringer alone or FCS-Ringer containing 2% normal mouse serum. After a 30-min incubation at 0°C, the mixtures of bacteria and antibody or bacteria and control diluent were injected intravenously. Immediately afterwards, injection samples of the inoculation mixture were plated to determine CFU. The number of CFU was not significantly affected by in vitro incubation with antibody.

**RESULTS**

**Susceptibility to pneumococcal infection.** To determine whether the immunodeficiency of the C × D male mice would affect susceptibility to pneumococcal infection with strains other than WU2, LD₉₀ determinations were made for both normal mice (CBA/J, C × D females, D × C males, Swiss females) and the immunodeficient (xid) C × D males by using four additional S. pneumoniae strains given intravenously. Table 1 shows the mortality data for type 3 strain A66 in C × D male and C × D female mice. These data indicate that the LD₉₀ of A66 in C × D males is less than 10 CFU and is about 10⁴ CFU in C × D females. Figure 1 summarizes the LD₉₀ data obtained with five different S. pneumoniae. The values for LD₉₀ were 200- to 2,000-fold lower in

**TABLE 1. Susceptibility of xid and normal mice to type 3 A66 S. pneumoniae**

<table>
<thead>
<tr>
<th>Mice*</th>
<th>Ratio of alive to dead mice given an S. pneumoniae dose² of:</th>
<th>10¹</th>
<th>10²</th>
<th>10³</th>
<th>10⁴</th>
<th>10⁵</th>
<th>10⁶</th>
<th>LD₉₀⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td>C × D ♂</td>
<td>1:5</td>
<td>1:7</td>
<td>0:2</td>
<td>0:2</td>
<td>&lt;10¹</td>
<td></td>
<td>&lt;10¹</td>
<td></td>
</tr>
<tr>
<td>C × D ♀</td>
<td>2:0</td>
<td>2:0</td>
<td>7:3</td>
<td>6:8</td>
<td>4:7</td>
<td>0:2</td>
<td>1:3 × 10⁴</td>
<td></td>
</tr>
</tbody>
</table>

* C × D ♂, xid male CBA/N × DBA/2 mice; C × D ♀, immunologically normal female CBA/N × DBA/2 mice.

² Injected intravenously in 1% FCS-Ringer.

Calculated by the method of Reed and Muench (13). The susceptibilities of the two types of mice were shown to be different (P < 0.01) by calculating a Yates corrected 2 × 2 chi-square (7.9) by pooling the data between the LD₉₀ values. Two live and 16 dead C × D mice were compared with 15 live and 11 dead C × D ♀ mice.
**FIG. 1.** LD$_{50}$ values of *S. pneumoniae* strains in xid (C × D male, δ, WU) and normal (C × D female, ♀) mice. There were at least 5 mice at the inoculation doses immediately above and below the calculated LD$_{50}$ values. The LD$_{50}$ values of WU2, A66, type 6A, and type 19F were found to be significantly different in C × D δ and C × D ♀ mice at $P < 0.01$ for WU2 and A66 at $P < 0.05$ for 6A and 19F. These $P$ values were obtained by analyzing all data at or between the two LD$_{50}$ values by using a 2 × 2 chi-square.

$xid$ than in normal (C × D female) mice. The type 5 strain gave approximately the same LD$_{50}$ (about 30 CFU) in both xid and normal mice. The LD$_{50}$ values in the three other normal mouse strains were similar to those obtained with the C × D females (data not shown).

**Binding of IgM anti-PC antibody to pneumococcal strains.** Pneumococci have PC in their C carbohydrates (7) and their F antigens (6). To determine whether this PC is accessible to anti-PC antibody, we determined the ability of varying amounts of bacteria of several *S. pneumoniae* strains to bind a $^{125}$I-labeled IgM hybridoma antibody (22.1A4) to PC. As a positive control, R36A, an unencapsulated type 2 bacterium was used. Nonspecific binding to bacteria was determined with *L. monocytogenes*. As shown in Fig. 2, all pneumococcal strains bound a significant amount of antibody when compared with *L. monocytogenes*. At a concentration of $3 × 10^6$ bacteria, the encapsulated pneumococci bound an average of 15% of the available anti-PC antibody, whereas *L. monocytogenes* bound 0%. An equivalent number of unencapsulated pneumococci bound 55% of the available antibody. About 300 times as many encapsulated pneumococci were required to show binding equivalent to that of the unencapsulated strain.

**Protection against infection with type 3 and type 1 *S. pneumoniae* by using hybridoma antibody to PC.** To examine the ability of antibody to protect mice from pneumococcal infection, we injected immunologically normal mice with about $10 \times \text{LD}_{50}$ of pneumococci that had been incubated with antibody, diluent, or diluent plus 2% normal mouse serum. The normal mouse serum was included to compensate for the fact that some of the hybridoma antibodies were used as ascitic fluid at dilutions of 1:50 or greater. The 2% normal mouse serum was not expected to be protective since it would contain only about 0.02 µg of antibody in 0.2 ml, a value well below the protective levels (3).

In experiments with type 3 strain A66, 5 of 5 mice protected with 20 µg of IgG3 anti-PC and 5 of 5 control mice protected with 20 µg of IgG3 antibody to the type 3 capsule were alive 3 days after infection (Fig. 3). All six unprotected mice and 4 out of 5 mice protected with 20 µg of IgM anti-PC antibody died. However, IgM anti-PC antibody may be at least partially protective against A66 since 100-µg doses protected 2 of 3 D × C male mice (data not shown). A similar experiment showed that 50 µg of IgG3 anti-PC antibody provided protection against 100 (about $10 \times \text{LD}_{50}$) type 1 *S. pneumoniae* (Fig. 4). Of 12 control mice given 1% FCS-Ringer diluent or anti-*Salmonella* antibody in place of anti-PC antibody, 10 died within 6 days.

**Attempts to protect against type 5 *S. pneumoniae*.** Several attempts to protect against infection with the type 5 *S. pneumoniae* have thus far proven unsuccessful. CBA/J or C × D females given 200 µg of either IgG3 or IgM anti-PC antibody died within 3 days postinfection, as did control mice receiving either 2% normal mouse serum diluent or Ringer lactate.

**DISCUSSION**

This study suggests that anti-PC antibody may be protective against many strains of *S. pneumoniae*. This conclusion is based on three types of data. In one set of experiments we showed that type 3 (strain A66), type 6A, and type 19F pneumococci were more virulent in xid mice than in normal mice. This finding was consistent with the protective role of natural anti-PC antibody demonstrated in an earlier study, where it has been shown that the extreme susceptibility of xid mice to type 3 strain WU2 can be eliminated with passive anti-PC antibody from the normal serum of non-xid mice (4).

In other experiments we showed that hybridoma antibody to PC was able to protect immunologically normal mice from lethal doses of type 1 and type 3 strain A66 *S. pneumoniae*. S. Szu, S. Clarke, and J. Robbins at the Bureau of
Biologics, Bethesda, Md., have likewise demonstrated that anti-PC antibody can protect mice from fatal infection with type 6A and type 4 strains (personal communication). Our binding studies demonstrated that 125I-labeled hybridoma antibody could bind all S. pneumoniae strains tested to a similar degree. The fact that 300 times as many encapsulated as unencapsulated bacteria were required to show equivalent anti-PC antibody binding indicates that the capsules are highly, but not completely, effective at masking the cell wall associated PC determinants. The availability of PC determinants on encapsulated S. pneumoniae probably accounts for the protection observed against type 1 and type 3 with anti-PC antibody.

Our experiments with a type 5 strain indicate that anti-PC antibody may not be equally protective against all S. pneumoniae strains since neither we nor Szu, Clarke, and Robbins have thus far been able to protect against type 5 S.
pneumoniae with anti-PC antibody. These data could be interpreted to indicate that either the high virulence of the strain masks any protective effects of anti-PC antibody or the lack of protective effects of natural anti-PC antibody results in the high virulence. Some support for the former interpretation comes from our binding studies, which showed that live type 5 S. pneumoniae, like other pneumococcal types, could be bound in vitro by IgM antibody to PC. This finding indicates that the lack of protection against type 5 is not due to PC being inaccessible to anti-PC antibody in this strain.

In this study we observed that IgM anti-PC antibody is less protective against type 3 strain A66 than either IgG3 anti-PC antibody or IgM anti-type 3 antibody. The difference in protection observed with IgM and IgG3 anti-PC antibody is almost certainly a reflection of the difference in isotype since both antibodies bear the TEPC-15 idiotype and thus would be expected to have virtually identical specificities (8). These findings confirm an earlier study with the type 3 strain WU2, which demonstrated that on a weight basis IgG3 antibodies were 40- to 90-fold more protective than IgM antibodies and that anti-type 3 antibodies were about 10 times more protective than anti-PC antibodies (3).

The results reported in this paper support the findings of previous studies demonstrating that anti-PC antibody could protect mice against type 3 S. pneumoniae strain WU2 (3, 4), and together with the unpublished results of Szu, Clarke, and Robbins, indicate that anti-PC antibody may be important in protection against infection with many types and strains of S. pneumoniae.

ACKNOWLEDGMENTS

We thank Latham Claffin, Kenneth Schroer, and Patricia Basta for providing the hybridoma antibodies; Phillip Baker for providing some of the mice; William H. Benjamin, Jr. and Joyce Lehmeyer for advice and assistance; and Ann Brookshire for typing the manuscript. We would especially like to thank S. Szu, S. Clarke, and J. Robbins of the Bureau of Biologies for allowing us to refer to their unpublished data.

This work was supported by Public Health Service grants AI 15986, T32AI07150, HD09732, CA 16673 and CA 13148 from the National Institutes of Health.

LITERATURE CITED