

March 1981

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ANTIPHOSPHOCHOLINE ANTIBODIES FOUND IN NORMAL
MOUSE SERUM ARE PROTECTIVE AGAINST INTRAVENOUS
INFECTION WITH TYPE 3 *STREPTOCOCCUS PNEUMONIAE**

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Although it has been clearly demonstrated that antibodies to pneumococcal capsules are protective in both mice and men (1, 2), the protective properties of antibodies to other pneumococcal constituents have been less well investigated. In the present report we describe results indicating that naturally occurring antibodies binding with the phosphocholine (PC)¹ determinant of the pneumococcal cell wall C-carbohydrate (3) are important in the protection of mice from experimental pneumococcal infections.

We made this observation while investigating what effects the immune deficiency of the CBA/N mouse might have on its susceptibility to pneumococcal infection. This strain carries an X-linked inability (*xid*) to produce normal humoral antibody responses to a group of thymus independent (TI-2), predominantly carbohydrate antigens (4), including type 3 pneumococcal capsular polysaccharide (5), trinitrophenyl-Ficoll (6), PC (7, 8), dextran (9), levan (8), and group A streptococcal carbohydrate (10). The CBA/N mouse is probably unable to make high levels of anticarbohydrate antibodies in general because the bulk of mouse anticarbohydrate antibodies is either IgM or IgG₃ (11), and CBA/N mice produce low levels of IgM (12, 13) and virtually no serum IgG₃ (13). In other respects, CBA/N mice appear immunologically normal because they make relatively normal antibody responses to T-dependent antigens (14–16), they have relatively normal levels of the other murine immunoglobulin isotypes (13), and they express normal cell-mediated immunity (6).

By infecting CBA/N mice with various pathogens, it should be possible to determine whether anti-carbohydrate antibodies play important roles in defense. Our studies have clearly demonstrated that CBA/N mice are highly susceptible to intravenous infection with type 3 pneumococci (17), but contrary to our expectations, we have found that naturally occurring antibody to PC rather than induced anti-type 3

* Supported by grants AI-15986, AI-00338, AI-14782, CA-16673, and AI-11635 from the National Institutes of Health, Bethesda, Md.

¹ Abbreviations used in this paper: BSA, bovine serum albumin; C × D, (CBA/N female × DBA/2 male) F₁ mice; D × C, (DBA/2 female × CBA/N male) F₁ mice; KLH, keyhole limpet hemocyanin; NAG, β-phenyl-N-acetyl-D-glucosaminide; NMS, normal mouse serum; PBS, phosphate-buffered saline; PC, phosphocholine.

capsular antibody was important for survival. Other investigators have shown that CBA/N mice are also especially susceptible to *Salmonella typhimurium*, *Ascaris suum*, and *Plasmodium yoelii* (18–20).

Materials and Methods

Mice. (CBA/N × DBA/2)F₁ (C × D) males and (DBA/2 × CBA/N)F₁ (D × C) males were obtained from the Rodent and Rabbit Production Unit of the National Institutes of Health, Bethesda, Md. BALB/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, Maine.

Bacterial Strains. A human isolate of type 3 pneumococci was obtained courtesy of Mr. John Courtney, Washington University School of Medicine. This isolate had been maintained over a period of 15 yr by annual passage through mice. After three additional mouse passages, strain WU1 was established from a smooth colony. From a subsequent mouse passage of WU1, we isolated a more virulent subline, WU2. Two additional mouse passages have not increased the virulence of WU2. Both sublines were shown to be type 3 by specific antisera. Group A streptococcus, J17A4 (21), was obtained from Dr. R. Lancefield, The Rockefeller University. *S. typhimurium*, SR-11 (22), was obtained from Dr. L. J. Berry, The University of Texas at Austin. *Streptococcus pneumoniae*, R36A (23), was obtained from Dr. A. Tomasz, The Rockefeller University. *Listeria monocytogenes*, LM-22, was obtained from Dr. D. McGregor, Cornell University. All cultures were stored at –70°C in media (see below), except for *S. pneumoniae* which was frozen in medium plus 10% glycerol. Freshly thawed cultures of *L. monocytogenes* and *S. typhimurium* were grown in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% yeast extract (Difco Laboratories). Streptococcal and pneumococcal cultures were grown in the same medium supplemented with 0.2% heparinized human blood. Pneumococci for infection were grown from a frozen stock as indicated above for ~6–8 h and harvested by centrifugation at 4°C while still in log phase. The bacteria were washed once and suspended in Ringers-lactate solution. Optical density (OD) at 420 nm was determined (OD of 1 ≅ 3 × 10⁸ cocci/ml). The bacteria were kept at 4°C from the time of harvest until injection. Immediately after injection, the inoculum was plated on blood agar to determine the number of colony-forming units per milliliter, and to examine colony morphology.

Protection of C × D Males with Normal Mouse Serum (NMS). Pools of NMS were obtained by bleeding about 40 8–12-wk-old C × D and D × C males. Harvested NMS was heat inactivated at 56°C for 40 min, filter sterilized, and stored at –20°C. About 200 live bacteria were added per 0.1 ml of NMS. After 30 min incubation on ice, an equal volume of cold Ringers was added, and mice were injected intravenously with 0.2 ml of the mixture. Injections were planned so that each cage contained about equal numbers of mice receiving D × C and C × D NMS.

Levels of Anti-PC Antibody in NMS. These values were determined by radioimmunoassay. The wells of vinyl microtiter plates (Cooke-Dynatech Laboratories, Inc., Alexandria, Va.) were coated overnight with 0.1 ml of PC-bovine serum albumin (BSA) at 10 µg/ml in phosphate-buffered saline (PBS). The PC-BSA was prepared as described elsewhere (24). The wells were rinsed, filled with 1% (BSA) in 0.005 M PBS, pH 7.4 (1% BSA-PBS), and allowed to stand for 3 h at room temperature. A 1:30 dilution of normal serum in 1% BSA-PBS was added to the first well and diluted out in subsequent wells with threefold serial dilutions. Plates were allowed to stand overnight at 4°C, and rinsed with PBS. Wells were then filled with 0.1 ml of 1% BSA-PBS containing 50,000 cpm of ¹²⁵I-labeled goat anti-mouse κ antibody (25). After an additional overnight incubation at 4°C, the wells were rinsed, cut from plates, and counted in a gamma counter. Maximum counts bound to the wells was ~30,000. For each serum we determined the dilution binding 15,000 cpm of the label. Anti-PC titers could be inhibited with 0.1% phosphocholine chloride. By comparing the titers of the normal sera with the titers of samples containing known amounts of hybridoma antibody, we calculated the amount of antibody in the NMS.

Absorption of NMS with Bacteria and Immunoabsorbents. Bacteria were grown in starter cultures as described above and then grown in 500 ml vol of Todd-Hewitt broth plus 0.5% yeast extract for 6–12 h. While the cultures were still in log phase, they were killed with formaldehyde (0.2%

final concentration, 12–24 h at room temperature) (26). Killed bacteria were harvested by centrifugation, washed twice with Ringers solution, once with 1% BSA-PBS, and suspended in 1% BSA-PBS. PC and β -phenyl-*N*-acetyl-D-glucosaminide (NAG) immunoabsorbents were prepared as described previously (27, 28). NMS were absorbed by adding 6×10^8 bacteria or 0.05 ml immunoabsorbent/ml of NMS and rotating at 4°C for 24–36 h. The absorbed sera were clarified by centrifugation and then sterilized by filtration before use.

Suppression of T-15 Idiotype. The anti-PC response of mice was suppressed by administration of monoclonal anti-T-15 antibody derived from the hybridoma GB4-10-4. Details on preparation, characterization, and suppressive characteristics of this monoclonal antibody will be described elsewhere. Briefly, this hybridoma was derived from a fusion of HOPC-8-immunized A/J lymph node cells with the myeloma P3 NS1Ag4-1. The anti-idiotypic antibody is $\gamma 1, \kappa$, its binding to T-15 is inhibited by PC-keyhole limpet hemocyanin (KLH) but not by PC and it is specific for idiotypic determinants on T-15 and H8 but not two other PC-binding myelomas, MOPC167 and MOPC511. Adult mice were suppressed by four daily injections of 100 μ g of purified antibody before challenge with pneumococcus. This dose has been shown to inhibit both the T-15 idiotype and total anti-PC response (Kearney, Barletta, Quan, and Quintans, manuscript in preparation).

Protection with Hybridoma Antibodies. IgM anti-type 3 hybridomas CA3-1, CC4-8, and DB6 have been described previously (29). IgM anti-PC hybridoma M2 was obtained from Dr. P. Gearhart, Carnegie Institute of Technology. Anti-*S. typhimurium* hybridoma, ST-1, was obtained from P. Basta, University of Alabama in Birmingham. Hybridoma antibodies from all tumors except M2 were raised as ascites in BALB/c mice. The M2 tumor was raised in C \times D male mice.

The amount of antibody in these fluids was estimated by determining the total levels of IgM (30) in the ascites fluids. The concentrations of antibody in these ascites fluids ranged from 5–20 mg/ml. Mice were injected intraperitoneally 1 and 2 d later with 0.1 ml of diluted ascites fluid containing 200 μ g of hybridoma antibody 2 h before intravenous inoculation of pneumococci. Control mice received 0.1 ml of a 1:50 dilution of BALB/cJ NMS.

Results

Susceptibility of *xid* Mice to Type 3 Pneumococci. To examine the effects of the *xid* allele on the susceptibility of mice to pneumococcal infection, we infected the reciprocal F₁ males obtained by crossing DBA/2 and CBA/N mice. Males derived from a CBA/N mother (C \times D) express the recessive X-linked immunodeficiency, whereas males with a DBA/2 mother (D \times C) are normal in their immune responsiveness (6, 12).

When these mice were infected intravenously, we could easily distinguish between *xid* normal and defective mice (Table I). When the infections were given intraperitoneally, even the immunologically “normal” D \times C males were highly susceptible, as expected (31, 32). We found that C \times D males could be killed with ~1,000–10,000 times fewer type 3 *S. pneumoniae* pneumococci than D \times C males or C \times D females.

TABLE I
*LD₅₀ of Pneumococci in *xid* Normal and Defective Mice*

Pneumococcal strain	Route of infection	Mice		
		C \times D♂	C \times D♀	D \times C♂
WU1	Intravenous	10 ⁴ *	ND‡	10 ⁷
WU2	Intravenous	10 ¹	10 ⁵	10 ⁵
WU2	Intraperitoneal	<10 ¹	10 ¹	10 ²

* LD₅₀. All deaths occurred between 1 and 5 d after infection.

‡ Not determined.

Many of the C × D males died within 2 d (Fig. 1). It seemed unlikely that the early deaths of the C × D males were the result of their inability to respond to the infection by the production of antibody, because even normal mice would not be expected to produce appreciable serum levels of antibody within the first 2 d (33, 34). One possible explanation was that C × D males lacked “naturally occurring” anticarbohydrate antibody cross-reactive with type 3 *S. pneumoniae*.

Protection of *xid* Mice with NMS. To determine whether normal mouse sera are protective, we incubated the infecting doses of pneumococci at 0°C with 0.2 ml of heat-inactivated NMS from either C × D or D × C male mice. This incubation step did not reduce the numbers of viable pneumococci, as determined by growth on blood plates. Mice were injected intravenously with 0.2 ml of the incubation mixture and injected intraperitoneally with additional 0.2-ml vol of the corresponding normal serum at 1 and 2 d after infection. From the data plotted in Fig. 2A and B, it is apparent that even these small amounts of D × C male serum can protect C × D males from infection with pneumococcal strains WU1 and WU2. No protection was seen with C × D male serum.

Specificity of Protective Antipneumococcal Antibody in NMS. To investigate the specificity of the protective antibody, we absorbed normal serum with formalin-killed bacteria. The results of this study are depicted in Fig. 3A, where it can be seen that absorption with either the encapsulated strain, WU2, or the nonencapsulated strain, R36A, removed most of the protective ability from the serum. Absorption with strain J17A4 had a much smaller effect. Because strain R36A is a genetically nonencapsulated type 2 pneumococcal strain, these data indicate that the protective antibodies in normal serum are not directed against the type 3 capsule of the pneumococcus but against some other component(s). Because both the WU2 and R36A strains should have PC in their cell walls (3), we suspected that the protective antibody might be directed against this determinant. As expected, pooled normal sera from C × D male mice had much lower levels of anti-PC antibody than sera from C × D females or D × C males (Table II) (35). The anti-PC antibody in D × C male sera was in fact largely removed by absorption with both of the pneumococcal strains. The significance of the residual

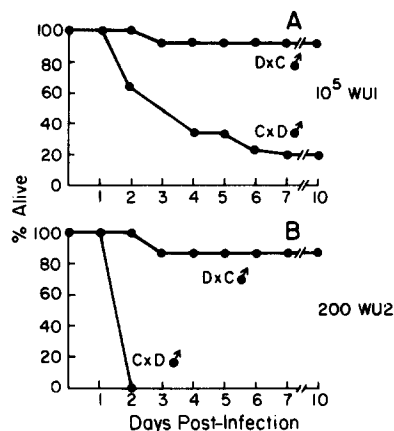


FIG. 1. Susceptibility of D × C and C × D males to type 3 *S. pneumoniae*. Mice were infected intravenously with either 10⁵ WU1 (A) or 200 WU2 (B) on day 0. Each group contained 10 or more mice.

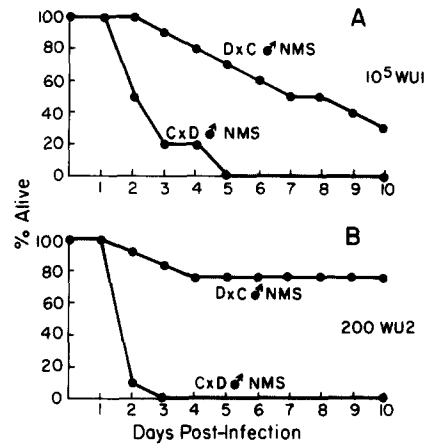


FIG. 2. Protection of C \times D males with NMS. Mice were infected intravenously with either 10^5 WU1 (A) or 200 WU2 (B) in 0.2 ml of heat-inactivated C \times D male or D \times C male NMS on day 0. The bacteria were incubated with the NMS for 30 min at 0°C before infection. Each group contained 10 or more mice.

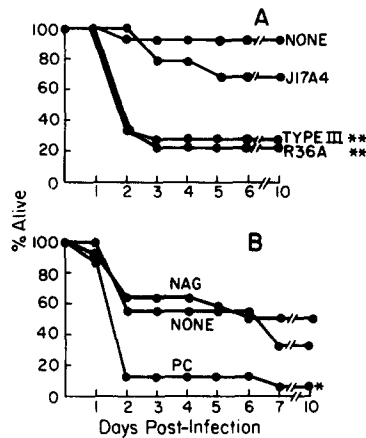


FIG. 3. Protection of C \times D males with absorbed D \times C male NMS. Preliminary experiments indicated that 1–2 OD U (420 nm) of formalized strain WU2 *S. pneumoniae* were required to absorb out most of the protective activity of 1 ml of D \times C male serum. In these experiments, D \times C serum was absorbed with 2 OD U/ml (10^8) killed bacteria (A) or 0.05 ml of immunoabsorbent (B) per milliliter of NMS. Live WU2 bacteria were then incubated in the absorbed NMS before injection as in Fig. 2. Bacteria used for absorption were WU2, type 3 *S. pneumoniae*, R36A, nonencapsulated (rough) *S. pneumoniae*, and J174A, group A streptococci. Immunoabsorbents used PC-Sepharose and β -phenyl-*N*-acetyl-D-glucosaminide-Sepharose. Each group contains nine or more mice. Significant differences from "none" at <0.03 and <0.01 are indicated by * and **, respectively.

anti-PC antibody is unclear. It may be due to low affinity antibody because it is not absorbed out by an excess of bacteria. The anti-PC antibody was only partially absorbed by group A streptococci. This partial absorption by group A streptococci correlates with the fact that this strain also partially removed protection from D \times C male sera.

To determine whether the protective antibodies in normal sera were directed against PC, we absorbed D \times C male serum with a PC-containing immunoabsorbent. As a control, the sera were absorbed with an immunoabsorbent containing NAG, the

TABLE II
Titers of Anti-PC Antibody in NMS

Serum pool		Anti-PC titer*
C × D male		6
C × D female		106
D × C male		113
	Inhibitor‡	
C × D male	0.1% PC	10
C × D male	0.1% Rhamnose	11
D × C male	0.1% PC	3
D × C male	0.1% Rhamnose	96
	Absorbent§	
D × C male	3.0×10^9 WU2	26
D × C male	1.0×10^9 WU2	25
D × C male	0.33×10^9 WU2	29
D × C male	0.1×10^9 WU2	61
D × C male	1.0×10^9 R36A	25
D × C male	1.0×10^9 J17A4	72
D × C male	1.0×10^9 Listeria	110
D × C male	PC-Sepharose	33
D × C male	NAG-Sepharose	107

* Dilution of antisera binding 50% of labeled anti- κ (see Materials and Methods). Each value is an average of two or more determinations. By using anti-PC hybridoma M2 as a standard, the concentrations of anti-PC antibody in C × D male, C × D female, and D × C male sera are 15, 258, and 265 ng/ml.

‡ Normal mouse sera were titered out in the indicated inhibitors diluted in 1% BSA-PBS.

§ Absorptions were done as described in Fig. 2. Numbers of bacteria for absorption were determined from the OD 420 nm of washed bacteria.

immunodominant group of streptococcal group A carbohydrate. The results shown in Figure 3B indicate that only the PC immunoabsorbent removed the protective ability. Thus it appears likely that anti-PC antibodies in normal sera play an important role in the resistance of mice to pneumococcal infection.

Pneumococcal Susceptibility of Mice Whose Anti-PC Antibody Response Has Been Suppressed. Because of the unexpected nature of the above observations, we sought to determine by a completely different method whether anti-PC antibodies were in fact essential for protection against pneumococcal infection. Most anti-PC antibodies in BALB/c mice bear a common idiotype, T-15 (36, 37). It has been shown previously that, in the adult, passive administration of antibodies reactive with the T-15 idiotype can suppress the production of most anti-PC antibodies (38, 39) without affecting responsiveness to other antigens. Thus, we used the anti-T-15 hybridoma, GB4-10-4 ($\gamma 1, \kappa$), to suppress the anti-PC responses of BALB/c mice. The dose of GB4-10-4 used has been shown to block induced anti-PC responses in BALB/c mice (Kearney, Barletta, Quan, and Quintans, manuscript in preparation) and in this study reduced the levels of naturally occurring anti-PC antibody to $<1/3$ of normal levels. These mice were infected with 5×10^3 WU2, a dose not normally fatal to BALB/c mice. As can be seen in Fig. 4, most of the suppressed mice and none of the control mice died of pneumococcal infection.

*Protection of *xid* Mice with Hybridoma Antibodies.* Finally, we examined the ability of

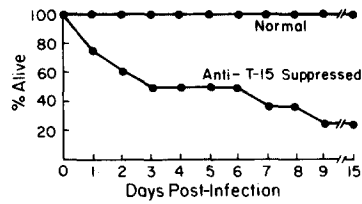


FIG. 4. Effect of anti-PC suppression on the resistance of BALB/cJ mice to *S. pneumoniae*. Anti-PC antibody production was suppressed by intraperitoneal injection of 100 μ g/d of GB4-10-4 anti-T-15 hybridoma. After a week of daily injections, mice were rested 6 d, bled on day 7, and infected intravenously on day 8 with 5×10^3 WU2. Each group contained eight mice.

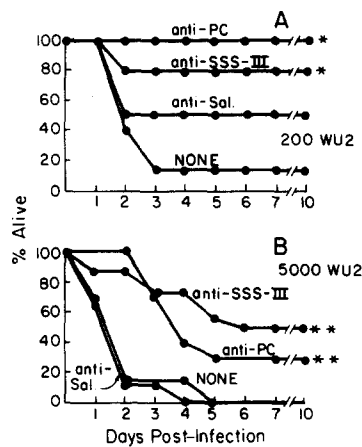


FIG. 5. Protection of C \times D males with passive antibody. Mice were injected intraperitoneally with 0.1 ml of diluted hybridoma-containing ascites fluid or 1:50 BALB/cJ NMS (none) 2 h before and 1 and 2 d after intravenous injection of 200 (A) or 5,000 (B) viable WU2. In B, each group contained eight or more mice. In A, the numbers of mice per group were 7 anti-PC; 15 anti-SSS-III; 4 anti-Salmonella; and 7 NMS. Significant difference from "none" at $P < 0.03$ or $P < 0.01$ is indicated by * or **, respectively.

IgM hybridoma antibodies to either type 3 polysaccharide, PC, or *S. typhimurium* to protect C \times D males from pneumococcal infection. Mice were injected intraperitoneally with 200 μ g of hybridoma antibody in 0.1 ml of diluted ascitic fluid 2 h before inoculation with either 200 or 5,000 colony-forming units of strain WU-2, *S. pneumoniae* (Fig. 5). To control for possible protection by naturally occurring antipneumococcal antibodies in the ascitic fluids, we injected a group of mice with a comparable dilution (1:50) of BALB/c NMS (Fig. 5). To further exclude the possibility that any protection obtained with the anti-PC hybridoma might be due to naturally occurring antibody, the M2-containing ascitic fluid was obtained from C \times D male mice carrying the tumor.

The results of these experiments demonstrated that the anti-PC hybridoma, M2, and the three IgM anti-type 3 hybridomas, CA3-1, CC4-8, and DB6, all gave protection. The three anti-type 3 hybridomas mediated similar levels of protection, and their pooled data are depicted. Protection was also observed with isolated M2 and CC4-8 (data not shown). The anti-Salmonella hybridoma showed significantly less protection. Although we do not yet know the minimum amounts of hybridoma

antibody required, it is now clear that as little as 20 $\mu\text{g}/\text{d}$ of M2 and CA3-1 antibody can provide complete protection.

Discussion

In these studies we used immunodeficient *xid* mice to examine the importance of anticarbohydrate antibody in the defense of mice against pneumococcal infection. We expected C \times D male mice to be highly susceptible to pneumococcal infection because they are unable to make antibodies to the capsule of the type 3 pneumococcus (5), and earlier reports had indicated that anticapsular antibodies were important for protection against pneumococcal infection (1, 31, 40). We were quite surprised when our results demonstrated that not only do antibodies in normal serum offer significant protection against pneumococcal infection, but these normal serum antibodies are reactive with the PC determinant of the cell wall rather than with the capsule.

We do not feel that our results negate any of the studies showing that anticapsular antibody is highly effective in the defense against pneumococcal infection. On the contrary, the role of anti-PC antibody may account for some of the nonspecific immunity to pneumococci (41, 42) and may delay the infection until protective levels of anticapsular antibody are generated.

If anti-PC antibodies do play an important role in the defense against pneumococci or other pathogens, it might explain in part why anti-PC antibodies show such extreme homogeneity of their specificity (43), idiotype (36, 44), and structure (45–47).

The naturally occurring anti-PC antibodies we find in normal sera are probably the result of immunostimulation with PC-containing environmental antigens. PC has been found on the surface of many microbes, including a number of different bacteria, fungi, and nematodes (48, 49). Naturally occurring anti-PC antibodies are commonly found in sera of mouse strains (35) other than CBA/N and are easily demonstrable in normal human sera (D. E. Briles and C. Forman, unpublished observations). The existence of anti-C carbohydrate antibody in human sera has been established previously (50).

There may be several reasons why protection with anti-PC antibody has not been reported before. Isolated C carbohydrate is poorly immunogenic (51), and even when an anti-PC response was induced, it may not have always raised the levels of anti-PC antibody sufficiently higher than the naturally occurring levels to cause a marked difference in protection. Furthermore, the early and dramatic successes of raising type-specific protective antibody to isolated pneumococcal capsules (1, 40) probably reduced interest in studies of protective antibodies to pneumococcal antigens other than capsular polysaccharides.

Although we know of no previous reports indicating that anti-PC antibodies are protective against pneumococcal infection, the older literature does contain several reports of immunizations with whole bacteria (52, 53) or bacterial fractions that yielded protection in rabbits across type barriers (54–56). The protection could be transferred with serum to other rabbits but not to mice (52). More recently, Thompson and Eisenstein (57) have shown that a vaccine composed of a subcellular preparation of rough type 3 pneumococci could protect mice from infection with encapsulated types 1, 2, or 3 pneumococci. It seems likely that at least some of these reports dealt with the effects of anti-PC antibody.

One of the most compelling findings was that of Dubos (58), who reported that if

autolysates of rough or smooth pneumococci were injected into rabbits, antisera could be produced that could protect mice against pneumococci regardless of their capsular type. Neither the nature of the antigen that induced these antibodies nor the antibody specificity was reported. However, it was known that autolysates of pneumococci contain C carbohydrate (59), and more recently it has been shown that PC is a major antigenic determinant of C carbohydrate (60). Choline has also been shown to be present in pneumococcal F antigen (61).

The fact that anti-PC antibodies are protective against encapsulated type 3 pneumococci is particularly interesting because all strains of *S. pneumoniae* have C carbohydrate in their cell walls (3, 51).

Summary

The antiphosphocholine (PC) antibody in normal mouse sera (NMS) provides protection against intravenous infection with encapsulated strain WU2 of type 3 *Streptococcus pneumoniae*. Mice unable to make anti-PC antibody, as a result of suppression with anti-T-15 idiotypic or inheritance of the *xid* gene of CBA/N mice, are highly susceptible to infection with strain WU2. Mice inheriting the *xid* gene can be protected with NMS from immunologically normal mice or with IgM hybridoma anti-PC antibody. The protective effect of NMS can be removed with PC-containing immunoabsorbents.

We would like to thank Dr. Patricia Gearhart and Ms. Patricia Basta, who provided two of the hybridomas used; Ms. Colynn Forman and Mr. Ulysses Finley, whose technical competence brought these studies to completion; Ms. Ann Brookshire, who typed the manuscript; and Mr. William Benjamin, Ms. Patricia Basta, Ms. Joyce Lehmyer, and Mr. Tony Marion, who took time from their own studies to provide advice, encouragement, and assistance during the course of these experiments. We are particularly grateful for suggestions and encouragement from Dr. John Robbins.

Received for publication 6 November 1980.

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