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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

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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 with 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_1 (C × D) males and females and (DBA/2 × CBA/N)F_1 (D × C) males were obtained from the Rodent and Rabbit Production Unit of the National Institutes of Health, Bethesda, Md. CBA/J 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 = 3×10^8 colony forming units [CFU]/ml). Dilutions were made in 1% heat-inactivated (56°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 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.

LD_{50} determinations. The number of pneumococci required to kill 50% of the infected mice (50% lethal dose, LD_{50}) was determined by infecting mice intravenously with doses of 10^1, 10^2, 10^3, 10^4, 10^5, or 10^6 pneumococci and recording the number of mice living for 10 days or more postinfection. The LD_{50} value was calculated by the method of Reed and Muench (13).

Hybridoma antibodies. Isolated anti-PC antibodies (immunoglobulin M [IgM], 22.1A4; IgG_3, 59.6C3) obtained from L. Claflin (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 (IgM, CA3-1; IgG_3, 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 contained 20,000 cpm of ^{125}I-labeled (11) IgM antibody to PC (22.1A4) and 20,000 cpm of $^{57}CoCl_3$ (as a volume marker) per 0.2 ml in 1% bovine serum albumin and 0.1 mM diethylenetriamine pentaacetic acid (DTPA) in phosphate-buffered saline (0.15 M NaCl, 0.0092 M K_2HPO_4, 0.0022 M KH_2PO_4, pH 7.2). This solution was filtered through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) immediately before use to remove aggregates. $^{57}CoCl_3$ 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):

$$\% \text{ bound} = 100 \times \left(\frac{\dfrac{\text{Final } ^{125}\text{I cpm}}{\text{Initial } ^{125}\text{I cpm}} - \dfrac{\text{Final } ^{57}\text{Co cpm}}{\text{Initial } ^{57}\text{Co cpm}}}{1 - \dfrac{\text{Final } ^{57}\text{Co cpm}}{\text{Initial } ^{57}\text{Co cpm}}} \right)$$

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 × LD_{50}) 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 injected 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, LD_{50} 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 LD_{50} of A66 in C × D males is less than 10 CFU and is about 10^4 CFU in C × D females. Figure 1 summarizes the LD_{50} data obtained with five different *S. pneumoniae*. The values for LD_{50} were 200- to 2,000-fold lower in

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

Mice[a]	Ratio of alive to dead mice given an *S. pneumoniae* dose[b] of:						
	10^1	10^2	10^3	10^4	10^5	10^6	LD_{50}[c]
C × D ♂	1:5	1:7	0:2	0:2			$<10^1$
C × D ♀		2:0	7:3	6:8	4:7	0:2	1.3×10^4

[a] C × D ♂, *xid* male CBA/N × DBA/2 mice; C × D ♀, immunologically normal female CBA/N × DBA/2 mice.
[b] Injected intravenously in 1% FCS-Ringer.
[c] 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 LD_{50} values. Two live and 16 dead C × D mice were compared with 15 live and 11 dead C × D ♀ mice.

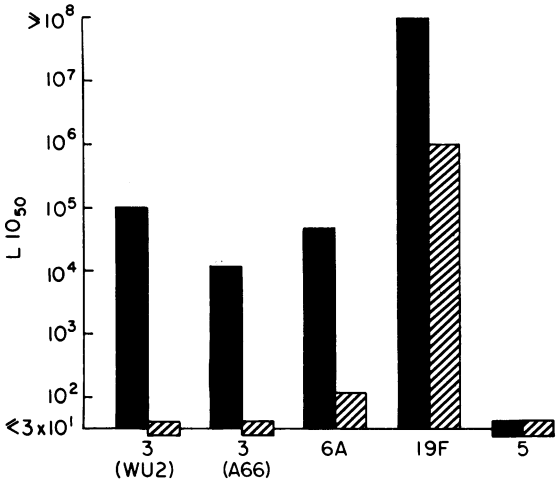

FIG. 1. LD_{50} values of *S. pneumoniae* strains in *xid* (C × D male, ♂, ▨) and normal (C × D female, ♀, ■) mice. There were at least 5 mice at the inoculation doses immediately above and below the calculated LD_{50} values. The LD_{50} values of WU2, A66, type 6A, and type 19F were found to be significantly different in C × D ♂ 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 3×10^8 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 × 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 IgG_3 anti-PC and 5 of 5 control mice protected with 20 μg of IgG_3 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 IgG_3 anti-PC antibody provided protection against 100 (about 10 × 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 IgG_3 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

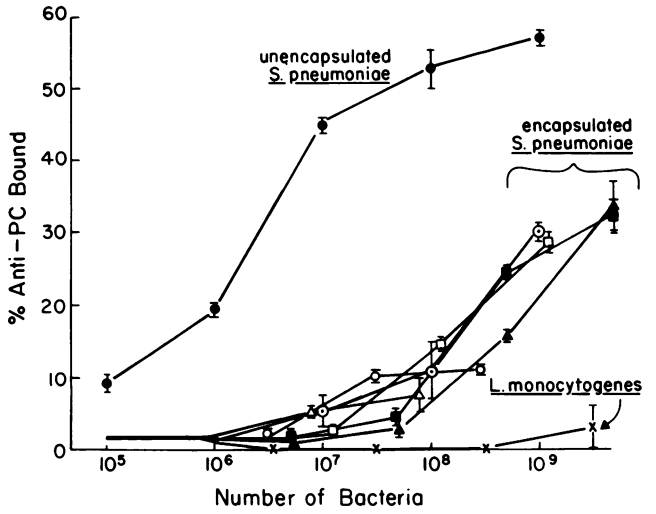

FIG. 2. Binding of ^{125}I-labeled IgM anti-PC antibody to *S. pneumoniae*. Strains used were R36A (●), type 1 (△), type 3 (strain A66) (□), type 3 (strain WU2) (○), type 5 (⊙), type 6A (■), and type 19F (▲). Nonspecific binding was determined with *L. monocytogenes* (X). Bars represent standard deviation of triplicate determinations.

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 ^{125}I-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.*

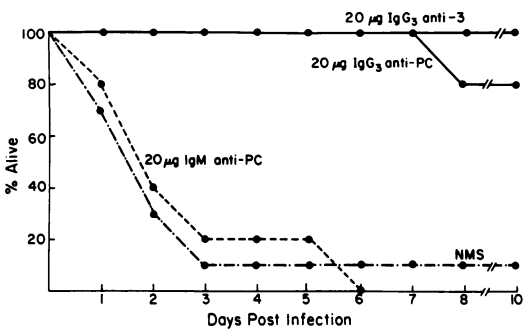

FIG. 3. Survival curves of mice protected against type 3 *S. pneumoniae* strain A66 with 20 μg of hybridoma antibody to PC. Control mice received either 2% normal mouse serum in diluent or 20 μg of antibody to the type 3 capsule. These data are from the Swiss mice in Table 1. Each curve represents the data from five mice except for the normal mouse serum curve, where data from six mice are shown. The survival curves for the mice protected with IgG_3 anti-type 3 or anti-PC antibody were significantly different from mice protected with normal mouse serum at $P < 0.004$, by using the two-sample rank test.

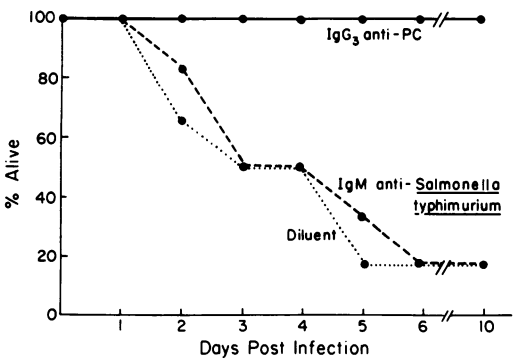

FIG. 4. Survival curves of Swiss female mice protected against type 1 *S. pneumoniae* with 50 μg of hybridoma IgG_3 antibody to PC. Control mice received either diluent (FCS-Ringer) or IgM antibody to *Salmonella typhimurium*. Each group contained six mice.

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 IgG_3 anti-PC antibody or IgM anti-type 3 antibody. The difference in protection observed with IgM and IgG_3 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 IgG_3 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*.

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LITERATURE CITED

1. **Au, C. C., and T. K. Eisenstein.** 1981. Nature of the cross-protective antigen in subcellular vaccines of *Streptococcus pneumoniae*. Infect. Immun. **31**:160–168.
2. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1944. Studies of the chemical nature of the substance inducing transformation of pneumococcal types. J. Exp. Med. **79**:137–158.
3. **Briles, D. E., J. L. Claflin, K. Schroer, and C. Forman.** 1981. Mouse IgG_3 antibodies are protective against infection with *Streptococcus pneumoniae*. Nature (London) **294**:88–89.

3a. **Briles, D. E., J. Lehmeyer, and C. Forman.** 1981. Phagocytosis and killing of *Salmonella typhimurium* by peritoneal exudate cells. Infect. Immun. **33**:380–388.

4. **Briles, D. E., M. Nahm, K. Schroer, J. Davie, P. Baker, J. Kearney, and R. Barletta.** 1981. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. J. Exp. Med. **153**:694–705.
5. **Briles, D. E., R. M. Perlmutter, D. Hansburg, J. R. Little, and J. M. Davie.** 1979. Immune response deficiency of BSVS mice. II. Generalized deficiency to thymus-dependent antigens. Eur. J. Immunol. **9**:255–261.
6. **Briles, E. B., and A. Tomasz.** 1973. Pneumococcal forssman antigen: a choline-containing lipoteichoic acid. J. Biol. Chem. **248**:6394–6397.
7. **Brundish, D. E., and J. Baddiley.** 1968. Pneumococcal C-substance, a ribitol teichoic acid containing choline phosphate. Biochem. J. **110**:573–582.
8. **Claflin, J. L., S. Hudak, and A. Maddlena.** 1981. Antiphosphocholine hybridoma antibodies. I. Direct evidence for three distinct families of antibodies in the murine response. J. Exp. Med. **153**:352–364.
9. **Dubos, R. J.** 1938. Immunization of experimental animals with a soluble antigen extracted from pneumococci. J. Exp. Med. **67**:799–808.
10. **Gotschlich, E. C.** 1971. A simplification of the radioactive antigen-binding test by a double label technique. J. Immunol. **107**:910–911.
11. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. **89**:114–123.
12. **MacLeod, C. M., R. G. Hodge, M. Heidelberger, and W. G. Bernhard.** 1945. Prevention of pneumococcal pneumonia by immunization with specific capsular polysaccharides. J. Exp. Med. **82**:445–465.
13. **Reed, L. J., and H. Muench.** 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. **27**:493–497.
14. **Schroer, K. R., K. J. Kim, D. F. Amsbaugh, P. W. Stashak, and P. J. Baker.** 1980. Lymphocyte hybridomas which secrete antibodies to the type III pneumococcal polysaccharide: idiotypic characterization, p. 178–180. *In* D. Schlesinger (ed.), Microbiology—1980. American Society for Microbiology, Washington, D.C.
15. **Thompson, H. C. W., and T. K. Eisenstein.** 1976. Biological properties of an immunogenic pneumococcal subcellular preparation. Infect. Immun. **13**:750–757.
16. **Tillett, W. S.** 1928. Active and passive immunity to pneumococcus infection induced in rabbits by immunization with R pneumococci. J. Exp. Med. **48**:791–804.
17. **Wood, B., and M. R. Smith.** 1949. The inhibition of surface phagocytosis by the capsular "slime layer" of pneumococcus type III. J. Exp. Med. **90**:85–99.