INFECTION AND IMMUNITY, Jan. 1983, p. 403–409
0019-9567/83/010403-07$02.00/0

Vol. 39, No. 1

Localization of Complement Component 3 on *Streptococcus pneumoniae*: Anti-Capsular Antibody Causes Complement Deposition on the Pneumococcal Capsule

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Received 26 July 1982/Accepted 21 September 1982

We have previously shown that complement component 3 (C3) deposited onto encapsulated *Streptococcus pneumoniae* by anti-capsular antibody (Ab) is a more efficient opsonin in vitro and in vivo than C3 deposited by anti-cell wall Ab (Brown et al., J. Clin. Invest. **69**:85–98, 1982). In the present study, we explored the cellular location of C3b molecules that differ in opsonic efficiency by using avidin-ferritin to localize biotinylated Ab and C3 molecules on *S. pneumoniae* for electron microscopy. Anti-cell wall Ab and C3b molecules deposited by this Ab on unencapsulated *S. pneumoniae* were localized to *S. pneumoniae* cell walls. Anti-capsular Ab and C3b deposited by this Ab were seen in clusters on encapsulated *S. pneumoniae* at a distance from the cell wall. However, no avidin-ferritin staining of encapsulated *S. pneumoniae* was seen on incubation with biotinyl–anti-cell wall Ab, biotinylated C3 fixed by anti-cell wall Ab, or nonimmune serum containing biotinyl-C3. In each case, uptake of the biotinylated component was proven by radioactivity measurements, since biotinylated Ab and C3 were also radiolabeled with ^{125}I. When avidin-ferritin did not bind to biotinylated components, Ouchterlony analysis indicated that C3 was bound to cell wall components on the encapsulated organisms. Thus, we conclude that, for encapsulated *S. pneumoniae*, opsonically efficient C3b molecules, deposited by anti-capsular Ab, are located on the *S. pneumoniae* capsule, whereas the opsonically inefficient C3b molecules deposited by anti-cell wall Ab or nonimmune serum are located on the cell wall. A major reason for the increased virulence of encapsulated compared to unencapsulated *S. pneumoniae* is that, in the absence of anti-capsular Ab, the *S. pneumoniae* capsule interferes with the recognition of cell wall-bound C3b molecules by phagocytic cell receptors.

It has long been known that the antigenic specificity of anti-*Streptococcus pneumoniae* antibodies (Ab) affects their biological function. Anti-capsular Ab have a protective effect in animal models of *S. pneumoniae* infection and a therapeutic effect in human disease, whereas Ab directed against cell wall constituents in general do not (6). We have recently reported data from an experimental model of *S. pneumoniae* bacteremia which showed that in vivo efficacy of anti-capsular Ab requires complement activation (3). However, in these experiments, protective anti-capsular Ab and nonprotective anti-cell wall Ab activated complement to similar extents. Further investigation showed that C3b deposited on *S. pneumoniae* by anti-capsular Ab and the classical complement pathway bound to the encapsulated organisms by anti-cell wall Ab. We speculated that this physiological difference between C3b molecules deposited by different Ab resulted from differences in location of deposition of C3b on *S. pneumoniae* organisms.

Recent work in our laboratory has demonstrated that C3 may be covalently linked to biotin without loss of functional activity (2) (biotinyl-C3). In the present study, we opsonized *S. pneumoniae* with Ab and biotinyl-C3 and then used avidin-ferritin to localize the C3b molecules on *S. pneumoniae*. The results of these experiments show that C3b is deposited in different locations by Ab with different specificities. Like nonimmune serum, immunoglobulin G (IgG) anti-cell wall Ab leads to cell wall deposition of C3b, whereas anti-capsular Ab leads to capsular C3 deposition. Thus, the site of deposition of C3b is determined by the specific-

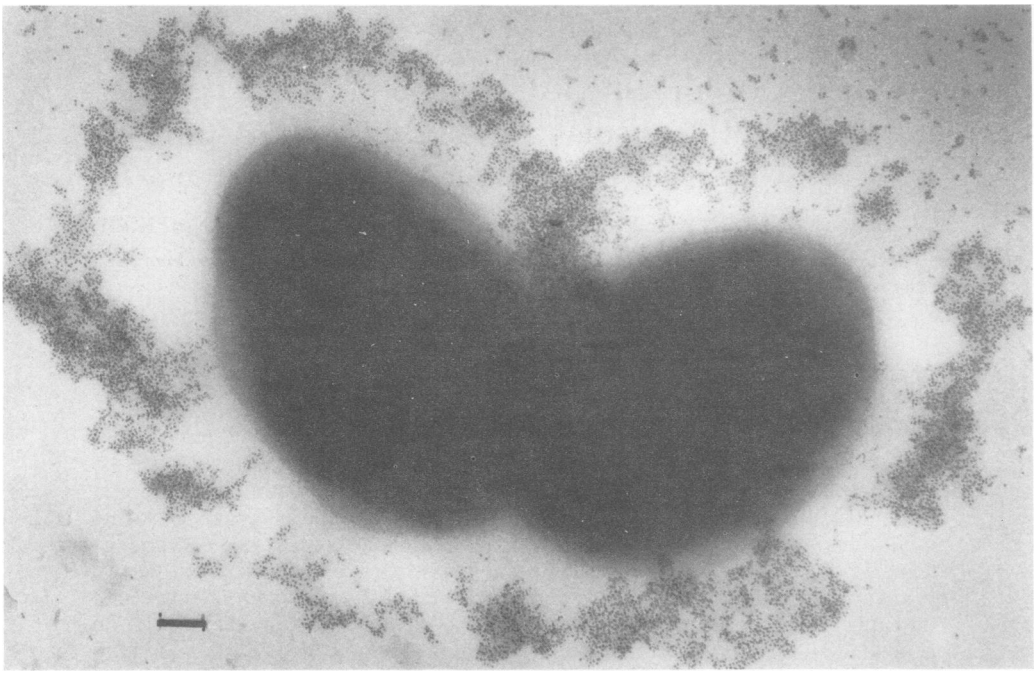

FIG. 1. *S. pneumoniae* type 7 incubated with biotinylated IgG anti-capsular Ab. The biotinylated Ab was localized by reaction with avidin-ferritin. Bar, 0.1 μm.

ity of the Ab responsible for complement activation and correlates with the opsonic efficiency of the bound C3b molecules.

MATERIALS AND METHODS

Pneumococci. *S. pneumoniae* type 7 (ATCC 6307), type 12 (ATCC 6312), and R36a (ATCC 27366) were obtained in lyophilized form from the American Type Culture Collection, Rockville, Md.; they were reconstituted and maintained as previously described (3). For these studies, *S. pneumoniae* was grown overnight in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), transferred to fresh media, and grown into log phase for approximately 4 h. The organisms were harvested, washed three times with normal saline, and then incubated for 20 min at room temperature in 0.8% glutaraldehyde in normal saline (Kodak Corp., Rochester, N.Y.). After four washes with normal saline, the organisms were stored at 4°C. These organisms were no longer susceptible to autolysis, and capsular integrity was well preserved, as assessed by the minimal loss of radiolabeled anti-capsular Ab on subsequent repeated washings. All glutaraldehyde-treated *S. pneumoniae* was used within 2 weeks of preparation.

Antibodies. IgG and IgM Ab with specificity for the capsules of *S. pneumoniae* type 12 or type 7 or for *S. pneumoniae* cell wall determinants were prepared as previously described (3). Capsular specificity of the Ab against type 12 and type 7 was demonstrated in the following ways: (i) binding of ^{125}I-labeled Ab to whole organisms was unaffected by prior absorbtion with R36a, an unencapsulated *S. pneumoniae*; and (ii) binding of Ab to whole *S. pneumoniae* organisms could be abolished by preincubation of the antisera with purified capsular polysaccharide of the appropriate type (kindly provided by James Hill, National Institute of Allergy and Infectious Diseases, Bethesda, Md.). Anti-capsular and anti-cell wall IgG were also biotinylated as previously described (1). Goat Ab to human C3 was prepared by injection of a goat with 100 μg of purified human C3 in complete Freund adjuvant, followed by two further injections over 6 months. The IgG fraction of goat anti-C3 plasma was purified by octanoic acid precipitation (9). Anti-Forssman Ab was prepared in rabbits by standard techniques (8).

Complement components. Guinea pig C1 was purchased from Cordis Laboratories, Miami, Fla. Functionally pure human C4 and C2 were prepared as previously described (5). Biochemically pure C3 was prepared and biotinylated as previously described (2). In some experiments, C3 and biotinyl-C3 were radiolabeled with ^{125}I via the Bolton-Hunter reagent, as previously described (3), to a specific activity of 10^5 cpm/μg without loss of hemolytic activity.

Sensitization of *S. pneumoniae*. Glutaraldehyde-treated *S. pneumoniae* organisms (1×10^9 to 2×10^9 colony-forming units per ml) were incubated with sufficient IgM or IgG Ab to bind approximately 1,000 molecules of IgM or 10,000 molecules of IgG per colony-forming unit, as determined by uptake of radiolabeled Ab. Organisms were then sensitized with C1, C4, C2, and C3, as previously described (3), except that human C4 at about 100 effective molecules per organism, human C2 at 100 sites per organism, and biotinyl-C3 at 500 to 1,000 sites per organism were

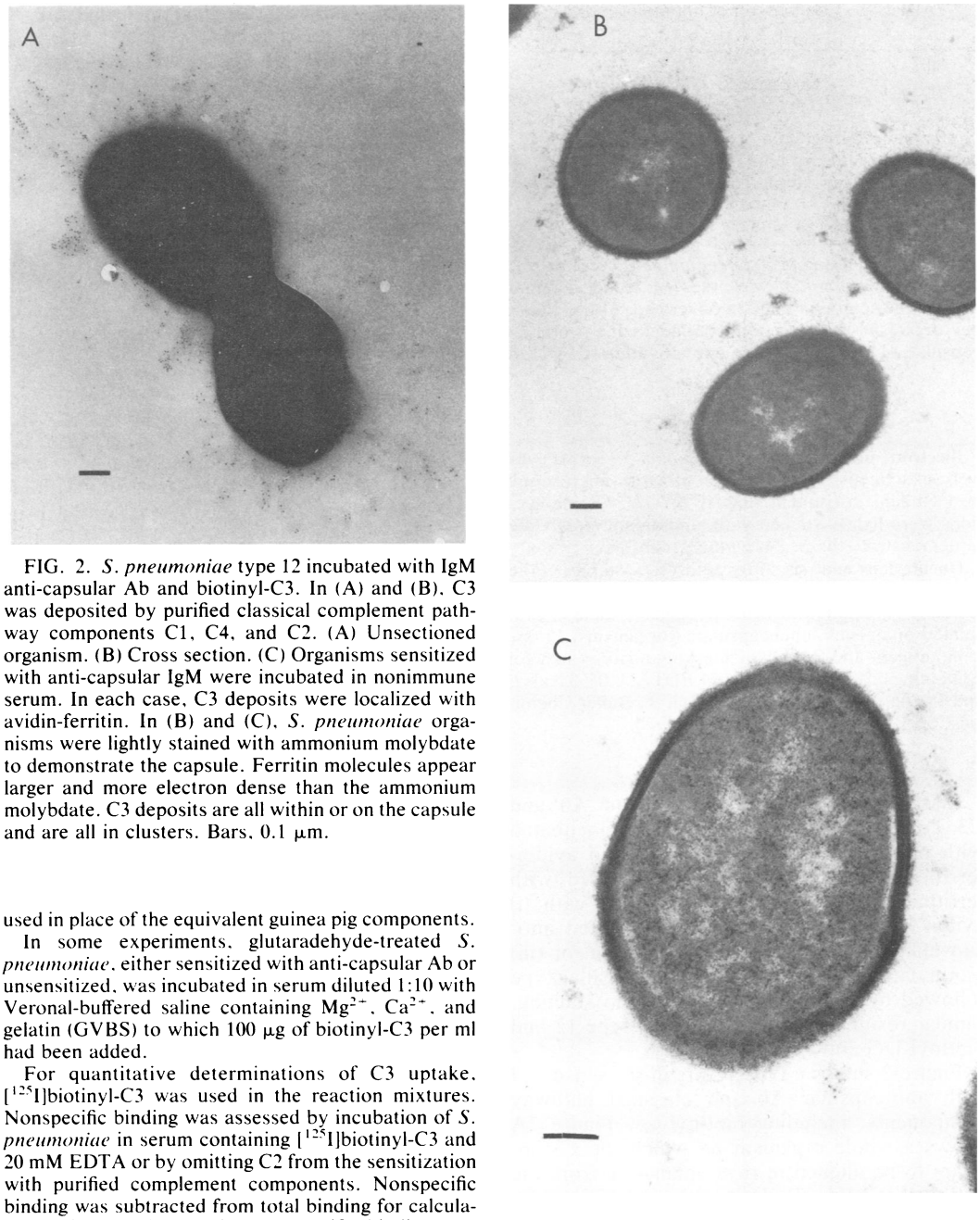

FIG. 2. *S. pneumoniae* type 12 incubated with IgM anti-capsular Ab and biotinyl-C3. In (A) and (B), C3 was deposited by purified classical complement pathway components C1, C4, and C2. (A) Unsectioned organism. (B) Cross section. (C) Organisms sensitized with anti-capsular IgM were incubated in nonimmune serum. In each case, C3 deposits were localized with avidin-ferritin. In (B) and (C), *S. pneumoniae* organisms were lightly stained with ammonium molybdate to demonstrate the capsule. Ferritin molecules appear larger and more electron dense than the ammonium molybdate. C3 deposits are all within or on the capsule and are all in clusters. Bars, 0.1 μm.

used in place of the equivalent guinea pig components.

In some experiments, glutaradehyde-treated *S. pneumoniae*, either sensitized with anti-capsular Ab or unsensitized, was incubated in serum diluted 1:10 with Veronal-buffered saline containing Mg^{2+}, Ca^{2+}, and gelatin (GVBS) to which 100 μg of biotinyl-C3 per ml had been added.

For quantitative determinations of C3 uptake, [^{125}I]biotinyl-C3 was used in the reaction mixtures. Nonspecific binding was assessed by incubation of *S. pneumoniae* in serum containing [^{125}I]biotinyl-C3 and 20 mM EDTA or by omitting C2 from the sensitization with purified complement components. Nonspecific binding was subtracted from total binding for calculations of molecular uptake; nonspecific binding was always less than 10% of specific binding.

In some experiments, glutaraldehyde-treated *S. pneumoniae* was incubated with biotinylated Ab without additional complement components. For Ouchterlony analysis of C3 binding, 5×10^9 live encapsulated organisms were incubated in 5 ml of serum diluted 1:10 in GVBS and washed three times with ice-cold normal saline. Autolysis was then allowed to proceed for 30 min at room temperature in 2% deoxycholate. This autolysate of opsonized *S. pneumoniae* was used as the antigen in Ouchterlony analysis of C3 deposition.

C3 localization. After sensitization of glutaraldehyde-treated *S. pneumoniae* with isolated classical pathway components or with serum, the organisms were incubated with avidin-ferritin (E-Y Laboratories, San Mateo, Calif.) for 30 min at room temperature with occasional agitation. Before use, avidin-ferritin was centrifuged for 10 min at 12,000 × *g* to remove aggregates. The organisms were then washed four times with phosphate-buffered saline and two times with 2% ammonium acetate before electron microscopy.

TABLE 1. Comparison of uptake of radiolabel with electron microscopic visualization of biotinylated, radiolabeled C3[a]

Incubation	^{125}I bound (cpm/10^9 CFU)	Molecules of biotinyl-C3 per CFU	Avidin-ferritin visualized by electron microscopy
Nonimmune serum alone	32,000	1,200	−
IgM anti-capsular Ab plus nonimmune serum	32,500	1,210	+
IgM anti-capsular Ab plus C1-C3	26,000	980	+
IgG anti-capsular Ab plus C1-C3	25,500	970	+
IgG anti-cell wall Ab plus C1-C3	29,000	1,100	−

[a] After incubation of *S. pneumoniae* type 12 with various anti-*S. pneumoniae* Ab, the uptake of [^{125}I]biotinyl-C3 onto *S. pneumoniae* was assessed. Either nonimmune serum or purified classical pathway components were used as the source of the C3 convertase. Although in each case equivalent numbers of C3 molecules were bound per organism, avidin-ferritin bound to the biotinyl-C3 only when *S. pneumoniae* was sensitized with anti-capsular Ab before complement activation. CFU, Colony-forming units.

Electron microscopy. *S. pneumoniae* organisms were examined by transmission electron microscopy in a Hitachi instrument, model HY-11C. Some sections were lightly stained with ammonium molybdate to demonstrate the *S. pneumoniae* capsule.

Ouchterlony analysis. After autolysis, samples of the sensitized, solubilized *S. pneumoniae* organisms were tested for precipitation with Ab against whole encapsulated organisms, unencapsulated organisms, Forssman antigen, and C3 by Ouchterlony analysis in a gel containing 0.9% agarose, 20 mM EDTA, 0.05% NaN_3, and 1% polyethylene glycol 4000 (J. T. Baker Chemical Co., Phillipsburg, N.J.).

RESULTS

Electron-microscopic localization of Ab and C3. Figure 1 shows a type 7 organism incubated with biotinylated anti-capsular Ab and avidin-ferritin. The capsule is densely stained with ferritin. Control organisms incubated with (i) avidin-ferritin alone, (ii) non-biotinylated anti-capsular Ab followed by avidin-ferritin, or (iii) biotinylated Ab against a different capsular type followed by avidin-ferritin showed no staining. Similar results were obtained with type 12 and biotinyl-IgG anti-type 12.

Figure 2 shows a type 12 organism sensitized with anti-capsular Ab and classical pathway components, including biotinyl-C3. Figure 2A shows a whole organism on which the C3 appears to be aligned in rays emanating from the cell wall. Figure 2B shows a cross section of a similarly sensitized organism showing that the ferritin is localized in small clusters, all at a distance from the bacterial cell wall. Similar results were obtained with type 7 organisms with anti-capsular Ab and biotinyl-C3. Controls incubated with (i) unbiotinylated C3 and (ii) biotinyl-C3 but no C2 showed no binding of avidin-ferritin.

Similar experiments with anti-cell wall IgG to sensitize encapsulated organisms did not show uptake of avidin-ferritin whether the biotin was bound to Ab or to C3. Encapsulated organisms were also incubated in human serum without detectable antibodies to type 7 or type 12 capsular polysaccharide (7) to which biotinyl-C3 had been added. In this situation, almost no binding of avidin-ferritin was detected, although occasional ferritin granules could be seen at the cell walls of sectioned organisms. In contrast, C3 biding to pneumococcal capsule could be detected if the organisms were incubated with anti-capsular Ab (either IgG or IgM) and then washed before incubation in nonimmune serum (Fig. 2C). To ensure that binding of the biotinylated components to the organisms was occurring under the conditions in which no ferritin was seen in association with *S. pneumoniae*, the biotinylated Ab and biotinylated C3 were radiolabeled with ^{125}I by the method of Bolton and Hunter. Comparison of the uptake of these radiolabeled components with radiolabeled unbiotinylated components showed that they bound to the organisms equally efficiently (data not shown). Anti-cell wall IgG mediated the binding of [^{125}I]biotinyl-C3 to *S. pneumoniae* as efficiently as anti-capsular IgG, as previously described (3). When *S. pneumoniae* was incubated in serum, equal amounts of [^{125}I]biotinyl-C3 were bound in the absence and presence of anti-capsular IgM, yet ferritin binding was seen only when the Ab was present (Table 1). Thus, the failure to demonstrate avidin-ferritin binding did not result from poor uptake of the biotinylated components when anti-cell wall IgG or nonimmune serum was used.

To determine whether avidin-ferritin was inhibited from binding to biotinyl-IgG or C3b bound to the cell wall, unencapsulated *S. pneumoniae* R36a was incubated with biotinyl-IgG anti-cell wall Ab (Fig. 3A) or with serum containing biotinyl-C3 (Fig. 3B). In both cases, avidin-ferritin was easily demonstrated to bind to the cell wall. Hence, cell wall binding of

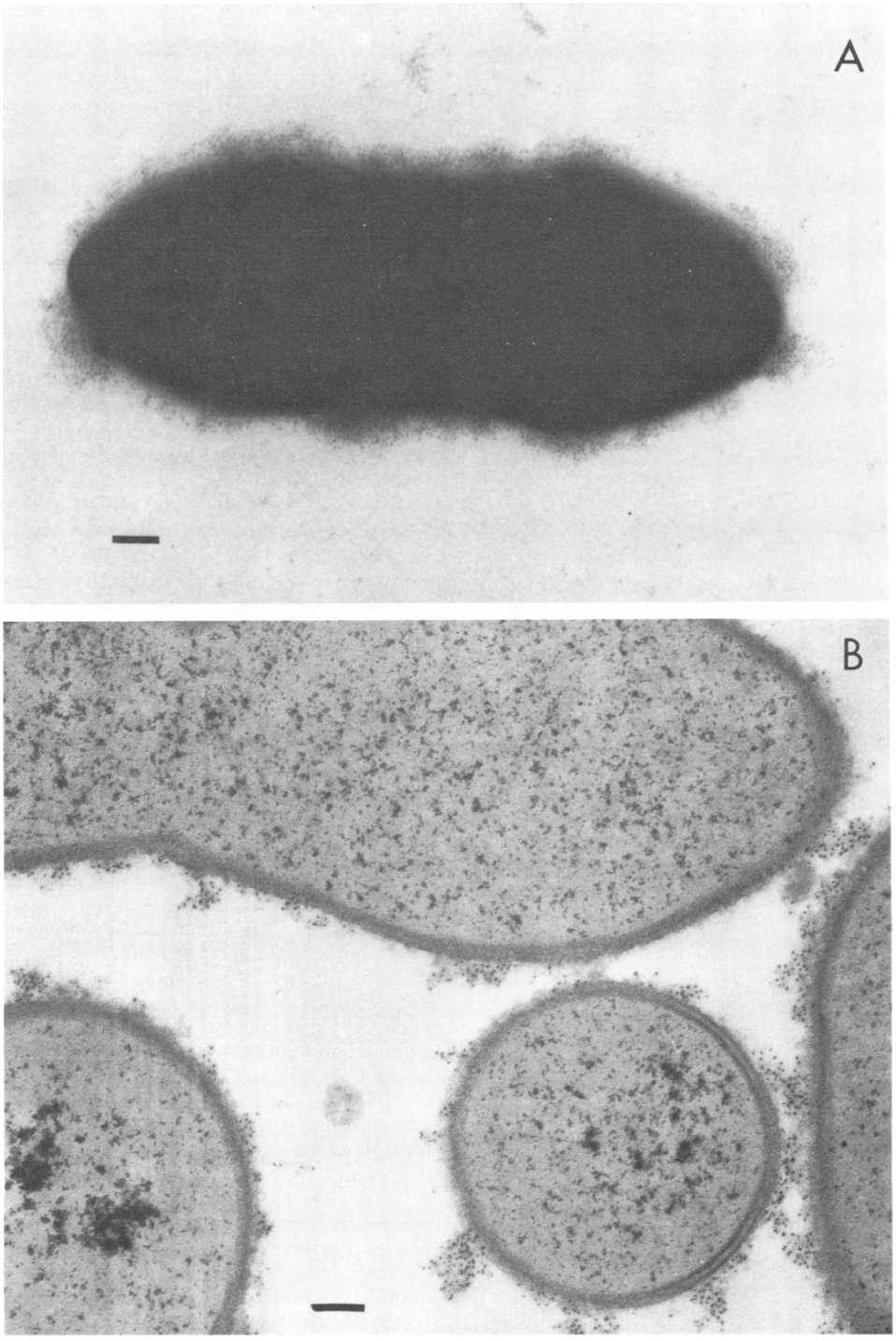

FIG. 3. Cell wall localization of Ab and C3 on unencapsulated organisms. Unencapsulated *S. pneumoniae* R36a incubated with either biotinyl-IgG (A) or serum containing biotinyl-C3 (B) was reacted with avidin-ferritin to localize Ab and C3. The ferritin is all deposited at the cell wall. Bars, 0.1 μm.

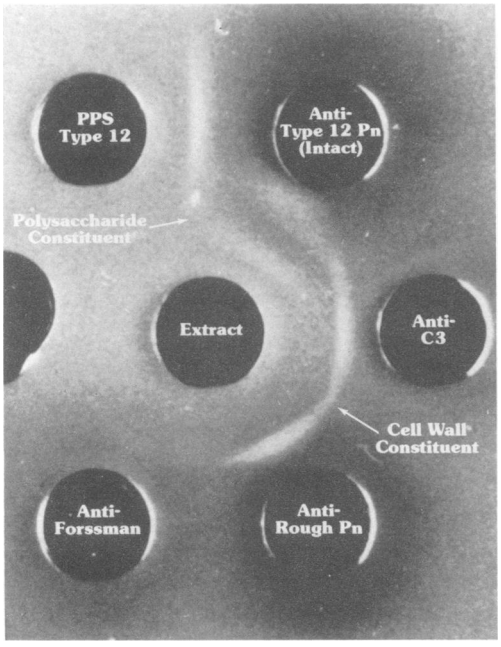

FIG. 4. Ouchterlony analysis of C3 deposition on encapsulated *S. pneumoniae* (Pn) in nonimmune serum. PPS type 12 is the purified capsular polysaccharide of *S. pneumoniae* type 12; it is used to identify the polysaccharide constituent of the *S. pneumoniae* anti-type 12 response. The precipitin line between anti-C3 and autolyzed *S. pneumoniae* makes a line of identity with Ab to *S. pneumoniae* cell walls but not Ab to *S. pneumoniae* capsule. C3 is deposited on the cell walls of encapsulated organisms when they are incubated in nonimmune serum. The Forssman antigen is present in *S. pneumoniae* cell walls (4), but anti-Forssman antibody is only weakly precipitating in this system.

biotinylated proteins did not inhibit the association of biotin and avidin. From these experiments, we concluded that the inability to see ferritin binding on encapsulated *S. pneumoniae* that was incubated with anti-cell wall IgG or nonimmune serum resulted from a failure of avidin-ferritin to penetrate the *S. pneumoniae* capsule. Therefore, immunochemical methods were used to localize deposited C3 under these circumstances. Samples of an autolysate of *S. pneumoniae* type 12 were used in double diffusion against antisera specific for cell wall antigens, capsular antigens, and C3. As shown in Fig. 4, the precipitin lines formed between the autolysate and anti-C3, antiserum to rough *S. pneumoniae*, and the noncapsular constituent of anti-type 12 (prepared against whole organisms) show complete identity. There was no difference in the Ouchterlony pattern when the pneumococcal autolysate was first centrifuged at 12,000 $\times$ *g* for 10 min to remove particulate material. Thus, C3 deposition by nonimmune serum occurs on the *S. pneumoniae* cell wall.

DISCUSSION

Winkelstein et al. have previously reported that C3 deposited by the alternative pathway in guinea pig serum is bound at the *S. pneumoniae* cell wall (11). These workers have also shown that purified *S. pneumoniae* cell walls, and indeed purified *S. pneumoniae* teichoic acid, can be initiators of alternative pathway activation in serum (14, 15). The work reported here extends Winkelstein's observation to show that, in nonimmune human serum with both classical and alternative complement pathways intact, C3 binds to the *S. pneumoniae* cell wall but not detectably to the capsule. This is consistent with the hypothesis that the alternative pathway is the primary mechanism for complement deposition on *S. pneumoniae* organisms in nonimmune serum (13). Additionally, Wilkinson et al. have shown that C3b is deposited on the cell wall of encapsulated staphylococci by alternative pathway activation in human serum (10). Thus, when these two encapsulated gram-positive organisms activate the alternative pathway, C3b is deposited at the cell wall below the capsule. Our previous work has shown that C3b bound to *S. pneumoniae* by anti-cell wall Ab interacts less efficiently with C3b receptors (3). Taken together, these data suggest that a major reason for the increased virulence of encapsulated compared with unencapsulated *S. pneumoniae* is that capsular polysaccharide interferes with recognition of cell wall-bound C3b by the C3b receptors of phagocytic cells.

We have also demonstrated that anti-capsular Ab mediates C3b deposition on the *S. pneumoniae* capsule. This effect may be achieved by either IgM or IgG anti-capsular Ab with purified proteins of the classical pathway. C3b is also deposited on the capsule when Ab-sensitized *S. pneumoniae* is incubated in serum with intact classical and alternative complement pathways. Since C3b will not be deposited onto the *S. pneumoniae* capsule efficiently in the absence of anti-capsular Ab, this represents a binding site for C3b which is available only when specific Ab is present. Anti-capsular Ab-mediated clearance of *S. pneumoniae* in vivo depends on complement activation (3). Hence, the greatly enhanced in vivo bacterial clearance caused by type-specific Ab must be mediated via these capsule-bound C3b molecules, and these must also be the molecules previously shown to bind very efficiently to C3b receptors.

The alternative pathway is activated by *S. pneumoniae* cell wall constituents whether or not a capsule is present, but the C3b deposited is an effective opsonin only in the absence of a

capsule. Hence, the great survival advantage of encapsulated *S. pneumoniae* in humans, the only natural reservoir for this organism, may be explained by the interference of encapsulation with normal host defense mechanisms. In this context, it is not surprising that most *S. pneumoniae* capsules do not activate the alternative pathway (12), since this would lead to deposition of C3b on the capsule. On the other hand, development of type-specific Ab restores to the host the ability to put C3b on the true outer surface of *S. pneumoniae*, so that complement can efficiently opsonize encapsulated bacteria for destruction.

ACKNOWLEDGMENTS

We thank Michael Frank for helpful advice, Donna Martin for excellent secretarial assistance, and Karen Leighty for excellent editorial assistance.

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