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Cross-Reactions between Pneumococci and Other Streptococci Due to C Polysaccharide and F Antigen

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By serological methods, all 83 known types of *Streptococcus pneumoniae* could be shown to possess C polysaccharide and F antigen. Cross-reactions due to these two antigens between pneumococci and a broad range of most other commonly encountered streptococci were examined. The presence of an antigen closely similar or identical to pneumococcal C polysaccharide was demonstrated in some strains of *Streptococcus mitior*. Therefore, we conclude that pneumococci cannot be identified serologically from mixed samples without culture. Streptococcal group C antiserum was found to cross-react with pneumococcal F antigen.

In 1930 Tillett et al. isolated a polysaccharide antigen from strains of *Streptococcus pneumoniae* that was not a capsular polysaccharide (28). This antigen was first named "C" substance and was later, more accurately, designated pneumococcal C polysaccharide (C-Ps). Since C-Ps was found in pneumococci of capsular types 1, 2, and 3 and in several strains of rough pneumococci, it was concluded that the C-Ps is a species-specific antigen common to all pneumococci (28). In addition to C-Ps, which is a cell wall constituent (29), pneumococci possess another antigen, which, apparently, is C-Ps covalently linked to a lipid moiety of the plasma membrane. It is called F antigen due to its "Forssman reactivity"; i.e., it is a heterophile.

Cross-reactions are known to occur between pneumococci and other nonhemolytic streptococci (1, 2, 15, 17, 18, 24a, 31). Some streptococci possess polysaccharide capsules which chemically are closely similar or identical to pneumococcal polysaccharide capsules (15), and they therefore give a clear-cut capsular reaction with pneumococcal typing antisera (15, 17, 18, 22). More often, cross-reactions between pneumococci and other streptococci are observed when testing is carried out by counterimmunoelectrophoresis (10, 27), coagglutination (co-A) (4, 5, 30), or latex agglutination (10, 20).

In the present study, we have examined pneumococci of all 83 presently known types for the presence of C-Ps and F antigen, and we have studied the nature of the cross-reactions between pneumococci and other streptococci.

MATERIALS AND METHODS

Bacterial strains. The 495 streptococcal strains examined are listed in Table 1. Of these, 358 strains were clinical isolates received at the Streptococcus Department, Statens Seruminstitut, Copenhagen, Denmark, for identification and for typing; 137 strains were stock strains from the Streptococcus Department, Statens Seruminstitut, or from culture collections. In addition, 23 strains of streptococci described by others as being cross-reactive with pneumococci were examined. These strains are listed in Table 2.

A pneumococcal C-mutant strain (CSR, SCS-2, clone 1) with a C-Ps capsule was used for the preparation of C-Ps (26). For co-A, a protein A-rich strain of *Staphylococcus aureus*, Cowan 1 (NCTC 8530; National Collection of Type Cultures, London, United Kingdom), was used.

Antisera. Antisera were raised by repeated intravenous injections in rabbits of Formalin-treated whole pneumococci, using the immunization schedule for production of diagnostic pneumococcal antisera described elsewhere (19). Anti-C-Ps antiserum was prepared and characterized as previously described (26). For production of streptococcal group C antiserum, antigen was prepared by heating bacterial cells for 1 h at 127°C. For use in immunoelectrophoresis, the immunoglobulin fractions were prepared from the antisera and from normal rabbit serum by precipitation with ammonium sulfate (1.8 M; pH 6.5) and ion-exchange chromatography (acetate buffer, pH 5.0; ionic strength, 0.05) on a DEAE-Sephadex A50 column (Pharmacia, Uppsala, Sweden).

co-A test. With some minor modifications, the co-A method described by others was used (5, 13). The Cowan 1 strain was grown for 16 h in 100 ml of trypsin broth, centrifuged, and suspended in 5 ml of saline. A 50-μl portion of antiserum (approximately 1 mg of immunoglobulin) was added. After 30 min of incubation at room temperature, the co-A reagent was ready for use. Because live staphylococci were coated with antibody, the reagents were stable only for a limited period, and new reagents were prepared every week.

The co-A test was performed on a glass slide by mixing a loopful of co-A reagent with a loopful of supernatant from a 16-h trypsin broth culture of the streptococcal strain to be examined. Distinct and immediate agglutination was registered as positive, and no or only weak agglutination occurring after several minutes was registered as negative. Trypsin broth and saline were used as negative controls.

co-A tests were performed with reagents prepared from anti-C-Ps antiserum and antistreptococcal group C antiserum.

Immunoelectrophoresis. Rocket and crossed immunoelectrophoreses of polysaccharides were carried out by using 1% agarose in Tris-Veronal buffer (pH 8.6) with an ionic strength of 0.02 as previously described (26, 27), applying antigen

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TABLE 1. Streptococcal strains

Type or group	Species	No. of strains examined	Representative strain(s)[a]
1–48 (total of 83 types)	*S. pneumoniae*	83	SSI, the 83 reference type strains
	S. pneumoniae[b]	142	
NT[c]	*S. pneumoniae*	4	ATCC 12213
NT	*S. pneumoniae*[b]	7	
A	*S. pyogenes*	1	NCTC 8198
A	*S. pyogenes*[b]	28	
B	*S. agalactiae*	2	NCTC 9993
B	*S. agalactiae*[b]	39	
C	*S. dysgalactiae*	1	NCTC 4335
C	*S. equi*	1	NCTC 9682
C	*S. equisimilis*	1	NCTC 7136
C	*S. zooepidemicus*	1	SSI CN 1393
C	*Streptococcus* sp.[b,d]	39	
D	*S. bovis*	2	ATCC 9809
D	*S. equinus*	1	NCTC 10386[e]
D	*S. faecalis*	1	NCDO 581
D	*S. faecium*	1	NCDO 942
D	*S. suis*	3	SSI 8074[e]
D	*Streptococcus* sp.[b, d]	8	
E	*S. lentus*	1	ATCC 12390
E	*S. lentus*[b]	5	
F, I–V[f]	*Streptococcus* sp.[d]	5	NCTC 10707
F	*Streptococcus* sp.[b, d]	5	
G	*S. anginosus*	2	NCTC 10713
G	*Streptococcus* sp.[b, d]	36	
H	*S. sanguis*	2	ATCC 12396
H	*S. sanguis*[b, d]	1	
K	*S. mitior*	1	NCTC 10232
K	*S. mitior*[b]	7	
L	*Streptococcus* sp.[d]	1	NCTC 10238
M	*S. mitior*	1	SSI Lindstrøm
N	*S. cremoris*	1	NCDO 607
N	*S. lactis*	1	NCDO 605
O	*S. mitior*	1	NCTC 8029
O	*S. mitior*[b]	6	
P	*Streptococcus* sp.[d]	1	SSI Thal & Moberg 34
Q	*S. avium*	1	NCTC 9938
R, S, and T	*S. suis* (see group D)		
U	*Streptococcus* sp.[d]	1	SSI U 49G
V	*Streptococcus* sp.[d]	1	SSI 5909
W	*Streptococcus* sp.[d]	1	SSI S.S.995
X	*Streptococcus* sp.[d]	1	SSI S.S.997
Z	*S. inae*	1	SSI S.S.1056
NG[g]	*S. acidominimus*	1	NCDO 2025
NG	*S. constellatus*	1	ATCC 27823
NG	*S. intermedius*	1	ATCC 27335
NG	*S. milleri*	2	NCTC 10708[e]
NG	*S. mitior*	1	ATCC 10557[h]
NG	*S. mitior*[b]	35	
NG	*S. morbillorum*	1	NCTC 11323
NG	*S. mutans*	1	NCTC 10449
NG	*S. raffinolactis*	1	NCDO 617
NG	*S. salivarius*	1	ATCC 13419
NG	*S. thermophilus*	2	NCDO 593

[a] ATCC, American Type Culture Collection, Rockville, Md.; NCDO, National Collection of Dairy Organisms, Reading, United Kingdom; NCTC, National Collection of Type Cultures, Colindale, London, United Kingdom; SSI, Statens Seruminstitut, Copenhagen, Denmark.
[b] Clinical isolates.
[c] NT, Nontypable, i.e., rough nonencapsulated strains.
[d] Strains that were grouped but not further specified.
[e] These strains are also included in Table 3.
[f] Ottens types I to V.
[g] NG, Nongroupable.
[h] This strain was originally deposited as *S. sanguis*.

preparations to the wells, and with anti-C-Ps or antistreptococcal group C antiserum incorporated in the antibody-containing gels. Due to the rather large volumes of antisera added to the intermediate gels, 2% agarose was used for their preparation.

Antigens. Supernatants from 16-h trypsin broth cultures were used as antigens in co-A tests. Todd-Hewitt and serum broths could not be used for the culture of antigen since these broths themselves gave a positive reaction with some of the co-A reagents.

Trichloroacetic acid (TCA) extracts were prepared as follows. Cells harvested from 50 ml of Formalin-killed (2%, vol/vol) 6-h-old trypsin broth cultures were suspended in 1.5 ml of TCA solution of various concentrations. The suspensions were incubated at 4°C for 16 h. After centrifugation, ethanol was added to the supernatants to a final concentration of 80% (vol/vol). After incubation overnight at 4°C, the precipitate was collected by centrifugation and dissolved in 100 μl of distilled water. Extractions with HCl and hot formamide were made according to the original descriptions by Lancefield (14) and Fuller (8), using 0.1 N HCl and formamide at 160°C, respectively. The TCA, Lancefield, and Fuller extracts were examined by rocket immunoelectrophoresis.

Purified C-Ps was prepared from autolysate of the pneumococcal C mutant strain as previously described (26). Pneumococcal F antigen was prepared from pneumococcal plasma membranes as follows. Pneumococci were collected from overnight trypsin broth culture (37°C) by centrifugation. The bacterial cells were autolyzed by suspension in 1/100 of the original volume of a 0.1% solution of deoxycholate in 0.15 M Tris buffer, pH 7.5. After lysis, the pH was adjusted to 5.0 with acetic acid and the bacterial debris was collected by centrifugation. The precipitates from several cultures of different types of pneumococci were pooled and washed three times with a large excess of 0.05 M acetate buffer, pH 5.0. From the insoluble fraction collected by centrifugation after the final washing the F antigen was prepared as described by others (7).

A 50-μg portion of F antigen was kindly supplied by Alexander Tomasz, The Rockefeller University, New York, N.Y., and dissolved in 100 μl of distilled water.

The F-antigen preparations were examined by crossed immunoelectrophoresis.

Trypsin broth. Trypsin broth was prepared essentially as described by Pope and Smith (23), the only modification being that the trypsin digestion was discontinued when an analysis had shown the amino-nitrogen content to be at least 1 mg/ml.

Chemicals. All chemicals used were of pure grade from commercial sources.

RESULTS

The presence of C-Ps in extracts of strains of different streptococcal species was first examined by means of rocket immunoelectrophoresis with anti-C-Ps antibody in the gels (Fig. 1). Although preliminary experiments showed that C-Ps could be extracted from pneumococci by both Fuller and Lancefield extractions, the most distinct precipitates were obtained with TCA extracts. Only TCA concentrations higher than 25% (wt/vol) were able to extract C-Ps, and the best results were obtained with 60% (wt/vol) TCA, which, therefore, was chosen for the present study. With purified C-Ps the detection limit of rocket immunoelectrophoresis was found to be 5 μg of C-Ps per ml.

TABLE 2. co-A test reactions for C polysaccharide in streptococcal strains

Strains[a]	No. of strains with positive reaction/ no. tested
Pneumococci	
Encapsulated	225/225
Nonencapsulated	11/11
Other streptococci	
Groups A–Z, except K, M, and O	0/196
Different nonhemolytic species, except *S. mitior*	0/11
S. mitior	
Groups K, M, and O and nongroupable strains	18/52

[a] Strains listed in Table 1.

We then compared Co-A of culture supernatants, performed with a reagent prepared with anti-C-Ps antiserum, with rocket immunoelectrophoresis of TCA extracts. A total of 87 pneumococci (representing 83 different types and 4 rough strains) were all positive in both tests, and 54 other streptococci (representing groups A to Z, except K, M, and O, and different nonhemolytic species except *Streptococcus mitior*) were all negative in both tests. Of 38 *S. mitior* strains (representing serogroups K, M, and O and nongroupable strains), 9 were positive in both tests and 29 were negative in both tests. Thus, the results obtained by the methods were in full agreement. The detection limit of co-A was found to be 1 μg of C-Ps per ml.

Thereafter, a larger number of strains was examined by co-A only because it was the easiest of the two methods and at least as sensitive as rocket immunoelectrophoresis. Altogether, 495 streptococcal strains were studied, and the results obtained are given in Table 2. All pneumococci tested, covering all known types as well as rough strains, were found to possess C-Ps. With the exception of some strains of *S. mitior*, all other streptococci were negative. The *S. mitior* strains that possessed C-Ps were found among both strains belonging to serogroups K, M, and O and nongroupable strains.

In addition, we examined 23 streptococcal strains described by others to cross-react with pneumococci and found only 6 of these strains, all *S. mitior*, to possess C-Ps (Table 3).

Rabbit antistreptococcal group C antiserum was shown to react with the F antigen of pneumococci by crossed immunoelectrophoresis. With anti-C-Ps antiserum in the upper gels and normal rabbit serum in the intermediate gels (controls), F antigen and C-Ps, applied to the wells of the lower gels, precipitated in the upper gels (Fig. 2A and C). When the intermediate gels contained antistreptococcal group C antiserum, the F antigen precipitated at a lower position (Fig. 2B), whereas the C-Ps precipitate hardly was affected by this antiserum (Fig. 2D). Identical results were obtained with the two different F-antigen preparations. Addition of C-Ps to the antiserum contained in the gel (10 mg of C-Ps per ml of antiserum) did not abolish the precipitation of F antigen by antistreptococcal group C antiserum (not shown).

Of 164 pneumococcal strains tested by co-A, with a reagent prepared with antistreptococcal group C antiserum, 152 strains, representing all types of pneumococci as well as some rough pneumococcal strains, gave positive reactions. Twelve strains, however, did not react; six of these were type 3. These results demonstrate that F antigen is an antigen common to all types of pneumococci.

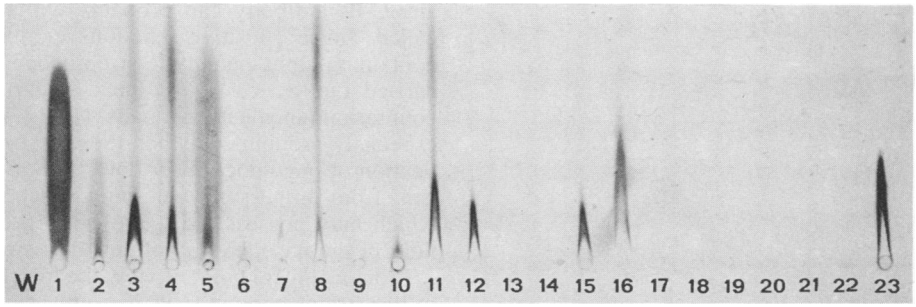

FIG. 1. Rocket immunoelectrophoresis of TCA extracts of pneumococci and streptococci. The gel contains 3 μl of anti-C-Ps antibody per cm^2 of gel area. The wells (W) contain 5-μl extracts of the following: five pneumococcal strains—C mutant (well 1), type 25 (well 2), type 6A (well 3), rough ATCC 27336 (well 4), rough ATCC 12213 (well 5); 11 strains of *S. mitior*—RA 101 (well 6), RA 96 (well 7), RA 94 (well 8), RA 92 (well 9), RA 91 (well 10), RA 88 (well 11); group O SSI 23899 (well 12), SSI 23764 (well 13), SSI 23700 (well 14), group O SSI 1360 (well 15), group M SSI Lindstrøm (well 16); one group G streptococcus, NCTC 10713 (well 17); three group C streptococci, SSI CN 1393 (well 18), NCTC 7136 (well 19), and NCTC 4335 (well 20); one group B streptococcus, NCTC 9993 (well 21); one group A streptococcus, NCTC 8198 (well 22); and as a positive control, 0.01% C-Ps (well 23).

DISCUSSION

Pneumococci are equipped with a powerful autolytic enzyme (muramyl-L-alanine amidase) that can hydrolyze the cell walls of the bacteria (16) and release cell wall components, including C-Ps, to the culture medium during growth. Therefore, the presence of C-Ps in pneumococci can simply be demonstrated by testing the supernatant of an overnight culture by co-A with a reagent prepared from anti-C-Ps antiserum. Streptococci, other than pneumococci, are not equipped with a very active autolytic enzyme and might conceivably only release cell wall material as a result of cell death in late growth phases. Representative strains of pneumococci and other streptococci were fixed by Formalin and extractions were made by treatment with cold TCA. By this method C-Ps being virtually free of protein and peptidoglycan can be prepared (29). The extracts were examined by rocket immunoelectrophoresis (Fig. 1) and compared with co-A of culture supernatants. Full agreement was found between the results obtained by the two methods. Thus, co-A was found to be as specific and as sensitive as rocket immunoelectrophoresis, and since co-A was the easiest method to perform, a larger number of strains were examined by this method.

TABLE 3. Streptococci reported to cross-react with pneumococci

Species[a]	co-A[b]	Strain no.[c]	Reference
S. equinus	−	NCTC 10386	10
S. milleri	−	NCTC 10708	10
S. milleri	−	CCUG 11668	10
S. milleri	−	SSI Hellberg	15, 18
S. milleri	−	SSI I.P.	18
S. milleri	−	SSI Sundell	15, 18
S. mitior[d]	+	CCUG 11773	24
S. mitior	+	RA 88	1
S. mitior	+	RA 91	1
S. mitior	−	RA 92	1
S. mitior	+	RA 94	1
S. mitior	+	RA 96	1
S. mitior	+	RA 101	1
S. mitior	−	SSI 321	15, 18
S. mitior	−	SSI 2015	18
S. mitior	−	SSI 6012	15, 18
S. salivarius	−	SSI A176 (=A92)	15, 17, 18
S. salivarius	−	SSI 4731	15, 18
S. salivarius	−	SSI 4907	15, 18
S. salivarius	−	CCUG 2654	10
S. suis	−	SSI 2524	22
S. suis	−	SSI 8074	22
S. suis	−	SSI 14636	15, 22

[a] Species designation given by the Streptococcus Department, Statens Seruminstitut, Copenhagen, Denmark.
[b] Carried out with a reagent prepared from anti-C-Ps antiserum.
[c] CCUG, Culture Collection, University of Göteborg, Göteberg, Sweden; NCTC, National Collection of Type Cultures; RA, Robert Austrian, University of Pennsylvania, Philadelphia; SSI, Statens Seruminstitut.
[d] All *S. mitior* strains listed are nongroupable.

C-Ps was found in all known types of pneumococci, and none of the 225 strains examined were lacking this antigen (Table 2). This result emphasizes that the conclusion of Tillett et al. (28) still holds: C-Ps is a common antigen of *S. pneumoniae*.

Besides pneumococci, only some strains of *S. mitior* were found to give positive reactions (Tables 2 and 3).

Of the 23 nonpneumococcal strains described by others to cross-react with pneumococci, only 6 nongroupable strains of *S. mitior* were found to possess C-Ps (Table 3). C-Ps has previously been described in acid extracts of five of these strains (1) that were labeled alpha-hemolytic streptococci. The sixth strain was reported by others to give a positive reaction in a C-Ps enzyme-linked immunosorbent assay (24a). Thus, some strains of *S. mitior* possess C-Ps, and C-Ps therefore is not a species-specific antigen of *S. pneumoniae* (11, 24, 24a, 28). In the enzyme-linked immunosorbent assay developed for detection of C-Ps, 36% of clinical isolates of alpha-hemolytic streptococci were found to give a positive reaction (24a); these strains were designated *Streptococcus mitis*, which according to the classification scheme currently in use in our department is synonymous with *S. mitior*. It was suggested that the observed cross-reaction was due to phosphorylcholine, a residue of the C-Ps (29), as a common antigenic determinant and not due to C-Ps itself. This postulate does not agree with our previous results, which showed that anti-C-Ps antiserum raised in rabbits was specific for C-Ps and did not react with other phosphorylcholine-containing polysaccharides (25). The C-Ps enzyme-linked immunosorbent assay (24a) is also based on rabbit anti-C-Ps antiserum, and we therefore assume that the reported cross-reaction is not due to phosphorylcholine per se but to a polysaccharide antigen present in the bacteria identical or closely similar to C-Ps. This antigen, on the other hand, also contains phosphorylcholine. Further examinations of some of our extracts that gave positive reactions

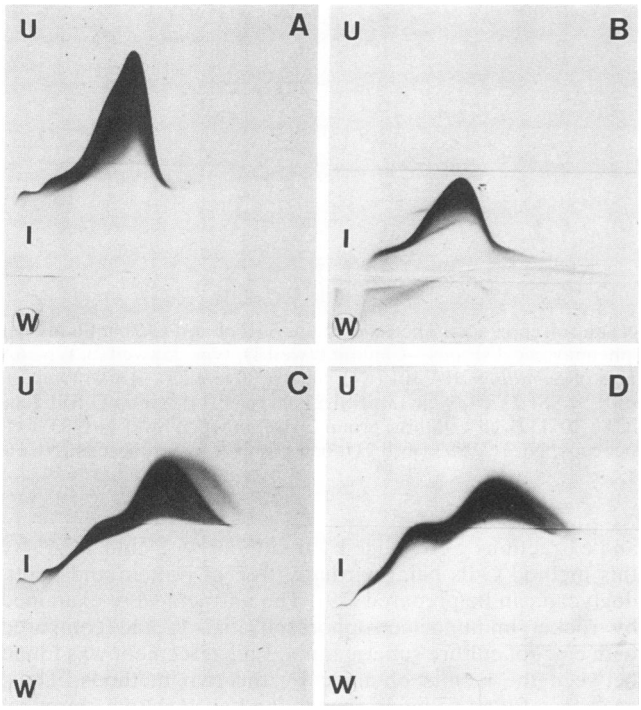

FIG. 2. Interaction between pneumococcal antigens and antistreptococcal group C antiserum examined by crossed immunoelectrophoresis. Upper gels (U): 4 μl of anti-C-Ps antibody per cm^2 of gel area. Intermediate gels (I): A and C, 60 μl of immunoglobulin from normal rabbit serum (controls); B and D, 60 μl of antistreptococcal group C antiserum per cm^2 of gel area. Wells (W) contain (A and B) 5 μl of 0.1% F antigen and (C and D) 3 μl of 0.1% C polysaccharide.

with the C-Ps co-A reagent, with a monoclonal antiphosphorylcholine antibody (25), showed the phosphorylcholine determinant to be present (not shown). This has also been shown by others (24) to be the case with some group O streptococci.

By the capsular reaction test and precipitation techniques, cross-reactions between pneumococci and other nonhemolytic streptococci due to structural similarities in capsular polysaccharides have been demonstrated for some strains of *Streptococcus milleri* (15, 18), *S. mitior* (15, 18), *S. salivarius* (15, 17, 18), and *S. suis* (22). These strains did not possess C-Ps (Table 3). A variety of clinical and stock strains of nonhemolytic streptococci was examined by others for cross-reactions with polyvalent pneumococcal antiserum (Omni-serum), using immunofluorescence, immunoelectrophoresis, and latex agglutination (10). As many as 68% of these strains, most of which were *S. mitis* (*S. mitior*), were positive in one or more of the tests. However, only 12% were positive in all three tests. Four of the strains, registered as positive by Holmberg et al. (10), were found by us (Table 3) and by others, including Holmberg (24a), not to possess C-Ps. Holmberg and co-authors consider the reaction of these four strains to be due to capsular polysaccharides.

Diagnostic pneumococcal type and group antisera are prepared by intravenous injections of whole bacterial cells (19); these, therefore, contain varying amounts of anti-C-Ps antibody (unpublished observation). The anti-C-Ps antibody does not interfere when typing of pneumococci is carried out by the capsular reaction test (25). However, the presence of anti-C-Ps antibody can probably explain some of the so-called "false"-positive reactions exhibited by some C-Ps containing alpha-hemolytic streptococci when the tests are carried out by co-A (4), latex agglutination (10, 20), or counterimmunoelectrophoresis (10, 27). This problem is especially pronounced when these tests are used for identification of pneumococci or detection of pneumococcal antigens from throat swabs or sputa, since strains of *S. mitior*, which may possess C-Ps, are often present in the normal flora of the nasopharynx of healthy humans.

From a clinical point of view the information that some *S. mitior* strains contain C-Ps is useful, since carriage of these strains may conceivably contribute to the development of antiphosphorylcholine antibody (9) and anti-C-Ps antibody (12, 21) found in humans. Likewise, some of the C-Ps, which has been demonstrated in sputum of some humans (11), may originate from these bacteria.

The observation that group F streptococci (types I to V) possess an antigen that cross-reacts with C-Ps (1) could not be confirmed in the present study with our own strains.

In our routine diagnostic laboratory, pneumococci have regularly been observed to react with a co-A reagent prepared with antistreptococcal group C antiserum, and this cross-reaction has also been reported by others (5, 20, 30). Since both streptococcal group C polysaccharide and pneumococcal F antigen are Forssman antigens (6, 7), we investigated whether this common feature could explain these cross-reactions. C-Ps and F antigen are quite similar and serologically cross-reactive, but the latter is unique in containing covalently bound fatty acid and in showing Forssman reactivity (3). Thus, both antigens are precipitated by anti-C-Ps antiserum (Fig. 2A and C) but only the F antigen is precipitated by antistreptococcal group C antiserum (Fig. 2B and D) due to its Forssman reactivity. Of the 164 pneumococcal strains examined for cross-reactions with antistreptococcal group C antiserum, 152 (representing all types) were positive. Six strains of type 3 and six other pneumococci were not found to cross-react with the anti-group C antiserum; this is in agreement with the findings of a previous study (20). Probably these strains also possess F antigen, but the F antigen may stick to the plasma membrane fractions and therefore not be released into the culture supernatant. Our results demonstrate that the cross-reaction between pneumococci and group C streptococci is due to the Forssman nature of the pneumococcal F antigen and that streptococcal group C antiserum reacts rather specifically with pneumococcal F antigen. This cross-reaction should not cause diagnostic problems, because of pronounced differences in the colonial morphology and pathogenicity of pneumococci and group C streptococci.

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