CAPSULATION OF PNEUMOCOCCUS WITH SOLUBLE C-LIKE (C_s) POLYSACCHARIDE

I. Biological and Genetic Properties of C_s Pneumococcal Strains

By DONALD L. BORNSTEIN, M.D., GERALD SCHIFFMAN, Ph.D., HARRIET P. BERNHEIMER, Ph.D., and ROBERT AUSTRIAN, M.D.

(From the John Herr Musser Department of Research Medicine, The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, and the Department of Medicine, State University of New York, Downstate Medical Center, Brooklyn, New York 11203)

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In 1949, Taylor (1) reported the recovery from a transformation reaction of a pneumococcus with a capsular antigen which differed both from that of the parent of the noncapsulated Type II strain which had served as the recipient cell and from that of the capsulated Type III strain which had served as the donor of deoxyribonucleates (DNA) in the system. In 1953, Beiser and Hotchkiss (2) described the isolation of several analogous strains of capsulated pneumococci from a transforming system employing the same reactants. Antisera prepared in rabbits with vaccines of these strains gave a positive capsular precipitin or Quellung reaction with homologous cells and with other capsulated variants recovered from the same transforming system which failed to react with Type III or Type II anticapsular serum. DNA from these unusual strains was shown also to be capable of transforming noncapsulated cells to the new capsular type. Further study of the novel capsular antigen failed to demonstrate a relationship between it and any of the 82 known pneumococcal capsular types. In subsequent experiments in our laboratories, a similar capsulated strain was recovered from a transforming reaction in which the recipient cells were noncapsulated variants of pneumococcus Type II; but, on this occasion, the DNA employed was extracted from capsulated Type I pneumococcal cells. Two hypotheses might account for the observations described. Uptake of foreign DNA might lead to recombination with or to rearrangement of resident DNA leading to the appearance of the novel capsular material; or the cells of the unusual capsular type might be spontaneous mutants selected during growth of cells in the transforming system. The experiments to be reported support the latter hypothesis and suggest that the novel capsule, composed of a C-like polysaccharide, may arise as a result of alteration of the normal mechanisms controlling the production of pneumococcal C or cell wall polysaccharide.

Materials and Methods

Nomenclature of Pneumococcal Variants.—The nomenclature of pneumococcal variants employed will be that described in earlier publications (3–5). Mutants of noncapsulated variants producing capsules of C-like polysaccharide will be designated by the symbols denoting the parental noncapsulated strain followed by the symbol C_s; i.e., S-$_{II}C_s$ is a variant of a noncapsulated strain of pneumococcus Type II which produces a capsule of soluble C-like polysaccharide. Binary and ternary capsulated strains having capsular components of C_s polysaccharide will be indicated in the following fashion: S-$_{III}C_s$SI-III is a ternary capsulated pneumococcus producing soluble capsular polysaccharides of Types C_s, I and III, the cell producing these polysaccharides having been derived from a noncapsulated mutant of pneumococcus Type III.

To indicate transformants, the letter T will be interposed between the designation of the parental strain and the character transformed; e.g., strain S-$_{III_4}$TC_s is a noncapsulated mutant of pneumococcus Type III which has been transformed to capsular type C_s.

Strains of Pneumococcus.—Noncapsulated variants derived from several capsular types of pneumococcus were used.

S-I phenotypes: S-$_{I_1}$ and S-$_{I_2}$ are described in reference 4. S-$_{I_4}$ is a noncapsulated mutant of the Type I pneumococcal strain SVI designated previously as strain I 192R.

S-II phenotypes: The noncapsulated variants of pneumococcus Type II employed were all sublines of a noncapsulated mutant, R36, of strain IID39S and are described in reference 5.

S-III phenotypes: The origin of strain S-$_{III_4}$ is given in reference 3, that of strains S-$_{III_5}$ and S-$_{III_6}$ in reference 5.

S-V phenotype: This strain is described in reference 5.

S-VIII phenotypes: S-$_{VIII_1}$ is strain VIIIR1H and S-$_{VIII_2}$ is strain VIIIR13 described in reference 6.

Strain S-$_{II}C_s$ was obtained from Beiser and Hotchkiss (2) and was designated strain T15BM by them.

S-$_{I_2}C_s$, S-$_{I_4}C_s$, S-$_{III_4}C_s$, S-$_{V}C_s$, S-$_{VIII_1}C_s$, and S-$_{VIII_2}C_s$: spontaneous mutants of strains S-$_{I_2}$, S-$_{I_4}$, S-$_{III_4}$, S-V, S-$_{VIII_1}$, and S-$_{VIII_2}$ producing capsules of soluble C-like polysaccharide.

Strains SI, SII, SIII, and SVIII are capsulated strains of pneumococcus Types I (SVI), II (D39S), III (A66), and VIII (B) described in reference 7. Strains SXVI, SXXVII, and SXXVIII were isolated from patients with pneumonia.

Media.—All strains were grown in beef heart infusion broth with Neopeptone (Difco Laboratories, Detroit, Mich.).

Isolation of Pneumococcal Variants Producing C_s Capsular Polysaccharide.—Noncapsulated variants of pneumococci were inoculated into 100 ml cultures of beef heart infusion broth with Neopeptone to which had been added 5% human plasma and 0.1 μg/ml of purified pneumococcal C polysaccharide. The culture was incubated at 37°C overnight and 5 ml of the supernatant fluid was centrifuged 5 min at 1000 rpm to precipitate small aggregates of agglutinated noncapsulated cells. The supernatant fluid of the 5 ml aliquot was used then to inoculate a second 100 ml culture. Variants producing C_s capsular polysaccharide were recovered usually after three passages of the type described. When noncapsulated strains with a high back mutation rate to parental capsular type were employed, antiserum to the homologous parental capsular antigen was included in the system.

Preparation of DNA and Techniques of Transformation.—The methods used were those described by MacLeod and Krauss (8) and by Bernheimer, Wermundsen, and Austrian (5).

Preparation of Anticapsular Sera.—Vaccines were made by the method described in reference 3.

Rabbits were immunized by intravenous injection of 1 ml of vaccine containing 50–200

μg of bacterial nitrogen four times a week for 2 wk and bled 6 days after the final injection of vaccine. This schedule of immunization was repeated until potent antisera were obtained.

Isolation of Filamentous Variants of Pneumococcus Producing C_s Capsular Polysaccharide.—The technique employed is described in reference 7.

Determination of M Protein in Pneumococcal C_s Capsular Variants.—The methods used for the preparation and absorption of antisera and for the extraction of M protein from pneumococcal cells are set forth in reference 6.

Preparation of C-Reactive Protein.—C-reactive protein was isolated from pleural or ascitic fluids of patients with neoplastic or inflammatory disease following precipitation of the protein with C_X polysaccharide prepared by the method of Anderson and McCarty (9). The precipitate was washed with iced normal saline containing 5 meq/liter of calcium chloride, resolubilized in 0.1 N sodium citrate and reprecipitated by dialysis against saline with calcium. After several reprecipitations, the precipitate was solubilized in citrate and was resolved by

TABLE I

Agglutination of Pneumococcal Strain S-$_{II}C_s$ by Homologous Antiserum before and after Absorption with Noncapsulated Pneumococci or with Homologous C_s Polysaccharide

Serum	Antiserum dilution								Saline control
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Anti-S-$_{II}C_s$	++++	++++	+++	++	+	+	±	−	−
Anti-S-$_{II}C_s$ absorbed with strain S-$_{II}$	±	−	−	−	−	−	−	−	−
Anti-S-$_{II}C_s$ absorbed with strain S-$_{III_4}$	++	++	±	−	−	−	−	−	−
Anti-S-$_{II}C_s$ absorbed with C_s polysaccharide	++	++	±	−	−	−	−	−	−

chromatography on DEAE–Sephadex A-50 in excess citrate. The final product was found to be free of detectable polysaccharide or of any contaminating serum proteins by immunodiffusion and by immunoelectrophoresis. Over 85% of the total nitrogen was precipitable with C polysaccharide. Immunization of rabbits with such purified preparations yielded monospecific antisera to a human C-reactive protein, producing only one band of identity with all purified samples of CRP tested, and giving no reaction with normal human serum.

EXPERIMENTAL

Serologic Evidence for the Relation of the Capsular Antigen of Pneumococcal C_s Variants to Pneumococcal C Polysaccharide.—Rabbits were immunized with a vaccine of pneumococcal strain S-$_{II}C_s$ to obtain potent antiserum to the capsular antigen of this strain. To render such antiserum type specific, it was absorbed with a vaccine of pneumococcus S-$_{II}$ to eliminate antibodies to other pneumococcal constituents. Following such treatment, the antiserum no longer agglutinated the cells of the vaccine strain S-$_{II}C_s$, used to immunize rabbits (Table I) and failed to give a positive capsular precipitin reaction as

it had prior to treatment. Absorption of the same antiserum with an heterologous strain of noncapsulated pneumococci, $S\text{-}III_4$, was followed by similar results. The findings suggested strongly that the capsular antigen of strain $S\text{-}IIC_8$ was related to a somatic antigen common to pneumococci derived from a variety of capsular types. A purified preparation of pneumococcal C polysaccharide was prepared (10), therefore; and the antiserum to pneumococcus $S\text{-}IIC_8$ was absorbed with this material. The results of this experiment were the same as those following absorption of the antiserum with vaccines of intact

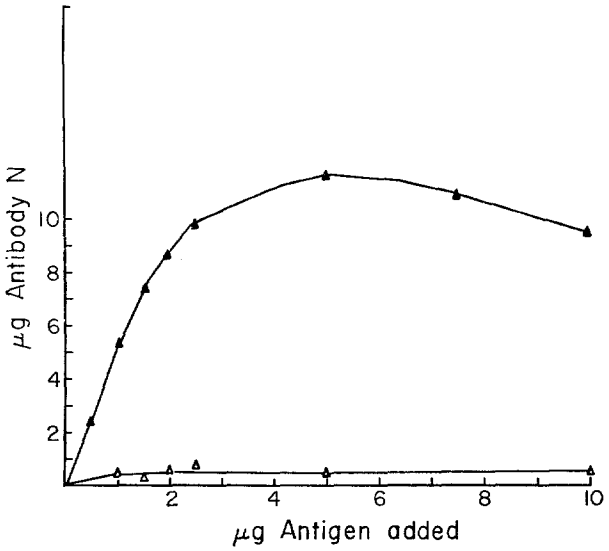

FIG. 1. Precipitation of pneumococcal C polysaccharide by antiserum to strain $S\text{-}IIC_8$. Precipitin curves obtained by adding C polysaccharide to antiserum before (▲——▲) and after (△——△) absorption with homologous C_8 polysaccharide. 100 μl of unabsorbed serum and 500 μl of absorbed serum were used.

noncapsulated pneumococci. Precipitation of C polysaccharide by antiserum to pneumococcus $S\text{-}IIC_8$ before and after absorption with C polysaccharide is shown in Fig. 1.

Antisera prepared by prolonged immunization of rabbits with noncapsulated pneumococcal strains $S\text{-}II$ and $S\text{-}III_4$ reacted with cells of strain $S\text{-}IIC_8$ in a fashion consistent with the findings noted in the preceding paragraph although sera from these rabbits were totally unreactive prior to immunization. Antisera to these two noncapsulated strains agglutinated strain $S\text{-}IIC_8$ (Table II) and each gave a positive capsular precipitin or Quellung reaction with the cells of strain $S\text{-}IIC_8$.

Capsular polysaccharide of strain $S\text{-}III_4C_8$, which produces a larger capsule

than strain S-$_{II}C_s$, was isolated from the supernatant fluid of cultures of the organism by precipitation with 1 volume of ethanol. No C polysaccharide is precipitated from supernatant fluids of cultures of noncapsulated pneumococci by this technique. Absorption of antisera to strains S-$_{II}$, S-$_{III_4}$, and S-$_{III_4}C_s$ with the capsular polysaccharide of strain S-$_{III_4}C_s$ obtained in this fashion resulted in loss of the ability of all three antisera to agglutinate strain S-$_{III_4}C_s$ (Table III). Loss of the ability of antisera to strains S-$_{II}C_s$ and strain S-$_{III_4}$ to precipitate C polysaccharide following absorption with the capsular polysaccharide of strain S-$_{II}C_s$ obtained in the same fashion is shown in Fig. 2.

TABLE II

Agglutination of Pneumococcal Strain S-$_{II}C_s$ by Antisera to Noncapsulated Pneumococci

Serum	Antiserum dilution								Saline control
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Anti-S-$_{II}$	+++	++	+	+	±	−	−	−	−
Anti-S-$_{III_4}$	+++	+++	++	++	+	±	−	−	−

TABLE III

Effect of Absorption of Antisera to Pneumococcal Strains S-$_{III}$ C_s, S-$_{III_4}$, and S-$_{II}$ with the Capsular Polysaccharide of Strain S-$_{III_4}C_s$ on Their Ability to Agglutinate Cells of Strain S-$_{III_4}C_s$

Antiserum	Antiserum dilution								Saline control
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
Anti-S-$_{III_4}C_s$	++++	++++	++++	++++	+++	++	+	−	−
Anti-S-$_{III_4}C_s$ absorbed with C_s polysaccharide	±	±	−	−	−	−	−	−	−
Anti-S-$_{III_4}$	+++	+++	+++	++	++	+	−	−	−
Anti-S-$_{III_4}$ absorbed with C_s polysaccharide	++	±	−	−	−	−	−	−	−
Anti-S-$_{II}$	+++	+++	++	+	±	−	−	−	−
Anti-S-$_{II}$ absorbed with C_s polysaccharide	±	−	−	−	−	−	−	−	−

The foregoing findings are all consistent with the conclusion that the C_s polysaccharide of these strains is related very closely to the C or cell wall polysaccharide of pneumococcus. Biochemical evidence of the close similarity of C and C_s polysaccharides will be presented in a subsequent report.

Reaction of Pneumococcal C_s Variants with Human C-Reactive Protein (CRP).—The serologic evidence indicative of similarity between the C and C_s polysaccharides suggested investigation of the reactivity of cells of pneumococcus S-$_{II}C_s$ with CRP. Microscopic examination of these cells mixed with CRP showed a positive capsular precipitin or Quellung reaction in the presence of calcium ions but not in the absence of the latter. The refractile properties of the capsule were indistinguishable from those obtained in the reaction with

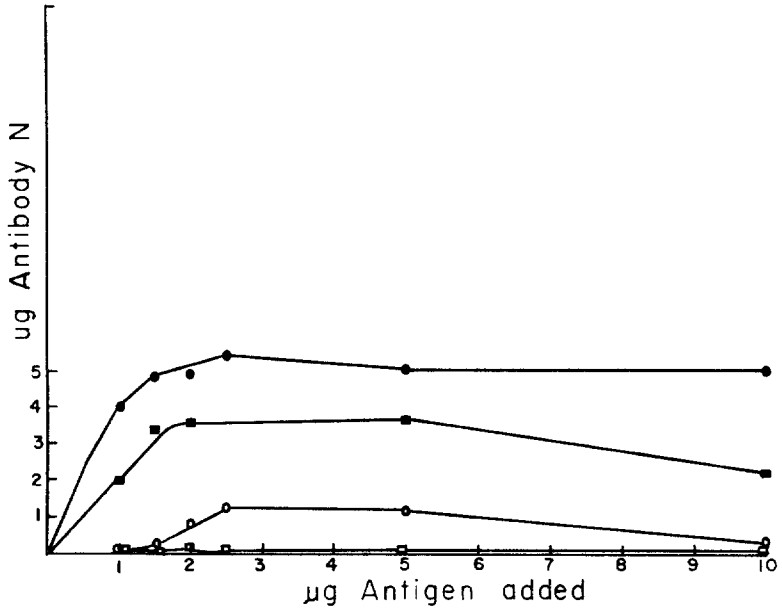

FIG. 2. Precipitation of pneumococcal C polysaccharide by antisera to noncapsulated pneumococcal strains S-$_{II}$ and S-$_{III_4}$ before and after absorption with C_s polysaccharide from strain S-$_{II}C_s$. Precipitin curves obtained by adding C polysaccharide to 100 µl of anti-S-$_{II}$ serum (■——■) or to 20 µl of anti-S-$_{III_4}$ (●——●) before absorption. Curves obtained with 100 µl of each antiserum after absorption with the C_s polysaccharide of strain S-$_{II}C_s$ are shown in open squares (anti-S-$_{II}$) and open circles (anti-S-$_{III_4}$).

type-specific antiserum. The same preparations of purified CRP gave also a positive capsular precipitin reaction with cells of pneumococcal types XVI, XXVII, and XXVIII (Table IV), confirming the observations of Löfström (11). These reactions, like those with cells of strains S-$_{II}C_s$ and S-$_{VIII_1}C_s$, were calcium dependent. Antiserum to strain S-$_{II}C_s$, unlike CRP, failed to react visibly with cells of the three other pneumococcal capsular types cited, and capsular antibody to each of the same three pneumococcal types gave no reaction with cells of strain S-$_{VIII_1}C_s$.

Cells of strain S-$_{II}C_s$ were also agglutinated by CRP in the presence of

calcium but not in its absence (Table V). The reaction was characterized by a very narrow range of agglutination by the CRP and when the standard dilutions used in agglutination tests with serum were employed, agglutination was observed in only 1 tube in which the CRP was diluted 1:320. When smaller increments of dilution were examined in this range, the phenomenom of agglutination by CRP could be demonstrated unequivocally. The agglomerates were distinctly more fragile than those produced by antiserum. Similar reac-

TABLE IV

Capsular Precipitin Reactions of Pneumococcal Types C_s, XVI, XXVII, and XXVIII with CRP and with Anticapsular Sera

Pneumococcal strain	Serological reagent					
	CRP with Ca^{++}	CRP without Ca^{++}	Anti-C_s	Anti-XVI	Anti-XXVII	Anti-XXVIII
S-$VIII_1C_s$	+	−	+	−	−	−
S XVI	+	−	−	+	−	−
S XXVII	+	−	−	−	+	−
S XXVIII	+	−	−	−	−	+

TABLE V

Agglutination of Pneumococcal Strain S-III_4C_s by Human C-Reactive Protein

Antigen	CRP dilution								Saline control
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	
S-III_4C_s	−	−	−	−	+	−	−	−	−
	1:200	1:240	1:280	1:320	1:360	1:400	1:440	1:480	
S-III_4C_s	−	+	++	++	++	+	±	−	−

tions were observed with bentonite particles coated with C_s polysaccharide. C_s polysaccharide obtained by precipitation of culture supernatant fluids of strain S-$II C_s$ with 1 volume of ethanol precipitated human CRP with the same efficiency as pneumococcal C polysaccharide when calcium was present (Fig. 3). These data also suggest the close relationship of C_s to C polysaccharide.

Origin of C_s Pneumococcal Strains.—The finding that C_s polysaccharide was related closely to the C or cell wall polysaccharide suggested that strain S-$II C_s$ might be a spontaneous mutant resulting from an alteration in the genetic control of the amount of C polysaccharide synthesized by the cell.

Most normal human and murine sera contain some antibody reacting with

pneumococcal C polysaccharide. Because human plasma or rabbit antisera to noncapsulated pneumococci are included in experiments designed to isolate capsulated transformants of pneumococcus, the selection of C_s capsular variants of pneumococcus in such experiments would appear at first to be unlikely. Examination of preparations of transforming DNA obtained by the methods employed in the experiments from which C_s variants were recovered originally showed them to contain 5–50 μg/ml of pneumococcal C polysaccharide, an amount sufficient to combine completely with all the anti-C antibodies present in most human plasmas and rabbit antipneumococcal sera. Because of these findings, attempts were made to isolate C_s variants from a variety of non-

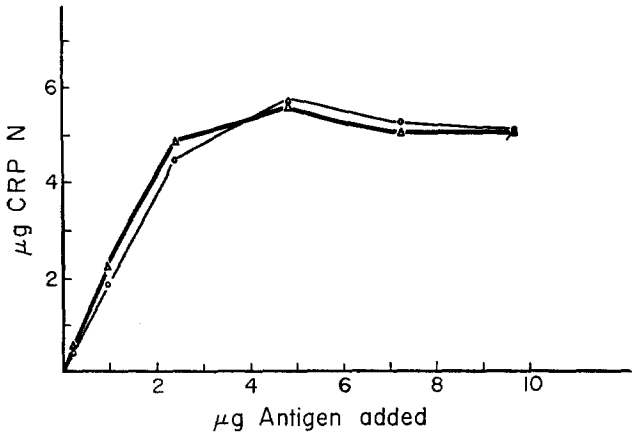

FIG. 3. Precipitation of pneumococcal C_s polysaccharide (△——△) and pneumococcal C polysaccharide (○——○) by human CRP.

capsulated pneumococcal strains by growing them in broth containing human plasma which agglutinated them in the presence of an excess of purified pneumococcal C or C_s polysaccharide which had been treated with DNAase. To cultures of the noncapsulated strains $S\text{-}_{I_2}$ and $S\text{-}_{III_4}$ which have high back mutation rates to their respective parental capsular types, antibody to the homologous capsular polysaccharide was added also to prevent the selection of capsulated Type I or Type III cells.

C_s variants were isolated from populations of seven noncapsulated pneumococcal strains: $S\text{-}_{I_2}$, $S\text{-}_{I_4}$, $S\text{-}_{II}$, $S\text{-}_{III_4}$, S-v, $S\text{-}_{VIII_1}$, and $S\text{-}_{VIII_2}$. The ease with which such mutants were recovered varied considerably from strain to strain and some required multiple passage in cultures of 100 ml before the mutant was isolated.

All C_s variants grew diffusely in broth containing human plasma in the presence or absence of added C polysaccharide, and all give a positive capsular

precipitin reaction with antibody to strain S-$_{II}C_s$. These observations demonstrate that C_s variants of pneumococcus are spontaneous mutants of this organism and that their initial discovery in experiments designed to effect pneumococcal capsular transformation was probably a fortuitous result of the neutralization of antibodies to pneumococcal C polysaccharide by the C polysaccharide present in the preparations of DNA employed.

Properties of C_s Pneumococcal Variants.—Pneumococci producing a capsule of C_s polysaccharide are stable and grow diffusely in broth like other strains of this species. On the surface of blood agar plates, they give rise to colonies which differ little in morphology from those of the noncapsulated variants from

TABLE VI

Inhibition of Capsulated, Noncapsulated, and C_s Pneumococcal Variants by Penicillin G

Parental pneumococcal type	Minimal bactericidal concentration of penicillin, *units/ml*		
	0.01	0.02	0.03
SI	S-I_2C_s	SI S-I_2	
SII	S-$_{II}$	SII S-$_{II}C_s$	
SIII		SIII S-III_4 S-III_4C_s	
SVIII		SVIII S-$VIII_1C_s$	S-$VIII_1$

which they are derived. In capsular precipitin tests, the size of the refractile halo around the cell varies from that seen in analogous reactions of pneumococcal Types I or IV with their homologous anticapsular sera to one which is just discernible.

The C_s variants undergo autolysis like other pneumococcal strains and are solubilized in the presence of surface-acting agents such as sodium deoxycholate. Because of the similarity of C and C_s polysaccharide and the association of the former with the cell wall mucopeptide of pneumococcus, the resistance of several C_s variants of pneumococcus to penicillin was investigated. The data are summarized in Table VI and demonstrate no increase in resistance of the C_s variants to the antibiotic over that of the noncapsulated strains from which they were derived.

Because of the close similarity of the C_s capsular material to the C or cell wall polysaccharide of pneumococcus, experiments were carried out to deter-

mine whether or not alteration in the mode of cellular separation after division would be accompanied by any change in the expression of the C_s capsule. All spontaneous mutants of pneumococcus bearing the C_s capsule were isolated originally as nonfilamentous variants. Cells of two strains, $S\text{-}_{III_4}C_s$ and $S\text{-}_{VIII_1}C_s$, were plated by the technique described earlier (7) for the isolation of filamentous variants of pneumococcus. After two to three transfers on solid media, colonies of each strain gave rise to marginal excrescences composed of filamentous cells which, in Gram-stained preparations, differed in no way from filamentous strains of pneumococcus described previously. Examination of the cells comprising the excrescences by the capsular precipitin or Quellung reaction showed the cells still to be producing capsules of C_s polysaccharide. DNA was prepared from cultures derived from single filamentous clones of strains $S\text{-}_{III_4}C_s$ and $S\text{-}_{VIII_1}C_s$. The preparations were capable of transforming nonfilamentous noncapsulated pneumococci to either the filamentous noncapsulated genotype or to the nonfilamentous C_s capsulated genotype. From these observations, it would appear that there is no relation between the mode of pneumococcal cellular separation after division and the production of C_s capsular polysaccharide.

Pneumococcal C_s variants derived from strains $S\text{-}_{II}$ and $S\text{-}_{III_4}$ were examined for the presence of M protein. Strain $S\text{-}_{II}C_s$ carries M protein type 2′, and strain $S\text{-}_{III_4}C_s$ carries M protein type 3 (Fig. 4 *a*–4 *d*). The production of C_s polysaccharide is not accompanied by any striking qualitative change in the production of this somatic antigen.

Occurrence of C_s Pneumococcal Variants in Nature.—The occurrence in man of pneumococcal C_s capsular variants cannot be assessed with certainty at the present time. Microscopic examination of spreads of 500 sputum specimens from patients with acute or chronic respiratory disease by the capsular precipitin or Quellung technique has shown one to contain rare coccal forms giving an unequivocally positive reaction. Unfortunately, this organism could not be recovered from cultures of the same sputum specimen made at the same time as the preparation for microscopy. In any event, pneumococcal C_s variants appear not to be common inhabitants of the human respiratory tract.

Virulence of C_s Pneumococcal Variants.—Pneumococci with capsules of C_s polysaccharide are lacking in virulence for several species of rodents. Injection of 1 ml of undiluted culture of strain $S\text{-}_{II}C_s$ intraperitoneally or subcutaneously into rats, guinea pigs, or rabbits failed to produce death or progressive localized lesions. Occasional deaths occurred in mice following injection of 1 ml of undiluted culture of strains $S\text{-}_{III_4}C_s$ or $S\text{-}_{VIII_1}C_s$, both of which have capsules of moderate size.

Phagocytosis of C_s Pneumococci by Human Polymorphonuclear Leukocytes.—Because of the lack of virulence of C_s pneumococcal variants for several species of laboratory animals, their phagocytosis by human polymorphonuclear leuko-

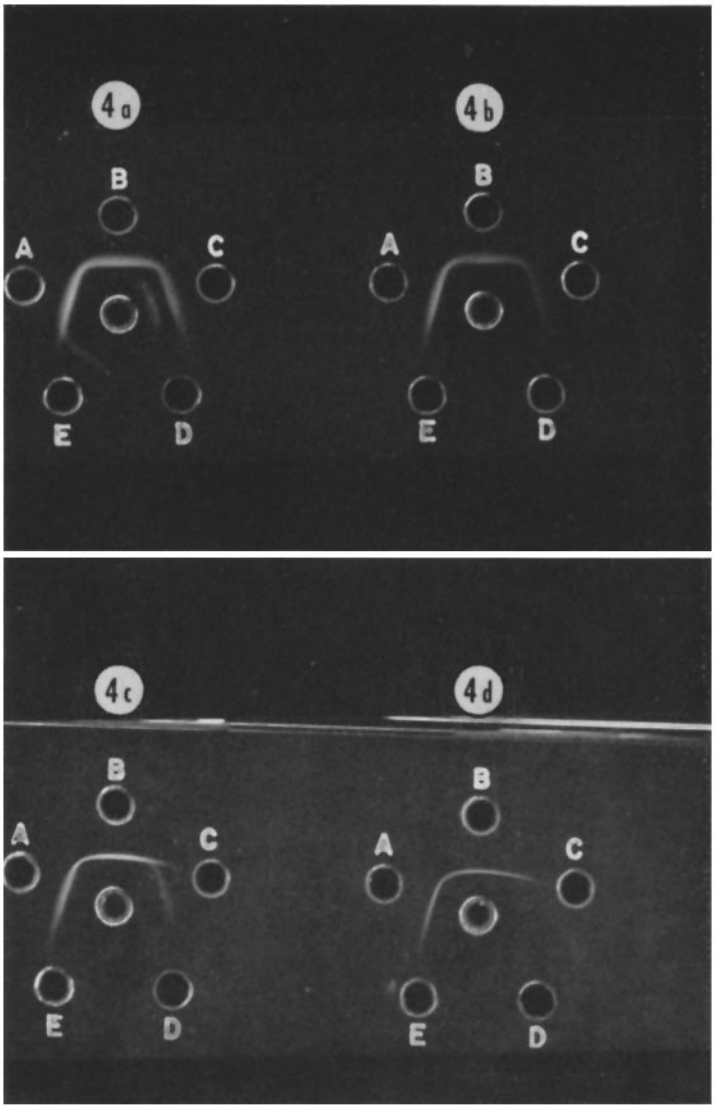

FIG. 4. *a*. Center well, anti-M2′ serum (unabsorbed); wells A and C, extracts of strain S-$_{II}C_s$; well B, extract of strain S-$_{II}$; well D, C polysaccharide; and well E, extract of strain S-$_{III_4}C_s$. *b*. Center well, anti-M2′ serum absorbed with strain S-$_{III_4}$. Outer wells as in 4 *a*. *c*. Center well, anti-M3 serum (unabsorbed); well A, extract of strain S-$_{III_4}C_s$; well B, extract of strain S-$_{III_4}$; well C, extract of strain S-$_{II}C_s$; well D, C polysaccharide; and well E, pepsin-treated extract of strain S-$_{III_4}C_s$. *d*. Center well, anti-M3 serum absorbed with strain S-$_{II}$. Outer wells as in Fig. 4 *c*.

cytes was investigated. 0.3 ml of fresh venous blood from a normal human subject containing 6.6 units of heparin was mixed with 0.2 ml of pneumococcal culture to give a ratio of 10 bacterial infectious centers per polymorphonuclear leukocyte. To separate tubes of the mixture, 0.1 ml of sterile physiologic salt solution, of a solution of C_s polysaccharide containing 50 μg/ml or of C_s anti-

TABLE VII

Phagocytosis of Capsulated, Noncapsulated, and C_s Pneumococcal Variants by Human Polymorphonuclear Leukocytes

Pneumococcal variant	Incubation time	Antibody N	C_s polysaccharide	Organisms ingested per polymorphonuclear leukocyte				
				0	1–3	4–6	7–9	10+
	min	*μg/ml*	*μg/ml*					
SVIII	10	0	0	97	3	0	0	0
	10	10 Anti-SVIII	0	30	18	17	18	17
	30	0	0	100	0	0	0	0
	30	10 Anti-SVIII	0	10	4	22	20	44
$S\text{-}VIII_1$	10	0	0	16	46	19	15	4
	10	0	8	17	46	24	8	5
$S\text{-}VIII_1C_s$	10	0	0	0	10	29	32	29
	10	0	8	13	50	31	3	3
	10	0	160	26	34	26	11	3
	10	160 Anti-C_s	0	4	14	40	26	16
	30	0	0	0	4	10	28	58
	30	0	8	0	7	23	33	37
	30	0	160	1	7	5	23	64
	30	160 Anti-C_s	0	2	4	6	10	78

serum was added. The tubes were sampled at zero time and at indicated intervals during incubation at 37°C in a roller drum. The samples were smeared on cover slips, stained with Wright's stain, and the bacteria in 100 polymorphonuclear leukocytes counted.

The results of experiments with pneumococcal strains SVIII, $S\text{-}VIII_1$, and $S\text{-}VIII_1C_s$ are shown in Table VII. In contrast to the capsulated Type VIII strain which was not phagocytized in the absence of added anticapsular antibody, cells of strains $S\text{-}VIII_1$ and $S\text{-}VIII_1C_s$ were ingested rapidly by the poly-

morphonuclear leukocytes in normal human blood. Because the serum of the donor of the blood used in this experiment contained 1–2 μg/ml of antibody N to pneumococcal C polysaccharide, the effect on phagocytosis of neutralization of this small amount of antibody with C_s polysaccharide was examined. As shown in Table VII, the neutralization of anti-C antibody resulted in a measurable slowing of phagocytosis of the C_s variant; whereas it had little effect, if any, on the phagocytosis of cells of the noncapsulated strain from which the C_s variant arose. The findings indicate that, although antibody accelerates slightly the phagocytosis of C_s pneumococcal cells, it is not essential to their ingestion by human polymorphonuclear leukocytes.

Genetic Studies of C_s Pneumococcal Variants.—DNA of C_s pneumococcal strains transforms readily competent noncapsulated variants of pneumococci derived from strains of several capsular types (Types I, II, III, and VIII) to the C_s genotype in the transforming systems cited in the section on Materials and Methods.

In an experiment in which DNA of the capsulated strain S-$_{III_4}C_s$ was used to transform cells of the noncapsulated strain S-$_{III_5}$, two capsulated variants were recovered, Type III cells and C_s cells. It is known from earlier experiments that strains S-$_{III_4}$ and S-$_{III_5}$ both possess the capsular genome of pneumococcus Type III with mutations at different sites in the cistron controlling the synthesis of uridine diphosphoglucose dehydrogenase (4). When either strain is transformed with the DNA of the other, genetic recombination leads to correction of the defect in the synthesis of Type III capsular polysaccharide and fully capsulated Type III pneumococci are recovered. Additional studies of the genes controlling normal capsular synthesis have shown that they occupy the same region of the pneumococcal chromosome regardless of capsular type (4). In the present experiment, in which transformation of strain S-$_{III_5}$ with DNA of strain S-$_{III_4}C_s$ led to the recovery of both capsulated Type III cells and C_s cells, it is apparent that the genetic locus responsible for the production of C_s capsular polysaccharide must occupy a site other than that controlling the synthesis of other pneumococcal capsular polysaccharides.

Further evidence for the foregoing conclusion has been obtained in transformation reactions in which binary and ternary capsular variants of pneumococcus have been isolated, one of the capsular components being C_s polysaccharide. These experiments were carried out with the technique described in reference 5 because C_s variants proved refractory to transformation in the transforming system described by MacLeod and Krauss (8). When pneumococcal strain S-$_{III_6}TC_s$ was exposed to the DNA of an intermediate capsular variant of pneumococcus Type III, S-$_{II}$T SIII$_2$, the binary capsulated strain, S-$_{III_6}TC_s$ SIII, which produced both C_s polysaccharide and normal amounts of Type III polysaccharide, was recovered. When the same recipient strain S-$_{III_6}TC_s$ was exposed to DNA of a streptomycin-resistant pneumococcus carrying the wild

Type I pneumococcal capsular genome, transformation of the streptomycin marker was observed, but no new capsular phenotypes were isolated. If DNA from a streptomycin-resistant strain carrying an atypical Type I capsular genome (5) was used, however, ternary capsular cells were recovered which produce C_s, Type I and Type III capsular polysaccharides and which give a positive capsular precipitin or Quellung reaction with specific Type I, Type III, and C_s capsular antisera. These experiments show conclusively that the genes controlling the synthesis of soluble C_s polysaccharide can exist in pneumococci producing one or two normal capsular polysaccharides and must occupy a position in the pneumococcal genome distinct from those controlling the latter.

DISCUSSION

The experiments described demonstrate that the novel capsular variant of pneumococcus isolated during the course of transformation reactions by Taylor (1) and by Beiser and Hotchkiss (2) was not the product of interaction between transforming DNA and the cells exposed to it but rather a spontaneous mutant. Recognition of the immunologic relatedness of the capsular polysaccharide of this mutant to pneumococcal C or cell wall polysaccharide has permitted the use of an appropriate selective environment to obtain similar mutants from a variety of noncapsulated pneumococcal strains derived from several capsular types in the absence of exogenous DNA. The demonstration of C polysaccharide in preparations of transforming DNA explains also why the C_s mutants were selected in the transforming systems from which they were first isolated. The chemical constitution of the C_s capsular polysaccharide is very similar to though not identical with, that of pneumococcal C polysaccharide, as anticipated from immunologic findings, and will be presented subsequently in greater detail.

Further evidence for the relatedness of C_s polysaccharide to pneumococcal C polysaccharide has been derived from the reactivity of the former with C-reactive protein. The capsular precipitin or Quellung reaction resulting from the interaction of pneumococcal cells producing capsules of C_s polysaccharide with CRP in the presence, but not in the absence of calcium ions adds another pneumococcal variant to the three manifesting this phenomenon described earlier by Löfström (11). Comparison of the chemical structures of the capsular polysaccharides of pneumococcal Types XVI, XXVII, and XXVIII with those of C and C_s polysaccharides may help elucidate further the nature of the chemical groups reacting with CRP. In the light of the observations of Gotschlich and Edelman (12), it is of interest that the three capsular polysaccharides reacting with CRP are believed to contain phosphate in their structures (13).

Genetic analysis of C_s pneumococcal variants has shown clearly that the genetic factor controlling the synthesis of soluble capsular C_s polysaccharide is located in a region distinct from that determining the production of previously

identified capsular polysaccharides of pneumococcus. This fact has permitted the isolation of binary capsulated pneumococci, one of the capsular components of which is C_s polysaccharide. It has permitted also for the first time, the isolation, following transformation, of ternary capsulated strains of the same species.

The precise nature of the mutation leading to the production of soluble C_s capsular polysaccharide has not been defined. Several hypothetical possibilities exist. Production of C_s polysaccharide might result from derepression of the control mechanism for the synthesis of C polysaccharide, from gene duplication or from a genetic alteration affecting the linkage of C polysaccharide to other cell wall constituents. Information currently available does not permit distinction among these three possibilities. Production of capsular material related to structural cell wall polysaccharides has not been a commonly recognized phenomenon in bacteria. The observations of Knox and Hall (14) in *Lactobacillus casei* var. *rhamnosus* describe a possible mechanism for capsule formation resulting from a disturbance of the coupling of cell wall polysaccharide to other cell wall constituents.

Whether or not C_s pneumococcal variants occur in nature is uncertain at the present time. It is conceivable that they might have very limited potential for survival over noncapsulated variants of pneumococcus but the difference in susceptibility to phagocytosis between C_s and noncapsulated variants is not marked. The availability of a potent serologic reagent which gives a positive capsular precipitin or Quellung reaction with C_s pneumococcal cells, however, makes feasible a continued search for such forms in mammalian secretions. Such sera provide also the most potent immunologic reagents for the detection of pneumococcal C polysaccharide in extracts of this organism and for the detection of cross-reacting polysaccharides in other strains of streptococci from the human respiratory tract (15).

SUMMARY

Capsulated mutants of pneumococcus producing a capsule of soluble polysaccharide related immunologically to the C or cell wall polysaccharide of pneumococcus have been isolated from several noncapsulated variants of this organism. The capsular material of these strains reacts with antisera both to homologous strains and to noncapsulated strains of pneumococcus and with human C-reactive protein. C-reactive protein has been shown to give a positive capsular precipitin or Quellung reaction with C_s pneumococcal variants and to agglutinate them.

The genetic locus which determines the production of C_s polysaccharide is situated in a region of the pneumococcal chromosome distinct from that controlling normal capsular polysaccharide synthesis. Binary and ternary capsulated pneumococci, one of the capsular components of which is C_s polysaccharide, have been isolated following DNA-mediated transformation.

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