INFECTION AND IMMUNITY, Nov. 1971, p. 596-604

Vol. 4, No. 5
Printed in U.S.A.

Type-Specific Antigens of Group B Type Ic Streptococci

HAZEL W. WILKINSON AND R. G. EAGON

Bacteriology Section, Center for Disease Control, Atlanta, Georgia 30333, and Department of Microbiology, University of Georgia, Athens, Georgia 30601

Received for publication 7 June 1971

The type-specific antigens of group B type Ic (old designation type Ii) streptococci were extracted, purified, and characterized by serological and chemical methods. The Ia antigen, shared by types Ia and Ic, is a polysaccharide composed of 69% galactose and 25% glucosamine (i.e., 31% *N*-acetyl-glucosamine). However, these monosaccharides failed to inhibit significantly the quantitative precipitin reactions between purified antigen and type Ia antiserum. Indications are that the immunodominant group of this antigen consists of more than a simple monosaccharide. The Ic antigen, shared by types Ib and Ic, is a protein unrelated to the X and R protein antigens. Ic antigen consists of two serologically active determinants, one of which is susceptible to both trypsin and pepsin digestion and the other to pepsin but not to trypsin digestion. Acrylamide gel electrophoresis of the partially purified Ic antigen resulted in the occurrence of both determinants throughout the length of the gel, as shown by double gel diffusion slides.

Group B streptococci are divided into types by the presence of four polysaccharide antigens in acid-heat extracts (15, 16) and are characterized further by the presence of two protein antigens occurring at random among the types and among strains that seem devoid of type polysaccharide (10, 27, 28). Antisera to the polysaccharide antigens, Ia, Ib, II, and III, were shown to afford passive protection in mice challenged with the homologous virulent streptococci, whereas the protein antigens R and X seem unrelated to virulence. An intermediate type I possesses antigens in common with types Ia and Ib (36), but no new antigen specific for type Ii has been found.

Lancefield (*personal communication*) has shown that absorption of type Ib antiserum with type Ii cells removes antibodies that precipitate with type Ii acid extracts but leaves homologous Ib antibodies. On the other hand, absorption of type Ia antiserum with type Ii cells removes all precipitating antibody. The antigen possessed in common by types Ib and Ii has been designated Ic according to the practice of nonfunctional nomenclature. For the same reason, the type designation Ii has been changed to Ic. The antigens responsible for the serological specificity of type Ic are described in this report.

MATERIALS AND METHODS

Strains. Group B streptococci representative of the various types were described previously (36). Group B strains containing the X protein antigen, 24/60 (original "compton" from Pattison), and the R protein antigen, 25/60 (also original "compton" from Pattison), were supplied by J. Jelinkova, Institute of Epidemiology and Microbiology, Prague. Other strains containing an R protein (17, 19, 35) were supplied by R. C. Lancefield, The Rockefeller University, New York. These were: a group C strain, B337, and five group A strains, types 3 (D58X), 28(T28/150A/2), 33(C107/24), 43(C126/21), and 48(C510).

Antisera. Immunization methods were described previously (36). Antisera specific for types Ia, Ib, and II were produced with formalinized vaccines of strains 090, H36B, and 18RS21, respectively. Antisera produced with the type III strain D136C contained antibodies to the R protein in this strain, as well as antibodies to the type-specific polysaccharide. Type Ic antisera, produced with immunizing strain A909, were made specific for the Ic antigen either by absorption with type Ia cells 090 or by collecting the antisera before the rabbits' response to the Ia antigen. Type Ib antisera were made specific for the Ib antigen by absorption with Ic cells A909 or, alternatively, specific for the Ic antigen by collection of the sera before the rabbits' response to the Ib antigen. Anti-R sera, lot W3096 from a rabbit immunized with group C strain B337 and lot R1907 from a rabbit immunized with group A strain T28/150A/5, came from R. C. Lancefield. Antisera to the B compton strains containing X and R protein antigens were supplied by J. Jelinkova.

Precipitin tests. Capillary precipitin tests were done by placing the acid-heat extracts or purified antigen suspensions above the antisera. Reactions due to

polysaccharide antigens were much stronger and more stable by this method than when antisera were placed above the antigens. Furthermore, reactions with protein antigens were not affected adversely. The typing reactions were allowed to incubate at room temperature overnight but were observed for precipitation within several minutes, after several hours, and again after overnight settling. The polysaccharide reactions were always strongest within 30 min, whereas protein reactions were strongest after incubation overnight. The strongest reaction observed during this time was taken to be indicative of a particular test.

Quantitative precipitin and quantitative hapten inhibition tests were based on spectrophotometric procedures, as described by McCarty and Lancefield (25).

Immunodiffusion. Agar-gel diffusion slides were described previously (36). Immunoelectrophoresis slides were prepared by the Scheidegger technique (30).

Susceptibility of antigens to enzymes and to TCA precipitation. Samples (0.03 ml) of acid-heat extracts were incubated with one drop each of 5% trypsin, 0.5% pepsin, 12% deoxyribonuclease (DNase), and 12% ribonuclease (RNase) and with an equal volume of cold 10% trichloroacetic acid (TCA). The enzymes were tested at their optimal *p*H and incubated for 2 hr at 37 C. The TCA sample was incubated for 2 hr at 4 C and centrifuged to remove the precipitate. After neutralization, extracts were tested for serological activity.

Extraction and purification of antigens. The procedure used by Lancefield and Freimer (18) was modified slightly. Type Ic (A909) cells were extracted by hot HCl, and the Ic protein antigen was separated from the Ia polysaccharide antigen by precipitation with four volumes of 10% TCA at 4 C (7). The Ic precipitate was partially purified by successive washes in 5% TCA (repeated three times and heated at 90 C for 15 min the second time to remove nucleic acid components), 95% ethanol, 3:1 ethanol-diethyl ether (repeated twice and heated at 60 C for 3 min each time), and diethyl ether. The Ia antigen was precipitated from the TCA supernatant fluid by adding four volumes of ethanol at 4 C and then was dissolved in physiological saline. It was purified by incubation with 20 μg each of RNase and DNase for 4 hr at 35 C, followed by dialysis against 0.01 M phosphate buffered saline (PBS) at 4 C for 2 days, and then incubation with 50 μg of trypsin per ml at 35 C overnight, followed by dialysis against PBS at 4 C for 2.5 days and distilled water at 4 C for 2 days. Residual proteins and nucleic acids were removed by precipitation with cold 5% TCA (final concentration). The TCA was removed by extraction four times with 5-ml volumes of diethyl ether. This procedure was repeated until no significant absorption occurred at 260 or 280 nm.

The purified polysaccharide type antigen was separated from the group B antigen by fractional ethanol precipitation. Very little antigen precipitated with 1 volume of ethanol; most type-specific antigen precipitated between 1.5 and 2 volumes. The group antigen could be completely removed in successive cycles; when this was accomplished, the 1.5- and 2-volume precipitates were combined. Thirty-two grams of lyophilized A909 whole cells yielded approximately 100 mg of lyophilized Ia antigen.

Thin-layer chromatography. Purified Ia antigen was hydrolyzed in 2 N HCl for 2 hr at 100 C, 4 N H_2SO_4 for 2 hr at 110 C, and 6 N HCl for 17 hr at 110 C. The HCl hydrolysates were dried in vacuo over P_2O_5 and NaOH pellets and washed repeatedly with distilled water. The H_2SO_4 hydrolysate was neutralized with $Ba(OH)_2$ and centrifuged, and the supernatant fluid was filtered and dried in vacuo over P_2O_5. Amino acids and hexosamines were detected with ninhydrin and Elson-Morgan sprays (26) via two-dimensional chromatography on cellulose plates with isopropanol-formic acid-water (40:2:10) and *t*-butanol–methyl ethyl ketone–water–NH_4OH (50:30:10:10) as solvents (11). Sugars and hexosamines were detected with naphthoresorcinol (21) and Elson-Morgan sprays on silica gel H borate-buffered, heat-activated plates with *n*-butanol–acetone–water (40:50:10) as the solvent. The wedged-tip technique was employed (33).

Acrylamide gel electrophoresis. The partially purified Ic antigen was subjected to electrophoresis by the method of Davis (2) and by the acetic acid modified method (20). Bands were detected with 1% naphthol blue-black in 7% acetic acid. Unstained gels subjected to electrophoresis were sectioned at measured intervals and used in agar-gel diffusion tests with Ic-specific antiserum.

Analytical methods. Protein was determined by the method of Lowry et al. (22) and with the Biuret reagent (13). Total hexose was measured by the anthrone reaction (32) and by the secondary cysteine reaction (13). Hexosamine was determined by the method of Dische and Borenfreund (3) and that of Ludowieg and Benmaman (23). Galactose was measured with a Worthington galactostat (29) and by using the secondary cysteine reaction (13). Glucose was determined with a Worthington glucostat (9) and rhamnose, by the method of Dische and Shettles (4). A microdetermination of phosphorus was made (1), and nitrogen was determined by Kjeldahl nesslerization (34) of the ashed material (31).

RESULTS

Cross absorption of type I antisera. Absorption of Ia, Ib, and Ic antisera was done to confirm the observation that type Ib antiserum absorbed with type Ic cells still precipitated with type Ib but not with type Ic acid extracts (R. C. Lancefield, *personal communication*). Table 1 shows that type Ia antiserum reacted before absorption with types Ia, Ib (slight), and Ic extracts. Absorption of the serum with Ia and Ic cells removed all precipitating antibody, whereas absorption with Ib cells removed only the Ib cross-reaction. This is consistent with the view that types Ia and Ic share a common antigen. Type Ib antiserum reacted with types Ib and Ic extracts before absorption but did not cross-react with type Ia extracts. Absorption with type Ib cells removed all detectable antibodies. The presence of two antigens in type Ib, one of which is shared by

TABLE 1. *Cross absorptions of group B type I antisera*

Antisera		Capillary precipitin reaction with HCl extracts of		
Rabbit immunized with	Serum absorbed with streptococcal cells	Strain 090, type Ia	Strain H36B, type Ib	Strain A909, type Ic[a]
Strain 090, type Ia	None	4+[b]	1+	4+
	Ia			
	Ib	3+		3+
	Ic			
Strain H36B, type Ib	None		4+	2+
	Ia		2+	±
	Ib			
	Ic		3+	
Strain A909, type Ic	None	4+	4+	6+
	Ia		3+	4+
	Ib	1+		2+
	Ic			

[a] Type Ic previously designated Ii.

[b] Strength of reaction based on scale of 1+ (weak) to 4+ (strong) and greater than 4+ (exceptionally strong).

type Ic, became apparent when absorption of Ib antiserum with Ic cells removed the precipitin reaction with Ic cells but not the one with homologous Ib cells. Type Ic antiserum reacted strongly with all three extracts until absorption with Ia cells, which removed the precipitin reaction with the Ia extract; with Ib cells, which removed the reaction with Ib extract; or with Ic cells, which removed all detectable precipitin reactions.

These results are consistent with the scheme shown in Fig. 1. Type Ia contains an antigen which type Ic also contains. Type Ib contains two antigens, one of which is shared by type Ic.

Enzyme susceptibility and TCA precipitation of Ic antigen. Preliminary attempts to extract and purify the Ia and Ic antigens from type Ic cells indicated that Ic might be a protein rather than a polysaccharide. Table 2 shows the effects of proteolytic enzymes and TCA precipitation on extracts of the various group B types. Extracts of types Ia, II, and III were unaffected by these agents. Treatment of type Ic extracts with trypsin, pepsin, or TCA eliminated the precipitin reaction with antiserum specific for the Ic antigen and with type Ib antiserum containing Ic antibodies. Strong reactions still occurred with type Ia antiserum. Similarly, type Ic extracts did not react with antisera containing antibodies specific for the Ic antigen after trypsin, pepsin, or TCA

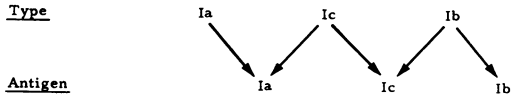

FIG. 1. *Diagram of the antigenic composition of group B, type I streptococci.*

treatment, whereas strong reactions occurred with type Ib antiserum which had been absorbed with A909 cells, type Ic. Negative precipitin reactions of enzyme- and TCA-treated Ib extracts with Ib, lot 3 antiserum may be explained by observations that this serum contains proportionately few antibodies specific for the Ib polysaccharide. Unabsorbed type Ic antiserum (not shown) reacted with all type I extracts, enzyme-digested or not, because antibodies to the Ia antigen often cross-react with the Ib antigen (16). The Ia/Ib cross-reaction is due to similarities of these two polysaccharide antigens, whereas the reactions of type Ic antiserum with types Ia and Ib extracts are due to shared antigens. Type Ic shares the polysaccharide Ia antigen with type Ia and the protein Ic antigen with type Ib.

Figure 2A shows that the Ic antigen is composed of two determinants, one susceptible to pepsin and trypsin digestion and the other to pepsin but not to trypsin. Both determinants precipitated with cold TCA. Both are present in the type Ic strain A909 and the type Ib strain H36B (Fig. 2B). The Ic antigen is also responsible for the reaction of two type II strains, SS869 and SS950, with Ib antiserum. This is the only heterologous type known to contain, occasionally, a Ic antigen, but this phenomenon occurs in less than 1% of group B strains (*unpublished data*).

Nonidentity of Ic antigen to X or R proteins. Table 3 shows that antiserum specific for the Ic antigen does not react with extracts of strains containing X or R proteins and that antiserum specific for the X protein and various antisera with R protein specificity do not react with the type Ic extract. These observations were the same when gel diffusion tests were run and when extracts of group A types 33 and 43 were used (not shown). Similarly, when type Ic was tested for reactions with T-agglutinating antisera and with M-precipitating antisera, the results were also negative.

Immunological relationships of type I antigens. Double gel diffusion tests (Fig. 3) were used to relate previous observations (36) to what is known now about the type I antigens of group B streptococci. A pepsin-treated acid extract of type Ib (H36B) was used so that only those reactions due to polysaccharide antigens would

TABLE 2. *Capillary precipitin reactions showing the effects of trypsin, pepsin, and trichloroacetic acid (TCA) on type antigens of group B streptococci*

HCl extracts			Antisera[a]					
Type	Strain	Treatment of extract	Type Ia	Type Ib lot 3[b]	Type Ic[c]	Type Ib[d]	Type II	Type III[e]
Ia	090	None	5+[f]					
		Trypsin	5+					
		Pepsin	5+					
		TCA	5+					
Ib	H36B	None		5+	4+	5+		
		Trypsin				5+		
		Pepsin				5+		
		TCA				5+		
Ic	A909	None	6+	5+	4+			
		Trypsin	6+					
		Pepsin	6+					
		TCA	6+					
II	18RS21	None					5+	
		Trypsin					5+	
		Pepsin					5+	
		TCA					5+	
III	D136C	None						5+
		Trypsin						4+
		Pepsin						4+
		TCA						4+

[a] Immunizing strains used for the production of type-specific antisera were as follows: 090 for type Ia, A909 for type Ic, H36B for type Ib, 18RS21 for type II, and D136C for type III.

[b] Same immunizing strain, same specificity as an earlier lot (36).

[c] Absorbed with 090 type Ia strain.

[d] Absorbed with A909 type Ic strain (gift of R. C. Lancefield). Different lot from the unabsorbed type Ib antiserum used for absorption experiments (Table 1).

[e] Absorbed with strain B337, group C Streptococcus containing an R antigen (gift of R. C. Lancefield).

[f] Strength of reaction based on scale of from 1+(weak) to 4+(strong) and greater than 4+(exceptionally strong).

appear. Type Ia or Ib antisera, made specific for the homologous strain by absorption with the heterologous one if necessary, were used in the serum wells shown in Fig. 3A. Precipitin bands occurred in the agar gel only between homologous antisera and extracts, i.e., type Ia antiserum with type Ia extract and type Ib antiserum with the type Ib extract (pepsin-treated). Antisera known to cross-react with the heterologous type I strain were used in Fig. 3B. Precipitin bands occurred between antisera and homologous extracts, as in Fig. 3A, and also between antisera and the heterologous type I extracts (type Ia antiserum with type Ib extract and type Ib antiserum with type Ia extract). Partial identity of the antigens is demonstrated by the occurrence of spurs between reactions due to homologous and heterologous antisera. On the basis of these experiments, it seems that (i) the "two Ib antigens" common to Ib and Ii described previously were actually the two Ic protein determinants, because they were eliminated by pepsin treatment and (ii) the "partial Ia antigen" described in Ib extracts was actually the Ib polysaccharide antigen which can be seen in Fig. 3B to form a line of partial identity with the Ia antigen when unabsorbed cross-reactive antisera were used.

Chemical composition of Ia antigen. Thin-layer chromatography of acid hydrolysates indicated that the purified Ia antigen was composed of galactose and glucosamine. These observations were confirmed in quantitative tests (Table 4) in which galactose was responsible for about 69% and glucosamine about 25% of the dry weight of

this antigen. The small amount of nitrogen (1.7%) is accounted for by the presence of glucosamine, which is probably *N*-acetylated. These values, corrected to include acetyl groups, would account for close to 100% of the total weight of the antigen. No rhamnose, glucose, phosphorus, amino acids, polyols, or lipids were detected in the purified Ia antigen.

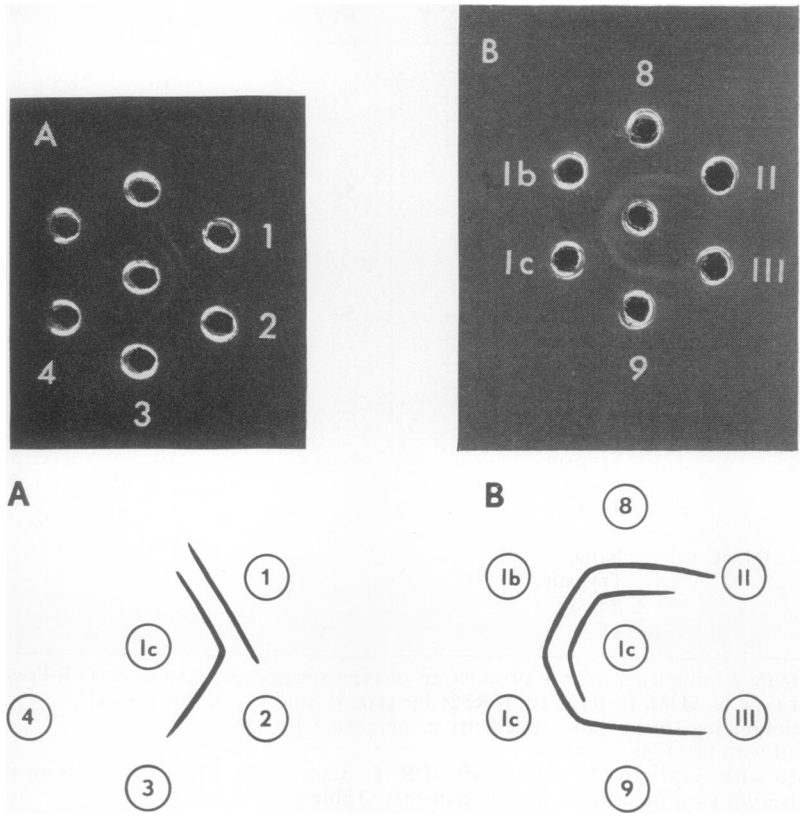

FIG. 2. *Double diffusion precipitin reactions of Ic antigens. A, effects of proteolytic digestion of HCl extracts of strain A909, type Ic. Peripheral wells contain: (1) untreated extract, (2) trypsin-digested extract, (3) pepsin-digested extract, (4) trichloroacetic acid-treated extract (see text for treatment). Center well contains antiserum prepared with A909, type Ic cells and specific for the Ic antigen. Identical results were obtained with HCl extracts of strain H36B, type Ib. B, Presence of Ic antigen in two group B, type II strains. Peripheral wells contain HCl extracts of strain A909, type Ic (Ic); strain H36B, type Ib (Ib); strain 18RS21, type II (II); strain D136C, type III (III); strain SS869, type II (8); and strain SS950, type II (9). Center well contains the same antiserum as in A.*

TABLE 3. *Nonidentity of Ic antigen to X or R protein antigens*

HCl extracts		Capillary precipitin reactions using antisera from rabbits immunized with				
Strain	Group/type	Strain A909, type Ic	Strain B compton containing X antigen	Strain B compton containing R antigen	Strain T28/150A/5, group A, containing R antigen	Strain B337, group C, containing R antigen
A909	B/Ic	5+[a]				
Compton	B/X		4+[a]			
Compton	B/R			5+[a]		2+
T28/150A/2	A/28R				4+[a]	2+
B337	C			2+	4+	5+[a]

[a] Reactions of antisera with homologous immunizing strains. Strength of reaction based on scale of from 1+(weak) to 4+(strong) and greater than 4+(exceptionally strong).

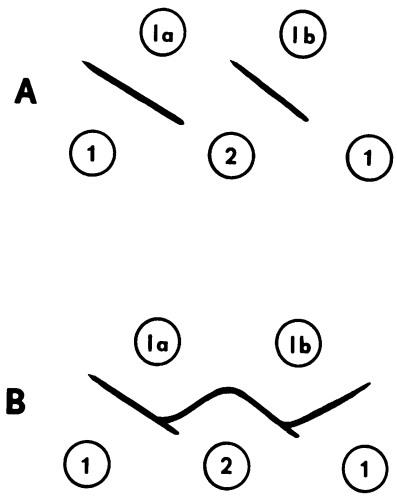

FIG. 3. *Double diffusion precipitin reactions of type Ia and type Ib HCl extracts with various lots of type Ia and type Ib antisera. Bottom wells contain HCl extracts: (1) strain 090, type Ia (2) strain H36B, type Ib. The H36B extract was digested with pepsin to remove precipitin reactions due to the Ic protein antigen. Top wells contain antisera produced by immunizing rabbits with strain 090, type Ia (Ia) and strain H36B, type Ib (Ib). A, Specific antisera were: (Ia) type Ia, lot 1, no absorption required; (Ib) type Ib, lot 3, absorbed with strain 090, type Ia. B, Cross-reactive unabsorbed antisera were: (Ia) type Ia, lot W3179; (Ib) type Ib, lot R1833.*

TABLE 4. *Chemical composition of type Ia-specific antigen from strain A909, type Ic cells*

Substance	Per cent (dry weight) of antigen
Total hexose	69.2
Galactose	69.0
Glucose	0
Rhamnose	0
Glucosamine	24.8
Galactosamine	<1
Nitrogen	1.7
Phosphorus	0

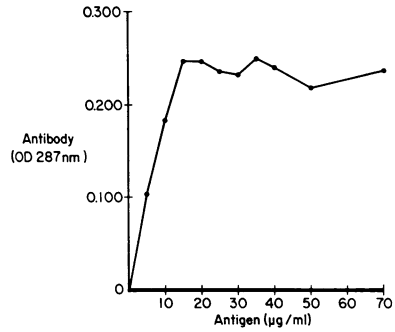

FIG. 4. *Quantitative precipitin test using the purified Ia antigen from A909, type Ic cells and type Ia antiserum prepared with 090, type Ia cells.*

Quantitative precipitin and hapten inhibition tests of Ia antigen. Figure 4 shows the quantitative precipitin test in which 10 to 70 µg of Ia antigen is plotted against optical density at 287 nm as a measure of antibody precipitated. An attempt was made to find the immunodominant group of the antigenic determinant by inhibiting the precipitin reaction by various substances (Table 5). The antigen is composed of galactose and glucosamine, but neither of these substances inhibited significantly. The only significant inhibition by a monosaccharide occurred with 20 mg of *N*-acetyl-galactosamine per ml. A 50-mg amount of this same substance, however, failed to inhibit to a greater extent than did 20 mg. In addition to the substances shown in Table 5, the following sugars exhibited no inhibition of qualitative precipitin tests: D-mannose, D-arabinose, muramic acid, methyl-α-D-galactoside, phenyl-β-D-galactoside, *o*-nitrophenyl-β-D-galactoside, and thiomethyl-β-D-galactoside. Furthermore, serological cross-reactions of Ia antigen with antisera specific for the following antigens composed of known immunodominant groups did not occur: streptococcal groups A [*N*-acetyl-D-glucosamine (24)] and C [*N*-acetyl-D-galactosamine (14)], pneumococcal types IV and XXVII [pyruvate (8)], and blood groups A (*N*-acetyl-D-galactosamine), B (D-galactose), and H (L-fucose) (12).

More than one lot of Ia-specific antiserum was used to test the inhibitory effect of galactose, *N*-acetyl-glucosamine, and *N*-acetyl-galactosamine. Results were the same, regardless of whether the immunizing strain had been 090, type Ia, or A909, type Ic.

The lack of significant inhibition by these substances suggested that the antigenic determinant was composed of more than one monosaccharide. Neutralized hydrolysates of 10 mg of the Ia antigen were used as potential hapten inhibitors of the precipitin reaction. The complete hydrolysate (2 N HCl, 2 hr, 110 C) did not inhibit, whereas a partial hydrolysate (1 N HCl, 2 hr, 65 C) inhibited 29% of the precipitin reaction. This was the mildest hydrolysis possible that rendered the antigen nonprecipitable with antiserum. Isolation and identification of oligosaccharide fragments from incomplete hydrolyses were not attempted because of the large quantity of antigen required for these experiments.

Absorption of types Ia and Ic antisera with purified Ia antigen. Cross-absorptions of type I antisera with whole cells (Table 1) showed that types Ia and Ic contained a common antigen.

That this antigen is the polysaccharide Ia antigen is shown in Table 6. Unabsorbed type Ia antiserum reacted with extracts of types Ia and Ic. Absorption of this antiserum with purified Ia antigen removed both of these precipitin reactions. Unabsorbed type Ic antiserum reacted with HCl extracts of types Ia, Ib, and Ic. When this antiserum was absorbed with purified type Ia antigen, the precipitin reaction with type Ia extract was removed but not the reactions with types Ib and Ic, both of which contain the Ic antigen.

Immunoelectrophoresis. The Ia polysaccharide antigen and the Ic protein antigen were subjected to electrophoresis by using Veronal buffer, *p*H 8.6. These were made visible by precipitation with homologous antisera. Figure 5 shows that the Ic antigen moved slowly toward the anode, and the Ia antigen moved slowly toward the cathode. This is similar to the immunoelectrophoretic pattern

TABLE 5. *Hapten inhibition of quantitative precipitin reaction between type Ia antiserum, produced against 090 type Ia cells, and purified Ia antigen from strain A909, type Ic*

Potential inhibitor[a]	Per cent inhibition
D-Galactose	0
D-Glucose	9
L-Rhamnose	7
D-Glucosamine	9
N-acetyl-D-glucosamine	7
D-Galactosamine	10
N-acetyl-D-galactosamine	26
D-Mannosamine	2
Sodium pyruvate	5
D-Galactose + sodium pyruvate	10
Antigen hydrolysate, complete	7
Antigen hydrolysate, partial	29
Lactose	0
Raffinose	0
Agarose hydrolysate	0

[a] Reaction mixture consisted of 20 mg of inhibitor (except for the antigen inhibitors which were 10 mg each prior to hydrolysis), 0.1 ml of type Ia antiserum, and 25 μg of purified Ia antigen, in a total volume of 1.0 ml.

TABLE 6. *Absorption of types Ia and Ic antisera with purified Ia antigen from strain A909 type Ic*

Antisera		Capillary precipitin reaction with HCl extracts of		
Rabbit immunized with	Serum absorbed with Ia antigen	Strain 090, type Ia	Strain H36B, type Ib	Strain A909, type Ic
Strain 090, type Ia	No	4+[a]		4+
	Yes			
Strain A909, type Ic	No	4+	4+	4+
	Yes		4+	3+

[a] Strength of reaction based on scale of from 1+ (weak) to 4+ (strong).

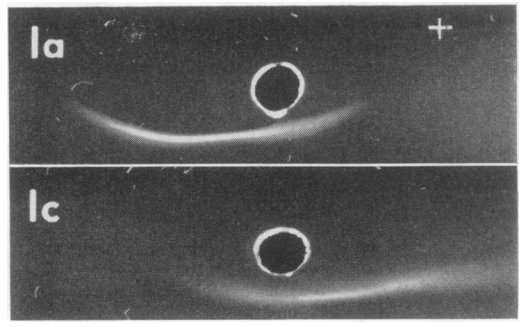

FIG. 5. *Immunoelectrophoresis of type Ic antigens, Ia polysaccharide, and Ic protein. Purified Ia antigen from strain A909, type Ic, was subjected to electrophoresis and then developed with type Ia antiserum, which had been produced with strain 090, type Ia, as immunogen. Top pattern shows movement of Ia antigen toward the cathode. Partially purified Ic antigen from strain A909, type Ic, was subjected to electrophoresis and then developed with type Ic antiserum, which had been produced with immunizing strain A909, type Ic. Bottom pattern shows movement of this antigen toward the anode.*

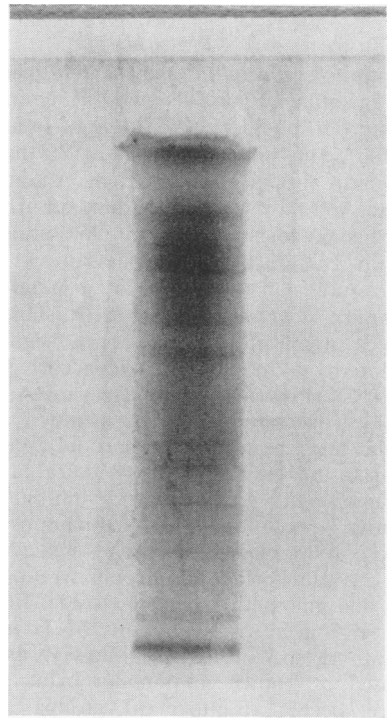

FIG. 6. *Acrylamide gel electrophoresis of the Ic protein antigen. Thirteen predominant bands occurred.*

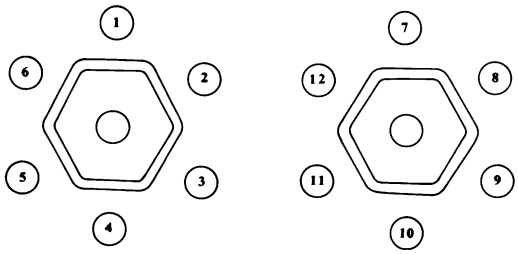

FIG. 7. *Double diffusion precipitin reactions of Ic protein antigen subjected to electrophoresis. An unstained acrylamide gel (duplicate of the stained gel shown in Fig. 6) was sliced at measured intervals and placed in the peripheral wells of an Ouchterlony slide. Central wells contained type Ic antiserum produced with A909, type Ic cells. The occurrence of two continuous precipitin bands between center and peripheral wells showed that the two Ic determinants occurred throughout the length of the acrylamide gel.*

of Lancefield and Freimer's purified, HCl-extracted type II polysaccharide (18).

Acrylamide gel electrophoresis. The lyophilized, partially-purified Ic antigen was composed of 81% protein when assayed by the biuret test; no carbohydrate or amino sugars could be detected. Acrylamide gel electrophoresis (Fig. 6) separated the preparation into 13 predominant bands. Serological reactivity of both determinants with antiserum specific for the Ic antigen occurred throughout the gel (Fig. 7). Variation of buffer or of quantity of antigen subjected to electrophoresis did not change these results.

DISCUSSION

Type Ic is differentiated from type Ia by the presence of a protein antigen Ic which it shares with type Ib. Pattison, Matthews, and Howell (27) and Pattison, Matthews, and Maxted (28) concluded that the protein antigens of group B streptococci are less stable and more likely to be involved in cross-reactions than the polysaccharide-type antigens which are related to virulence of the organism. Therefore, they recommended emphasizing the polysaccharide types and using the presence of two protein antigens, X and R, only as epidemiological tags for strains devoid of polysaccharide antigens. One might argue that the Ic antigen has the same significance as the X and R antigens except for two considerations. First, it occurs frequently and appears to be stable. Epidemiological data (*unpublished data*) from the Streptococcus Laboratory, Center for Disease Control, Atlanta, indicate that type I strains comprise 50% of the group B streptococcal types and that type Ic occurs frequently enough to make the differentiation of it from type Ia helpful from an epidemiological standpoint. The Ic antigen has occurred in all type Ib strains tested and has not been encountered in streptococci of other groups. Ib "monospecific" strains encountered previously (36) probably contained Ic and not Ib determinants. The only heterologous group B type in which the Ic antigen has occurred is type II. However, less than 1% of 675 group B cultures received for typing over a period of 3.5 years possessed the Ic/II antigenic complex. Second, Ic antigen appears to be serologically distinct from X or any of the R proteins commonly encountered. R protein was used originally to designate an antigen found in group A strains that has no relationship to virulence and therefore is different from the M protein, which is related to virulence and which elicits protective antibodies in suitable hosts. The relationship of Ic antigen to virulence and of Ic antiserum to protection is not known. Strain A909 was not virulent enough to use in mouse protection studies (R. C. Lancefield, *personal communication*).

The purified Ia antigen contained in A909 type Ic cells is a polysaccharide composed of galactose and glucosamine (probably acetylated). However, neither hapten inhibited the quantitative precipitin reaction between antigen and Ia-specific antiserum. These results were surprising, since Freimer (6) found 88% inhibition of the type II reaction with galactose, one of the sugars found in the type II polysaccharide. Slight inhibition of the Ia reaction with *N*-acetyl-galactosamine may indicate similarity in exposed hydrophobic groups. That no inhibition occurred with antigen hydrolysates containing maximal quantities of free sugars indicates that the immunodominant group is composed of more than a monosaccharide. That very mild hydrolysis yields products that do inhibit the quantitative precipitin reaction substantiates this assumption. The small yield of type antigen prohibited isolation and analysis of oligosaccharides from partial hydrolyses. Furthermore, the unavailability of oligosaccharides commercially prohibited further inhibition studies.

The partially purified Ic antigen is a protein with two determinants differing in susceptibility to trypsin and pepsin. The acrylamide gel electrophoretic pattern observed for the Ic protein is reminiscent of the pattern Fox and Wittner (5) obtained with M proteins in that serological activity occurs throughout the gel rather than being confined to certain bands. Similarly, it would not be surprising if acid extraction caused fragmentation of Ic protein, with all fractions containing both immunological determinants.

ACKNOWLEDGMENTS

The senior author wishes to thank Rebecca C. Lancefield for many helpful and inspiring discussions at The Rockefeller University and at the Center for Disease Control, Jiri Rotta for his interest and helpful suggestions, and R. C. Lancefield and Maclyn

McCarty for reviewing the manuscript. Also, we thank R. Lancefield, J. Rotta, and J. Jelinkova for strains and antisera.

LITERATURE CITED

1. Chen, P. S., Jr., T. Y. Toribara, and H. Warner. 1956. Microdetermination of phosphorus. Anal. Chem. **28**:1756–1758.
2. Davis, B. J. 1964. Disc electrophoresis. Ann. N.Y. Acad. Sci. **121**:404–427.
3. Dische, Z., and E. Borenfreund. 1950. A spectrophotometric method for the microdetermination of hexosamines. J. Biol. Chem. **184**:517–522.
4. Dische, Z., and L. B. Shettles. 1948. A specific color reaction of methylpentoses and spectrophotometric micromethod for their determination. J. Biol. Chem. **175**:595–603.
5. Fox, E. N., and M. K. Wittner. 1969. New observations on the structure and antigenicity of the M proteins of the group A streptococcus. Immunochemistry **6**:11–24.
6. Freimer, E. H. 1967. Type-specific polysaccharide antigens of group B streptococci. II. The chemical basis for serological specificity of the type II HCl antigen. J. Exp. Med. **125**: 381–392.
7. Greenberg, D. M. 1957. Preparation of samples of protein, amino acids, and other solids, p. 708. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 4. Academic Press Inc., New York.
8. Heidelberger, M., W. F. Dudman, and W. Nimmich. 1970. Immunochemical relationships of certain capsular polysaccharides of Klebsiella, pneumococci and Rhizobia. J. Immunol. **104**:1321–1328.
9. Huggett, A. St. G., and D. A. Nixon. 1957. Use of glucose oxidase, peroxidase, and O-dianisidine in determination of blood and urinary glucose. Lancet **2**:368–370.
10. Jelinkova, J. 1964. On the question of type identification of group B streptococci and their distribution in human and bovine material. Symposium on *Streptococcus pyogenes* (Jena 7–10 Oct. 1963, Germany). Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig. **196**:722–723.
11. Jones, K., and J. G. Heathcote. 1966. The rapid resolution of naturally occurring amino acids by thin-layer chromatography. J. Chromatogr. **24**:106–111.
12. Kabat, E. A. 1968. Structural concepts in immunology and immunochemistry, p. 1180 Holt, Rinehart & Winston, Inc., New York.
13. Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochemistry, 2nd ed., p. 533–560. Charles C Thomas Publisher, Springfield, Ill.
14. Krause, R. M., and M. McCarty. 1962. Studies on the chemical structure of the streptococcal cell wall. II. The composition of group C cell walls and chemical basis for serologic specificity of the carbohydrate moiety. J. Exp. Med. **115**:49–62.
15. Lancefield, R. C. 1934. Serological differentiation of specific types of bovine hemolytic streptococci (group B). J. Exp. Med. **59**:441–458.
16. Lancefield, R. C. 1938. Two serological types of group B hemolytic streptococci with related, but not identical, type-specific substances. J. Exp. Med. **67**:25–40.
17. Lancefield, R. C. 1958. Occurrence of R antigen specific for group A type 3 streptococci. J. Exp. Med. **108**:329–341.
18. Lancefield, R. C., and E. H. Freimer. 1966. Type-specific polysaccharide antigens of group B streptococci. J. Hyg. **64**:191–203.
19. Lancefield, R. C., and G. E. Perlmann. 1952. Preparation and properties of a protein (R antigen) occurring in streptococci of group A, type 28 and in certain streptococci of other serological groups. J. Exp. Med. **96**:83–97.
20. Larsen, S. A., C. D. Webb, and M. D. Moody. 1969. Acrylamide gel electrophoresis of group A streptococcal cell walls. Appl. Microbiol. **17**:31–33.
21. Lato, M., B. Brunelli, G. Cuiffini, and T. Mezzetti. 1968. Bidimensional thin-layer chromatography of carbohydrates on silica gel impregnated with boric acid. J. Chromatogr. **34**:26–34.
22. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
23. Ludowieg, J., and J. D. Benmaman. 1967. Colorimetric differentiation of hexosamines. Anal. Biochem. **19**:80–88.
24. McCarty, M. 1958. Further studies on the chemical basis for serological specificity of group A streptococcal carbohydrate. J. Exp. Med. **108**:311–323.
25. McCarty, M., and R. C. Lancefield. 1955. Variation in the group-specific carbohydrate of group A streptococci. I. Immunochemical studies on the carbohydrates of variant strains. J. Exp. Med. **102**:11–28.
26. Partridge, S. M. 1948. Filter-paper partition chromatography of sugars. I. General description and application to the qualitative analysis of sugars in apple juice, egg white, and foetal blood of sheep. Biochem. J. **42**:238–248.
27. Pattison, I. H., P. R. J. Matthews, and D. G. Howell. 1955. The type classification of group B streptococci, with special reference to bovine strains apparently lacking in type polysaccharide. J. Pathol. Bacteriol. **69**:51–60.
28. Pattison, I. H., P. R. J. Matthews, and W. R. Maxted. 1955. Type classification by Lancefield's precipitin method of human and bovine group-B streptococci isolated in Britian. J. Pathol. Bacteriol. **69**:43–50.
29. Roth, H., S. Segal, and D. Bertoli. 1965. The quantitative determination of galactose—an enzymic method using galactose oxidase, with applications to blood and other biological fluids. Anal. Biochem. **10**:32–52.
30. Scheidegger, J. J. 1955. Une micro-méthode de l'immuno-électrophorèse. Int. Arch. Allergy Appl. Immunol. **7**:103–110.
31. Schiffman, G., E. A. Kabat, and W. Thompson. 1964. Immunochemical studies on blood groups. XXX. Cleavage of A, B, and H blood-group substances by alkali. Biochemistry **3**:113–120.
32. Scott, T. A., Jr., and E. H. Melvin. 1953. Determination of dextran with anthrone. Anal. Chem. **25**:1656–1661.
33. Stahl, E. 1965. Wedge-tip technique, p. 35–36. *In* E. Stahl (ed.), Thin-layer chromatography. Academic Press Inc., New York.
34. Varley, H. 1960. Kjeldahl nesslerization method, p. 142. *In* H. Varley (ed.), Practical clinical biochemistry, 2nd ed. Interscience Publishers, Inc., New York.
35. Wiley, G. G., and P. N. Bruno. 1970. Cross-reactions among group A streptococci. III. The M and R antigens of type 43 and serologically related streptococci. J. Immunol. **105**:1124–1130.
36. Wilkinson, H. W., and M. D. Moody, 1969. Serological relationships of type I antigens of group B streptococci. J. Bacteriol. **97**:629–634.