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Conformational Aspects Critical to the Immunospecificity of the Type III Group B Streptococcal Polysaccharide†

Harold J. Jennings,* Czeslaw Lugowski,‡ and Dennis L. Kasper

ABSTRACT: Immunization of rabbits with group B type III *Streptococcus* organisms induces two distinct populations of antibodies with a specificity for determinants on the native capsular polysaccharide antigen of these organisms. Some of the structural and conformational features of the two determinants responsible for the formation of these antibodies were elucidated by ^{13}C NMR and serological studies on the native type III polysaccharide and some of its structurally modified analogues. The specificity of the determinant corresponding to the major population of antibodies is dependent on the presence of sialic acid residues on the native type III antigen, and although these residues are not an integral part of the determinant, they exert conformational control over it. The carboxylate groups of the sialic acid residues are an important factor in this control mechanism which could possibly involve intramolecular hydrogen bonding. The terminal sialic acid residues control the orientation of the penultimate β-D-galactopyranose residues with respect to the backbone of the native antigen. The orientation of these residues is critical to the determinant because the determinant is probably small and is located precisely at the junction of the same β-D-galactopyranose residues with the backbone of the native type III antigen. The determinant corresponding to the other population of antibodies is not sialic acid dependent. This determinant is located on the backbone of the native antigen in the vicinity of the other determinant but on the opposite side to the oligosaccharide branches. In this position, its conformation is unaffected even by the removal of the oligosaccharide branches from the native antigen.

Lancefield (1933, 1934, 1938) characterized two polysaccharide antigens obtained from group B *Streptococcus*: a group antigen common to all strains and the type-specific capsular polysaccharides that distinguished four major serotypes designated Ia, Ib, II, and III. Because these type-specific polysaccharides were isolated by the hot hydrochloric acid extraction of whole streptococcal organisms, they were shown to be immunologically incomplete and to form a lower molecular weight core to the complete native antigens which contain additional terminal sialic acid residues (Baker et al., 1976; Kasper et al., 1979; Tai et al., 1979). Jennings et al. (1980b) have demonstrated that the type III core antigen is structurally identical with the type 14S *pneumoniae* polysaccharide having terminal branch β-D-galactopyranose residues, while in the native type III antigen, all of the β-D-galactopyranose residues are masked by terminal sialic acid.

Antisera raised to type III group B streptococci were demonstrated to contain two antigenically distinguishable populations of antibodies (Kasper et al., 1979; Jennings et al., 1980b). One population had an exclusive specificity for the native type III antigen while the other was specific for the core antigen. It was postulated that this latter specificity was due to determinants terminating in β-D-galactopyranose residues which were generated on the organisms used as vaccines by the unintentional loss of sialic acid from the organism-associated native polysaccharide (Jennings et al., 1980b).

The work described in this paper confirms the exclusive specificity of the first population of antibodies for the native type III antigen but demonstrates that the major population of the core-specific antibodies is not specific for terminal β-galactopyranose residues and is precipitated by the native antigen. Because of the importance of antibodies to the native type III antigen in human immunity to group B streptococcal disease (Baker & Kasper, 1976; Kasper et al., 1979), the determinants responsible for the formation of these antibodies have been structurally elucidated.

Experimental Procedures

Materials. Strain M732 of group B *Streptococcus* designated type III was isolated from a child with meningitis. The strain was grown in a modified Todd-Hewitt broth, and the

† From the Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada, and Channing Laboratory, Harvard Medical School, and the Department of Medicine, Peter Bent Brigham Hospital, Boston, Massachusetts 02115. *Received December 8, 1980.* This is National Research Council of Canada Publication No. 19531. D.L.K. is the recipient of U.S. Public Health Service Research Career and Development Award 1KO400126. Supported in part by U.S. Public Health Service Research Grant 1A172538.

* Correspondence should be addressed to this author at the Division of Biological Sciences, National Research Council of Canada.

‡ Present address: Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, 53-114 Wroclaw, Poland.

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native type III polysaccharide was extracted from the organisms with a neutral-buffered 0.01 M EDTA[1] solution and subsequently purified as previously described (Kasper et al., 1979; Jennings et al., 1980b). The core type III polysaccharide was extracted from the whole group B streptococcal organisms with hot 0.2 M HCl according to the method of Lancefield & Freimer (1966) and was purified as previously described (Jennings et al., 1980b). Rabbit antisera to killed whole-cell vaccines of the prototype (M732) group B *Streptococcus* were made according to the method of Lancefield (1934) and were kindly supplied by Dr. Carol J. Baker, Baylor College of Medicine, Houston, TX. Hyperimmune rabbit antisera to broth-grown type XIV *Streptococcus pneumoniae* were also prepared by the method of Fischer et al. (1978).

Analytical Methods. Free sialic acid was determined by the thiobarbituric acid method (Warren, 1959; Aminoff, 1961). The sialic acid was removed from the native antigen by mild acid hydrolysis (0.05 M H_2SO_4 at 80 °C for 80 min) and purified with a Dowex 1 (formate form) anion-exchange column (Martensson et al., 1958). D-Galactose was determined enzymatically by the method of Fischer & Zopf (1964).

Gas–liquid chromatography (GC) was performed on a Hewlett-Packard 5830A instrument equipped with a flame ionization detector and a Model 18850A electronic integrator. The glass columns (180 × 0.15 cm) used contained the following liquid phases on Gas Chrom Q: (i) 3% OV-225 at 210 °C for alditol acetates and at 190 °C for partially methylated alditol acetates; (ii) 3% OV-17 at 200 °C for amino-sugar derivatives; and (iii) 3% OV-1 at 175–250 °C, program rate 4 °C/min, for methyl ester pertrimethylsilyl ether derivatives of sialic acid and its heptulosonic acid analogue. Combined gas chromatography–mass spectrometry (GC–MS) was carried out on a Finnegan 3100D instrument using columns (i), (ii), and (iii) and an ionization potential of 70 eV.

^{13}C NMR spectra were recorded at 37 °C in 10-mm tubes on a Bruker CXP 300 instrument at 75.47 MHz in the pulsed Fourier-transform mode with complete proton decoupling. Chemical shifts are reported in parts per million (ppm) downfield from external tetramethylsilane, and the ^2H resonance of deuterium oxide was used as a field frequency lock signal.

Glycose Analysis. Analysis of the glycose constituents of the type III streptococcal polysaccharides was carried out by using GC analysis essentially by the method of Dmitriev et al. (1975) as previously described (Jennings et al., 1980a). Sialic acid and its heptulosonic acid analogue were liberated from the native and oxidized type III polysaccharide according to the method of Suttajit & Winzler (1971), and their methyl ester pertrimethylsilyl ether derivatives were prepared by the method of Kamerling et al. (1975) and analyzed by GC–MS using column (iii).

Methylation Analysis. Polysaccharides were methylated according to the method of Hakomori (1964), and the methylated preparations were purified, hydrolyzed, and acetylated as previously described (Jennings et al., 1980a). The specifically methylated alditol acetates were then analyzed by GC–MS using columns (i) and (ii) (Lindberg, 1972).

Modifications of the Native Type III Streptococcal Polysaccharide. (a) Reduction of the Sialic Acid Residues. The carboxyl groups of the sialic acid residues of the native type III polysaccharide were reduced to hydroxymethyl groups essentially by the method of Taylor & Conrad (1972). A solution of the polysaccharide (26 mg in 2 mL of water) was adjusted to pH 4.75 with 2 M HCl, and following the addition of EDC (Pierce Chemical Co., Rockford, IL) (10 mol equiv per mol equiv of sialic acid), the pH of the reaction was continuously adjusted to 4.75 with 1 M HCl until the reaction had ceased (2 h). At this stage, 5 drops of octanol (to prevent foaming) were added followed by solid $NaBH_4$ (final solution approximately 1.5 M with respect to $NaBH_4$), and the solution was stirred overnight. The pH of the solution was then adjusted to 4.5 with cold 5% acetic acid, and the reduced native antigen was purified by gel filtration on a Sephadex G-25 column (2.6 × 80 cm) equilibrated with 0.01 M NH_4HCO_3 solution at pH 8.2. The yield of the lyophilized reduced native type III polysaccharide was 23 mg.

(b) Periodate Oxidation of the Terminal Sialic Acid Residues of the Native Type III Polysaccharide. The sialic acid residues of the native type III polysaccharide were oxidized with sodium metaperiodate to their heptulosonic acid analogue according to the method of Suttajit & Winzler (1971). To 5 mL of polysaccharide solution (1 mg/mL) in PBS was added 0.05 M sodium metaperiodate solution to give a molar ratio of periodate to sialic acid of 4 to 1. The reaction mixture was allowed to stand in an ice bath in the dark for 2 h, at which time excess periodate was destroyed by ethylene glycol (6 mol per mol of periodate). The resultant solution was dialyzed against PBS and reduced with sodium borohydride. Excess borohydride was destroyed by adding cold 50% acetic acid to pH 4.5, and the solution was dialyzed against 0.01 M NH_4HCO_3 in the cold. Lyophilization of the resultant solution yielded 4 mg of the oxidized polysaccharide.

(c) Selective Removal of the Terminal Galactose Residues of the Incomplete Core Polysaccharide. In preparation for their removal, the terminal β-D-galactopyranose residues were first oxidized to their 6-aldehyde derivatives by the method of Avigad et al. (1962). The core polysaccharide (60 mg) in 0.02 M sodium phosphate buffer (pH 7.0, 10 mL) was treated under a layer of toluene with D-galactose oxidase (EC 1.1.3.9, Worthington, 450 units) and horseradish peroxidase (EC 1.11.1, Worthington, 180 units). After 110 h at room temperature, the solution was then extracted with an equal volume of 80% aqueous phenol. Phase separation was promoted by centrifugation, and the aqueous layer was removed, dialyzed, and lyophilized to 50 mg of the aldehydic derivative of the core polysaccharide. This latter polysaccharide was treated with 5 mL of 0.5 M NaOH at 80 °C for 1 h, made momentarily acidic (pH 4), dialyzed, and lyophilized to yield 30 mg of degalactosylated core polysaccharide. This modified polysaccharide was re-N-acetylated in an aqueous solution buffered with $NaHCO_3$ with acetic anhydride and was treated with $NaBH_4$ to reduce residual aldehyde groups arising from the oxidation of some of the main-chain β-D-galactopyranose residues. Following adjustment to pH 4.5, the solution was dialyzed and lyophilized to yield 30 mg of the type III backbone antigen.

Serological Methods. (a) Immunodiffusion. Double radial immunodiffusion was performed as described by Ouchterlony (1962) in 0.9% agarose gels in PBS containing 2% poly(ethylene glycol) (M_r 4000). Slides for immunoelectrophoresis were prepared by the method of Scheidegger (1955) using a barbital buffer (pH 8.6) and a field strength of 40 M per slide for 4 h at 24 °C.

(b) Quantitative Microprecipitation. These experiments were carried out by using 50-μL aliquots of type III serum and reacting this serum with increasing concentrations of each

[1] Abbreviations used: EDTA, ethylenediaminetetraacetic acid; PBS, 0.01 M phosphate-buffered physiological saline; EDC, 1-ethyl-3-[(dimethylamino)propyl]carbodiimide hydrochloride; RABA, radioactive antigen binding assay.

Table I: Glycose Analysis of the Modified Polysaccharides from Type III Group B *Streptococcus*

polysaccharide	molar ratio			
	sialic acid	galactose[a]	glucose[a]	glucosamine[a]
native	0.81[b]	2.0	1.0	1.0
native (oxidized with periodate)	0.82[b]	2.0	1.0	1.0
reduced native	0.01	2.0	1.0	1.0
core	0.00	2.0	1.0	1.0
backbone	0.00	1.0	1.0	1.0

[a] Molar ratios accurate to ±5%. [b] Underestimated, but a value of 1.0 is indicated by the failure to detect terminal galactose residues in the methylation analysis (Table II).

antigen in 0.15 mL of PBS for determination of equivalence points (Kabat & Mayer, 1961). Subsequently, another aliquot of this serum was preabsorbed at equivalence point with the reduced native type III polysaccharide by incubating 50 μL of this serum with the equivalence antigen concentration for 24 h at 4 °C. After removal of the precipitates by centrifugation, a second antigen was added at concentrations similar to those used for the initial equivalence determination. These were incubated for 24 h, and the precipitate was removed by centrifugation and the quantity of antibody protein in the pellet determined by the method of Lowry et al. (1951). To check for remaining low-affinity antibodies after equivalence-point absorption of the type III serum with the reduced native antigen, we performed a second absorption with the same antigen at higher concentrations, 20, 40, 80, and 160 μg.

Inhibition Experiments. Experiments using serotransferrin as an inhibitor to the type III streptococcal antiserum and its homologous native polysaccharide were performed by using the quantitative microprecipitation method. Aliquots (50 μL) of the type III antiserum were incubated for 2 h at 37 °C with increasing amounts (0.1–160.0 μg) of serotransferrin. Quantitative precipitin curves were then established as before between the inhibited sera and the native polysaccharide at equivalence.

Experiments using the type Ia group B streptococcal polysaccharide and the type III core polysaccharide as inhibitors to the antibodies specific for the native type III polysaccharide and their homologous native polysaccharide were performed by a RABA inhibition method. Type III antiserum was absorbed with the core type III polysaccharide at equivalence. After incubation at 37 °C for 2 h and at room temperature for 24 h, the insoluble immune complexes were removed by centrifugation. The absorbed serum was divided into four aliquots (5 μL), and to three of these in PBS was added either 300 ng of the type Ia native polysaccharide, 300 ng of the type III native polysaccharide, or 300 ng of the core type III polysaccharide. PBS was added to the fourth aliquot as a control. Following incubation (2 h at 37 °C), 150 ng of intrinsically labeled (^{3}H-labeled) native type III polysaccharide was added to each, and the standard RABA method was performed as described by Baker & Kasper (1976). The percentage inhibition by the various polysaccharides of the immune complexes formed between the intrinsically labeled native type III antigen and the type III absorbed antiserum was determined with

$$\% \text{ inhibition} = 100 - \frac{\text{cpm in inhibited serum}}{\text{cpm in PBS control}}$$

Results

Structural Modifications of the Native and Core Type III Polysaccharides. The polysaccharides were structurally modified by chemical and enzymatic procedures, and to ensure that a complete and specific modification had occurred, we subjected the modified polysaccharides to compositional analysis (Table I) and methylation analysis (Table II). The average molecular weights of the original and modified polysaccharides were determined by gel filtration. The structures of all but the reduced and oxidized native type III antigens are shown in Figure 4 in the section on ^{13}C NMR spectroscopy.

Reduction of the Native Type III Polysaccharide. The carboxyl groups of the native type III polysaccharide (Figure 4) were reduced to hydroxymethyl groups essentially by the method of Taylor & Conrad (1972). This reduction was achieved with only minimal diminution in the molecular weight of the original native polysaccharide, the K_{av} on a Sepharose 4B column increasing from 0.52 to 0.58 following reduction. That the polysaccharide was neutral was ascertained by immunoelectrophoresis using type III antiserum. The reduced polysaccharide did not migrate whereas the native type III polysaccharide experienced a considerable anodic migration. This evidence is also consistent with the compositional data in which only insignificant quantities of sialic acid were detected in the reduced type III antigen (Table I). However, the detection of 2,3,4,6-tetra-*O*-methylgalactose in the mole ratio of 0.08 to 1.00 with respect to 2,3,6-tri-*O*-methylglucose in the methylation analysis of the reduced type III polysaccharide indicated that 8% of the terminal sialic acid residues of the native type III polysaccharide had been removed during the reduction. Again, as previously established (Jennings et al., 1980b), no 2,3,4,6-tetra-*O*-methylgalactose could be detected in the methylation analysis of the native type III polysaccharide (Table II).

Periodate Oxidation of the Native Type III Polysaccharide. Controlled periodate oxidation of the native type III polysaccharide (Figure 4) followed by reduction with $NaBH_4$ gave a modified polysaccharide which had undergone no detectable diminution in molecular size (Sepharose 4B column) and which had a glycose analysis identical with that of the original native type III polysaccharide (Table I). The fact that the sialic acid assays of the native and oxidized polysaccharides were identical

Table II: Methylation Analysis of the Modified Polysaccharides from Type III Group B *Streptococcus*

molar ratios of methylated derivatives	molar ratios of polysaccharides				
	native	native reduced	native oxidized	core	backbone
2,3,4,6-tetra-*O*-methylgalactose	0.0	0.08	0.0	1.0	0.0
2,4,6-tri-*O*-methylgalactose[a]	1.0	0.9	0.9	1.0	1.0
2,3,4-tri-*O*-methylgalactose[a]	1.0	1.0	1.0	–	–
2,3,6-tri-*O*-methylglucose[a]	1.0	1.0	1.0	1.0	1.0[b]
3-mono-*O*-methyl-*N*-methyl-*N*-acetylglucosamine	+[c]	+	+	+	–
3,4-di-*O*-methyl-*N*-methyl-*N*-acetylglucosamine	–[c]	–	–	–	+

[a] Molar ratios accurate to ±5%. [b] Ratio was 0.8 before final reduction of the modified product. [c] +, nonquantitative response; –, not detected.

Table III: Chemical Shifts of the Linkage Carbons of the Native and Modified Type III Streptococcal Polysaccharides[a]

polysaccharide	linkage carbons							
	A1	B1	C1	D1	E2	A4	B3	C4
native type III	103.9[b]	103.9[b]	103.6[c]	103.2[c]	101.1	79.8	83.4	78.3
reduced native type III	103.9[b]	103.9[b]	103.9[b]	103.9[b]	103.0	79.8	83.3	79.1
core type III	103.9[b]	103.9[b]	103.9[b]	103.9[b]		79.8	83.3	79.1
backbone type III	104.0[d]	104.0[d]	104.0[d]			79.8	83.2	UA[e]

[a] In parts per million from external tetramethylsilane. [b] Unresolved broad signals. [c] Tentative assignments. [d] Unresolved narrow signals. [e] UA, unassigned, but chemical shift < 76.0 ppm.

can be attributed to both sialic acid and its heptulosonic acid analogue yielding identical chromophores in the thiobarbiturate assay. In order to establish the degree of oxidation of the sialic acid residues, a GC–MS method was utilized which can discriminate between the above compounds (Suttajit & Winzler, 1971). This method indicated that 90% of the sialic acid residues had been oxidized to their heptulosonic acid analogue. Virtually no removal of the sialic acid residues occurred during the oxidation because as in the case of the native antigen, methylation analysis of the oxidized native type III antigen yielded no detectable 2,3,4,6-tetra-*O*-methylgalactose (Table II). It is interesting to note that despite the above evidence we were unable to account for a full molar quantity of sialic acid and its heptulosonic acid analogue in the repeating unit of the oxidized polysaccharide (Table I). This was also shown to be the case in the thiobarbiturate estimation of sialic acid in the native type III polysaccharide (Jennings et al., 1980b).

Hydrolysis of the Native Type III Polysaccharide. The terminal sialic acid residues were removed from the native polysaccharide by hydrolysis (Jennings et al., 1980b). No sialic acid could be detected in the core polysaccharide (Figure 4) (Table I), and methylation data (Table III) indicated that the ratio of terminal β-D-galactopyranose to interchain β-D-glucopyranose was 1 to 1.

Removal of the Terminal β-D-Galactopyranose Residues from the Core Polysaccharide. The enzymatic oxidation of the primary hydroxyl groups of the terminal β-D-galactopyranose residues of the core polysaccharide rendered them highly susceptible to elimination by treatment with alkali. Momentary acid treatment of the modified polysaccharide then selectively removed these residues from the core antigen to yield the backbone polysaccharide (Figure 4). Preliminary methylation analysis of the product (Table II) indicated that 1.2 mol of galactose per mol of glucose had been removed. However, this was found to be due to underestimation of the total residual methylated interchain galactose residues. This occurred when, on the prolonged exposure of the core polysaccharide to the D-galactose oxidase, some (20%) of the interchain galactose residues were also oxidized. On reduction of the enzymatically treated core polysaccharide, however, with $NaBH_4$, the expected ratio (1 to 1) of 2,4,6-tri-*O*-methylgalactose to 2,3,6-tri-*O*-methylglucose was restored. Although the molecular size of the backbone polysaccharide had decreased substantially (Sepharose 4B column), it still remained in a polymeric form.

Serological Properties of the Modified Polysaccharides. The native type III polysaccharide and its various structural modifications were compared for serological specificity against a type III group B streptococcal antiserum.

(*a*) *Immunodiffusion Studies.* Double diffusion studies involving the above antiserum against the native polysaccharide, reduced native polysaccharide, and core antigen are shown in Figure 1. As previously demonstrated (Kasper et al., 1979; Jennings et al., 1980b), spurring between the native and core polysaccharides indicated the presence of at least two populations of antibodies in the antiserum, one having an exclusive specificity for the native polysaccharide. Reduction of the sialic acid residues of the native polysaccharide eliminated this latter specificity from the modified antigen because, like the core antigen, it spurred with the native polysaccharide and despite the presence of few β-galactopyranose residues gave a precipitin line of identity with the core polysaccharide. Also, in an additional experiment, serotransferrin failed to precipitate the type III streptococcal antiserum despite the fact that it exhibits a large degree of structural homology in its glycose moiety with that of the native type III polysaccharide (Jennings et al., 1980b).

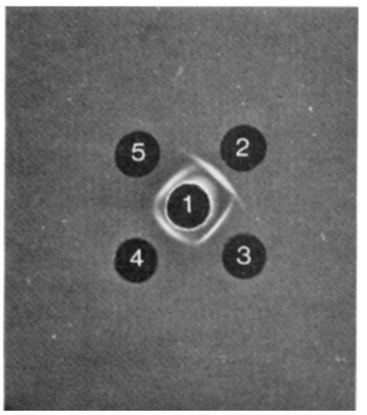

FIGURE 1: Immunodiffusion in agar of type III group B streptococcal antiserum (well 1) with the native type III polysaccharide (well 2), reduced native type III polysaccharide (well 3), and core type III polysaccharide (wells 4 and 5).

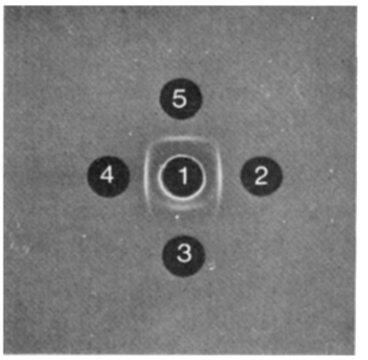

FIGURE 2: Immunodiffusion in agar of type III group B streptococcal antiserum (well 1) with the native type III polysaccharide (wells 2 and 4), the core type III polysaccharide (well 3), and the periodate-oxidized native type III polysaccharide (well 5).

While reduction of the sialic acid residues of the native polysaccharide destroyed its antigenicity against native polysaccharide-specific antibodies, the removal of both C8 and C9 from the glycol side chains of the majority of the sialic acid residues did not, and the modified polysaccharide still retained this specificity. In double diffusion experiments (Figure 2) using the group B streptococcal antiserum, the oxidized polysaccharide gave a complete line of identity with the native

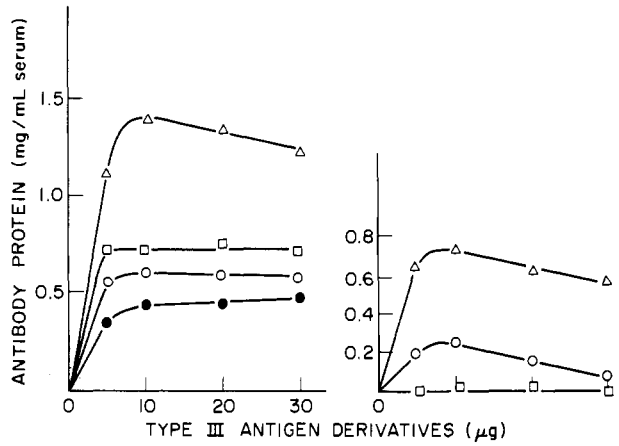

FIGURE 3: Quantitative precipitin analysis of the native type III polysaccharide (△), the reduced native type III polysaccharide (□), the core type III polysaccharide (○), and the backbone type III polysaccharide (●) with type III group B streptococcal antiserum (left) and the same antigens with the same antiserum previously absorbed at equivalence with the reduced native type III polysaccharide (right).

polysaccharide, while the core antigen exhibited its usual heavy spurring with the same native antigen. In similar immunodiffusion experiments (not shown), the backbone polysaccharide also reacted with the group B streptococcal antiserum, whereas unlike the core antigen (Jennings et al., 1980b), it reacted only weakly with a pneumococcal type 14 antiserum. This evidence and the fact that the two antigens gave only a partial line of identity with the former antiserum suggested that the population of antibodies specific for the core polysaccharide could be further divided on the basis of differing structural specificities. Confirmatory evidence for this was obtained in the following microprecipitation experiments.

(*b*) *Microprecipitation Experiments.* The precipitin curves are shown in Figure 3, and as anticipated, the native polysaccharide precipitated the largest amount of antibody from the type III group B streptococcal antiserum, thus indicating that it is the most complete antigen. Of the modified polysaccharides, the backbone polysaccharide precipitated the least while both the core and reduced native polysaccharides gave intermediate results, both precipitating nearly equal amounts of antibody at equivalence. These results confirm the presence of two definable antibody populations in the antiserum, one with a specificity for the native antigen and one for the backbone antigen. They still do not exclude the presence of a third, smaller antibody population with β-galactopyranose specificity.

Type III serum was absorbed with the reduced native antigen at the equivalence point, and after removal of the precipitates, the absorbed serum was reacted with the native, core, and native reduced antigens, and the quantitative precipitin curves are shown in Figure 3. After absorption with the reduced native antigen, only a small fraction of antibody remained which would precipitate the core antigen, confirming their essentially identical immunospecificity. The remaining antibodies are probably specific for β-D-galactopyranose determinants. A distinct population of antibody remained after absorption with native reduced antigen which reacted with the native antigen, confirming the ability of the native determinant to be of wider immune specificity than after reduction. All antibody which reacted with the reduced native antigen was removed by this single absorption with reduced native antigen because a second absorption was done with reduced native antigen at higher antigen concentrations and no further antibody was removed from the serum. Therefore, it is unlikely

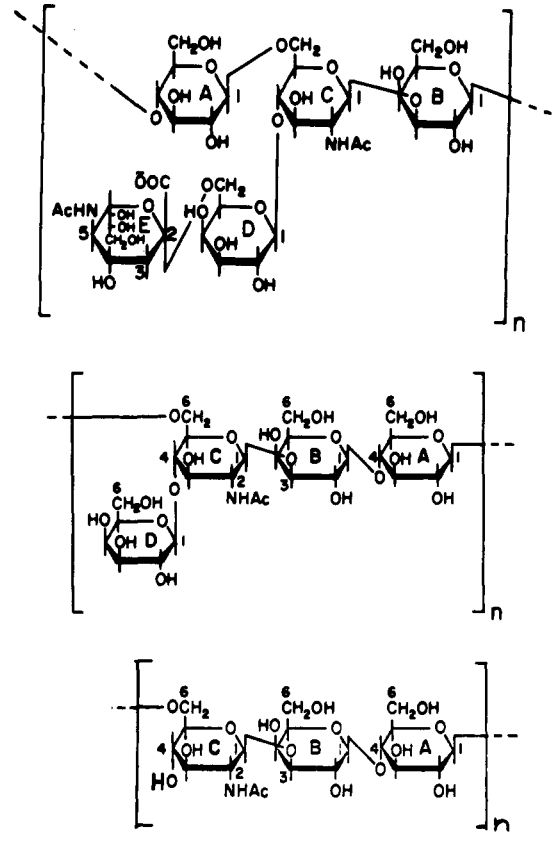

FIGURE 4: Structures of the repeating units of the native (top), core (middle), and backbone (bottom) antigens from type III group B *Streptococcus*.

that low-affinity antibodies were reacting with the native reduced antigen.

Inhibition Experiments. The high degree of specificity of the population of antibodies exclusive to the native type III polysaccharide was also confirmed by inhibition experiments using the native type III polysaccharide and its specifically absorbed homologous antiserum. By use of the radioimmunoassay binding technique, the native type III polysaccharide was able to inhibit the above serological reaction to the extent of 98% whereas the native type Ia polysaccharide gave no detectable inhibition despite the presence of terminal sialic acid residues on both of their respective side chains (Jennings et al., 1980b,c). Thus, the sialic acid residues of the native type III polysaccharide are not immunodominant, and neither, in fact, is the whole branch chain, because in addition, serotransferrin, which contains a common terminal α-D-NeuAc*p*-(2→6)-β-D-Gal*p* disaccharide with the native type III antigen (Jennings et al., 1980b), did not inhibit the precipitation of the native type III polysaccharide with its homologous antiserum. The core type III polysaccharide also showed only a small degree of inhibition (12.3%) on the radioimmunoassay binding experiments using the native type III polysaccharide and its absorbed homologous antiserum. While this experiment demonstrates that the core type III antigen is a poor inhibitor in this system, it does not rule out the possibility of the presence of a small number of low-affinity antibodies in the absorbed antiserum which are capable of binding to both the native and core type III antigens.

^{13}C NMR Spectroscopy. For a correlation of the serological specificity of the native type III antigen with its structural or conformational features, a comparison of its ^{13}C NMR spectrum with those of the reduced native, core, and backbone polysaccharides was made. Except for the reduced native antigen, the structures of the other antigens are depicted in

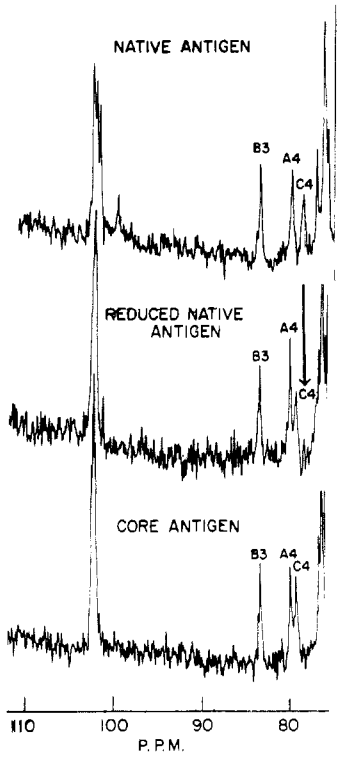

FIGURE 5: Fourier-transformed ^{13}C NMR spectra (75.47 MHz) in the region 75–110 ppm of the native (top), reduced native (middle), and core (bottom) type III antigens of group B *Streptococcus*. All spectra were taken with quadrature detection with an acquisition time of 0.54 s and a spectral width of 15 kHz. The number of free induction decays was approximately 50 000 for each spectrum.

Figure 4. The region of the spectra of particular interest was that between 78.0 and 104.0 ppm, shown in Figure 5, as this includes the signals of the anomeric and ring-linkage carbons of the sugar residues of the native and modified polysaccharides. The chemical shifts and assignments of these signals are also listed in Table III. Displacements in these signals are indicative of conformational changes in the torsion angles of bonds linking the different sugar residues of oligo- and polysaccharides (Jennings & Smith, 1978), and an examination of the signals in the spectra of the native and core antigens indicates that the signal at 78.3 ppm is significantly displaced to 79.1 ppm on removal of the terminal sialic acid residue while the linkage signals at 83.4 and 79.8 ppm have identical chemical shifts (83.4 and 79.8 ppm) in the spectra of both antigens. To unambiguously assign these linkage signals, it was first necessary to assign the linkages of the common backbone following the removal of terminal galactopyranose residues from the core antigen. In this process, the C4 signal of the backbone should undergo a considerable upfield displacement in comparison with C4 of the core and native antigens due to the removal of these residues (Jennings & Smith, 1978), and providing there are no accompanying conformational disturbances in the backbone on the removal of these residues, as is indicated by serological data, the C4 signal of the native and core antigens should be assignable by difference. Of the three linkage signals of the core antigen at 79.1, 79.8, and 83.4 ppm, only the signal at 79.1 ppm was displaced in the spectrum of the backbone antigen. Although this displacement could not be measured due to overlapping signals, it could be ascertained that it was an upfield displacement of more than 3 ppm, and on this basis, it was possible to assign the signal at 79.1 ppm to C4 of the core antigen. In a similar way, the signal at 78.3 ppm in the spectrum of the native antigen was assigned to C4 of the native antigen. The two remaining observable linkage carbon signals of the backbone antigen at 83.4 and 79.8 ppm, which were also present in the spectra of all the polysaccharides (Figure 5), could then be assigned to B3 and A4, respectively, on the basis of previous assignments (Jennings et al., 1980b). Another significant downfield displacement (>0.4 ppm) of one of the anomeric signals in the native antigen following its reduction or removal of its sialic acid residues was the signal at 103.2 ppm. It is possible and certainly consistent to propose that this signal is due to D1 linked directly to C4. In this position, it would also be sensitive to torsion angle changes previously proposed to occur in this linkage. It is interesting to note that on reduction of the native antigen the serological equivalence of the reduced native and core antigens was also matched by an equivalence of the chemical shifts of the common linkage carbons of the two antigens.

Discussion

Antisera raised to group B type III *Streptococcus* organisms in rabbits had been shown to contain two distinctive populations of antibodies associated with type specificity (Kasper et al., 1979; Jennings et al., 1980b). One population had an exclusive specificity for the native antigen and was dependent on the presence of its sialic acid residues, while the other population had a specificity for the core antigen. It was proposed that this latter core specificity was due to determinants terminating the β-D-galactopyranose residues generated by the unintentional removal of sialic acid from the cell-associated native polysaccharide. While microprecipitation experiments using the native and core type III polysaccharides largely confirmed the above results, experiments using the degalactosylated antigen (backbone antigen) demonstrated that in fact the major portion of the core-specific antibodies has a specificity associated with a determinant on the backbone of the core antigen. Thus, this determinant is also an integral part of the native antigen although the antigenicity of the determinant is not dependent on the presence of the terminal sialic acid residues of the native antigen. It is interesting to note, however, that the presence of these sialic acid residues on the native type III antigen is probably implicated in the immunogenicity of this backbone determinant. This is because serological experiments indicate that most of the antibodies to pneumococcal type 14 organisms, which have a capsular polysaccharide identical in structure with that of the type III core antigen, were found to be specific for determinants terminating in β-D-galactopyranose residues; only a minor proportion of antibodies with type III backbone specificity was detected. Baker & Kasper (1976) and Kasper et al. (1979) have demonstrated the essential role of the native type III polysaccharide in the development of human immunity to type III group B streptococcal disease. Whether the backbone determinant also plays a role in human immunity has not been fully established, but certainly the initial immune response is to the sialic acid dependent determinant in rabbits (Jennings et al., 1980b). An attempt was made to structurally define this latter determinant. Serological studies indicate that despite the dominant peripheral location of the sialic acid residues of the native type III antigen they are not themselves immunodominant. This was established when the related native type Ia antigen, also containing terminal α-D-linked sialic acid residues, did not inhibit the homologous serological reaction of the native type III antigen. In order to accommodate sialic acid in the determinant, it was therefore necessary to envisage a determinant of larger size, and an attempt was made to determine its size by using classical inhibition techniques (Kabat & Mayer, 1961). One might normally anticipate that

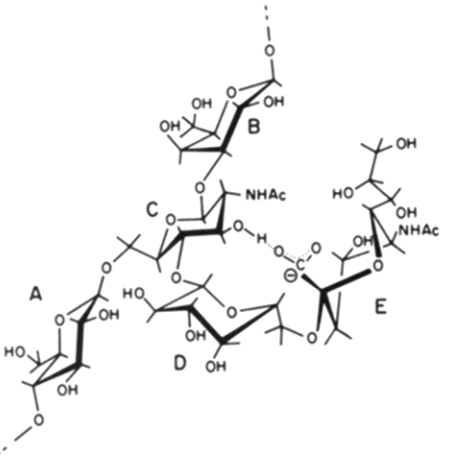

FIGURE 6: Proposed conformation of the repeating unit of the type III polysaccharide antigen of group B *Streptococcus*.

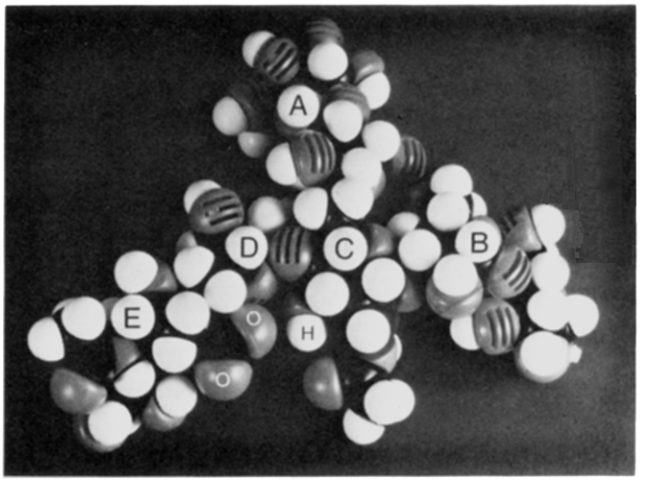

FIGURE 7: Space-filling model (CPK) of the repeating unit of the native type III group B streptococcal polysaccharide.

the disaccharide 6-*O*-α-D-[*N*-acetylneuraminyl]-β-D-galactopyranose, which constitutes the branches of the native type III antigen, should be a strong determinant. However, on structural grounds, one could also predict that it would be an unfavorable determinant because it is a part of rabbit (Leger et al., 1978) and human (Spik et al., 1975) serotransferrin. This prediction proved to be correct when in serological studies serotransferrin did not inhibit the homologous serological reaction of the native type III antigen. Therefore, in classical terms, one would expect this determinant to include residues from the backbone of the native type III antigen. Inclusive of the sialic acid residue, this would now constitute a large determinant but still possibly a feasible one, using the evidence of Kabat & Mayer (1961) on the proposed upper limit of linear glucopyranose determinants. However, it is unlikely that this determinant can extend very far along the backbone of the native type III antigen because the type III core antigen also proved to be a very poor inhibitor of its homologous serological reaction. Thus, by using classical inhibition techniques, it was not possible to define the determinant responsible for the specificity of the native type III antigen. In order to explain the above negative results, one can only postulate the conformational dependence of this determinant in which the terminal sialic acid residues must play an important role. Evidence for such a conformational change was obtained by a comparison of the chemical shifts in the ^{13}C NMR spectra of the native and core antigens. Chemical shift displacements involving linkage carbons can be indicative of interglycose conformational change (Jennings & Smith, 1978), and only one anomeric signal and one linkage signal, assigned to C4 of the 2-acetamido-2-deoxy-β-D-glucopyranose residues (C) of the native type III antigen, undergo such a displacement. Thus, the sialic acid residues of the native antigen control the torsion angles of the glycosidic bond between the penultimate β-D-galactopyranose (D) residues and the backbone 2-acetamido-2-deoxy-β-D-glucopyranose (C) residues (Figure 4). This evidence indicates a conformational change involving the branches of the native type III antigen rather than more extensive conformational changes in its backbone and is consistent with the serological evidence in which the antigenicity of the backbone determinant was found to be independent of the presence of terminal sialic acid residues. It is possible that this conformational control could involve interactions between the branches and backbone of the native antigen to form ring structures and that hydrogen bonding through the carboxylate groups of the sialic acid residues could be a factor in their formation (Figure 6). Certainly, reduction of these carboxylate groups to hydroxymethyl groups caused the reduced native type III antigen to behave both serologically and spectroscopically like the core antigen, which is supportive of the above hypothesis and in any event demonstrates the importance of the charged carboxylate group to this conformational control. A space-filling model (CPK) shown in Figure 7 suggests that such a structure is plausible and that this hydrogen bonding might occur between the carboxylate group of the sialic acid residues (E) and the hydroxyl group at C3 of the 2-acetamido-2-deoxyglucopyranose residue (C). However, serotransferrin could also form an identical ring-type structure and yet does not inhibit the homologous serological reaction of the native type III antigen. Obviously, this serological specificity must involve additional backbone sugar residues although the fact that the core antigen is also a poor inhibitor of the above serological reaction would suggest only a limited participation by these residues. With this evidence, it is conceivable that the determinant need not be too large to achieve specificity and in fact that sialic acid need not be directly involved in the determinant but simply function as an external factor in the conformational control of the determinant. This is certainly consistent with all the serological data and is supported by the inability of the removal of a large part of the glycol chain of all the sialic residues to change the specificity of the native type III antigen. Space-filling models (CPK) suggest that the determinant is probably located at residues C and D with a possible involvement of residue A. It is interesting to note that the backbone determinant must also be located in the same general area as the type specificity of all the group B streptococcal core antigens is probably derived from the variable linkages between their A and C backbone residues (Figure 6) (Jennings et al., 1980b; H. J. Jennings, C. Lugowski, and D. L. Kasper, unpublished experiments). The fact that the antigenicity of this determinant is independent of the branches of the native type III antigen means that it must be located on the opposite side of the native antigen to the sialic acid dependent determinant (Figure 6).

It is interesting to speculate on the role of this type of conformational control by sialic acid in other molecules. Certainly, the isomeric type Ia antigen also has a specificity dependent on its terminal sialic acid residues (Jennings et al., 1980c). It is also probably a feature of glycoproteins having terminal sialic acid residues. These glycoproteins are ubiquitous on all cell surfaces where they are probably involved in recognition processes which trigger different biological transformations (Jeanloz & Codington, 1976).

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Structure Elucidation of Marker Glycolipids of Alloantigen-Activated Murine T Lymphocytes†

K. Reinhold Gruner, Ronald V. W. van Eijk,‡ and Peter F. Mühlradt*

ABSTRACT: DBA/2 mouse spleen cells were stimulated in vitro by (a) alloantigen (mitomycin-treated CBA/J splenocytestes), (b) the T cell mitogen concanavalin A (Con A), and (c) the B cell mitogen lipopolysaccharide (LPS). The cultures were pulsed for 10 h with ^{14}C-labeled galactose and glucosamine. Radiolabeled glycosphingolipids (GSL's) were extracted from the cells and the neutral GSL's isolated and analyzed by high-performance thin-layer chromatography. Two of the radioactive neutral GSL's, 9 and 12a, were found to be prominent in the alloantigen-stimulated cells but not in T cells stimulated by Con A. GSL 9 was also present as a minor component in LPS-stimulated B lymphocytes. GSL's 9 and 12a were purified by preparative column chromatography on Iatrobeads. The sequence and anomeric linkages of the carbohydrate moiety of these glycolipids were determined by successive degradation with exoglycosidases. The structures were shown to be Gal(α1-x)Gal(β1-x)GlcCer (glycolipid 9) and GalNAc(β1-x)Gal(α1-x)Gal(β1-x)GlcCer (glycolipid 12a), respectively. The latter glycolipid may serve as a marker for alloantigen-activated T cell subpopulations.

Glycosphingolipids (GSL's)[1] of cells involved in immune processes have recently become of interest because of their potential involvement in functional interactions between immune cells and/or accessory cells. A systematic study of the GSL's of these cells, particularly in an activated state, may eventually help to understand the possible functions of these cell surface molecules.

Antibodies directed against gangliotetraosylceramide (Gg_4Cer) have been reported to react specifically with murine

† From the Gesellschaft für Biotechnologische Forschung mbH, Braunschweig-Stöckheim, Federal Republic of Germany. *Received December 16, 1980.*

‡ Present address: Reijksinstitut voor de Volksgezondheid, 3720, Bilthoven, The Netherlands.

[1] Abbreviations used: Con A, concanavalin A; C-M-W, chloroform-methanol-water; FCS, fetal calf serum; GSL, glycosphingolipid; GlcCer, glycosylceramide; *i*-Gb_3Cer, isoglobotriaosylceramide; *i*-Gb_4Cer, isoglobotetraosylceramide; Gb_4Cer, gangliotetraosylceramide; LPS, lipopolysaccharide.

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