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CHEMICAL AND IMMUNOCHEMICAL STUDIES ON LIPOPOLYSACCHARIDES FROM PYOCIN 103-SENSITIVE AND -RESISTANT *Neisseria gonorrhoeae*[*,†]

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ABSTRACT

The chemical and immunochemical properties of lipopolysaccharides (LPS) isolated from pyocin 103-sensitive and -resistant *Neisseria gonorrheae* were investigated. Marked differences were found in immunochemical behavior of LPS from pyocin-sensitive gonococcal strain JW31 and its isogenic pyocin-resistant variant JW31R. JW31 LPS readily precipitated wheat-germ agglutinin, soybean lectin, and rabbit anti-*Streptococcus faecalis* or horse anti-type 14 pneumococcal antibody. In contrast, JW31R LPS precipitated only soybean lectin. The combining-site specificity of anti-*S. faecalis* cross-precipitated by JW31 LPS, or type 14 pneumococcal capsular polysaccharide, was examined by hapten inhibition, and lactose found to be the most potent inhibitor. Horse anti-pneumococcal type 14 antibodies, cross-precipitated by JW31 LPS and streptococcal lactose polymer, exhibited heterogeneity with respect to combining site specificity. Gel filtration of LPS-derived core oligosaccharide showed both strain JW31 and JW31 R to possess R-type lipopolysaccharide with cores having a M_r ~1800. JW31R LPS contains more galactose but less hexosamine than JW31 LPS.. Both JW31 and JW31R core oligosaccharides possess D-glucosamine and D-galactosamine, probably *N*-acetylated, as the only nonreducing end-groups, and (1→4)-linked D-glucose residues. Chemical data support immunochemical findings which indicate that lactose units occur as a structural feature of JW31 gonococcal LPS.

INTRODUCTION

Pseudomonas aeruginosa strain PA103 produces a bacteriocin (pyocin 103) capable of inhibiting the growth of *Neisseria gonorrhoeae*[1]. Electron microscopic studies[1] show that pyocin 103 interacts directly with the cell surface of sensitive

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strains of *N. gonorrhoeae*, whereas microorganisms resistant to the inhibitory action of pyocin show no pyocin–cell surface interaction.

Pyocin-sensitive *N. gonorrhoeae* strains and their matched, isogenic pyocin-resistant variants have been shown to differ in their ability to be agglutinated by lectins[2]. In particular, pyocin-sensitive strains were agglutinated by the 2-acetamido-2-deoxy-D-glucose-specific lectin, wheat-germ agglutinin (WGA)[3,4], whereas their isogenic pyocin-resistant variants failed to agglutinate[2]. In addition, a lipopolysaccharide (LPS) purified from the pyocin-sensitive gonococcal strain JW31 was shown to function as a receptor for pyocin, whereas LPS purified from its isogenic-resistant variant strain JW31R showed no pyocin-receptor activity[2]. The nature of structural alteration(s) responsible for the loss of pyocin-receptor activity by JW31R LPS was not established. Since JW31R organisms were not agglutinated by WGA and quantitative lectin-binding experiments showed strain JW31R to possess fewer WGA-binding sites, it was suggested that LPS changes associated with pyocin resistance may involve a loss of structure, or an alteration affecting 2-acetamido-2-deoxy-D-glucose residues[2].

In the present study, the chemical and immunochemical properties of a lipopolysaccharide isolated from pyocin-sensitive strain JW31 and its pyocin-resistant variant JW31R were examined by use of lectins and antisera raised to polysaccharide antigens of known structure.

EXPERIMENTAL

Materials and methods. — *Neisseria gonorrhoeae* strains JW31 and JW31R were grown in liquid GCP medium[2] and the lipopolysaccharides isolated by hot-phenol extraction as previously described[2]. Cultures of pyocin 103-resistant strain JW31R gonococci, used to isolate JW31R LPS, were grown in the continuous presence of excess pyocin 103 to eliminate pyocin-sensitive revertants. Lipid-free oligosaccharides were isolated from purified LPS by acetic acid hydrolysis as described by Perry *et al.*[5].

Lactose polymer from *S. faecalis* strain N was provided by Dr. J. H. Pazur[6]. Pneumococcal type 14 capsular polysaccharide was isolated from cell-free, culture supernate essentially as described by How[7] for type I polysaccharide. The final product was treated with ribonuclease and deoxyribonuclease (5 mg each), emulsified with 1-trichloro-2-trifluoroethane, centrifuged, and precipitated from the separated water-layer by adding 95% ethanol (3 vol.)

Analytical methods. — 3-Deoxy-2-ketooctonic acid (KDO) was estimated by the method of Karkhanis *et al.*[8], and heptose by the method of Wright and Rebers[9]. D-*glycero*-L-*manno*-Heptose was provided by Dr. P. A. Rebers and D-*glycero*-D-*gluco*-heptose was purchased from Sigma Chemical Co. The specific extinction coefficient ($\Delta_{Abs} \cdot \mu g^{-1}$ of heptose · mL) for D-*glycero*-L-*manno*-heptose (0.0052), was lower than that for D-*glycero*-D-*gluco*-heptose (0.0088). The difference in absorbance at 505 nm and 545 nm was determined for each test sample, and

the heptose content estimated by use of the extinction coefficient for D-*glycero*-L-*manno*-heptose standards assayed in the same experiment.

Hexosamine, D-glucose, and D-galactose content of LPS and lipid-free oligosaccharides was estimated on samples hydrolyzed with M hydrochloric acid for 8 h at 100° in sealed glass tubes, unless otherwise stated. Following hydrolysis, the tubes were cooled, opened, and dried under reduced pressure in the presence of phosphorus pentaoxide and potassium hydroxide.

Total hexosamine content was estimated by a modification of the procedure described by Levvy and McAllen[10]. Lipid-free oligosaccharide (150 μg), LPS (200 μg), or hexosamine standard solutions (20–60 μg) were hydrolyzed, and then quantitatively transfered to clean test tubes (final volume 0.6 mL). A stock solution of *p*-dimethylaminobenzaldehyde reagent was prepared as described by Reissig *et al.*[11]. Molar extinction coefficients obtained for 2-acetamido-2-deoxy-D-glucose under these conditions ranged from 20 700 to 20 900 $Abs._{585\ nm} \cdot mol^{-1} \cdot L^{-1}$, in excellent agreement with values reported by Reissig *et al.*[11]. The molar extinction coefficient of 2-acetamido-2-deoxy-D-galactose was only 31% of that of 2-acetamido-2-deoxy-D-glucose. The total hexosamine content of unknowns was estimated from the $Abs._{585\ nm}$ obtained from samples by use of an extinction coefficient for 2-acetamido-2-deoxy-D-glucose standards assayed in the same experiment.

Hydrolyzates of LPS, or lipid-free oligosaccharides, (200–300 μg) were reconstituted in water and analyzed for D-glucose and D-galactose content with glucose/fructose and lactose/galactose Enzymological Assay Kits (Boehringer Mannheim). Phosphorus was estimated by the method of Ames[12].

Gel filtration. — Ascending gel filtration through Sephadex G-25 (Pharmacia Fine Chemicals, fine grade) was done in a column (1.5 × 58.2 cm) equilibrated with 10mM ammonium acetate buffer, pH 6.8, at 4° and a flow rate of 11.4 mL/h (6.5 cm/h). Fractions (1.0 mL) were collected automatically (LKB, Ultrorac 7000), and a sample of each fraction was analyzed for total hexose content by the phenol–sulfuric acid method[13]. The column (2.5 ×80.7 cm) of Bio-Gel P-6 (200–400 mesh, Bio-Rad Laboratories) used for gel filtration was equilibrated with 50mM ammonium acetate buffer, pH 7.0, at 4°, and the flow rate set at 18.4 mL/h (3.75 cm/h). Fractions (1.66 mL) were collected and an aliquot of each fraction was analyzed for total hexose content by the phenol–sulfuric acid method[13]. Columns of Sephadex G-25 and Bio-Gel P-6 were calibrated for molecular-weight estimation with the isomaltodextrin series of oligosaccharides described by Martineau *et al.*[14].

Methylation analysis. — Each lipid-free oligosaccharide from the LPS samples of gonococcal strains JW31 and JW31R was exhaustively methylated with methyl sulfinyl carbanion reagent prepared by the method of Hakomori[15]. The permethylated oligosaccharide was hydrolyzed, reduced, and acetylated, and the alditol acetate derivatives of *O*-methyl-substituted monosaccharides were examined by gas-liquid chromatography as described by Perry *et al.*[5].

Lectins. — Wheat-germ lectin (WGA) was purified by affinity chromatog-

raphy as described by Marchesi[16]. Soybean (*Glycine max.*) lectin was purchased fromn E-Y Laboratories. Precipitation of WGA (30 μg) by strain JW31 and JW31R gonococcal LPS was done at 0° in a total volume of 0.25 mL. Total nitrogen content in washed precipitates was estimated by the ninhydrin method[17].

Quantitative precipitation. — Anti-*Streptococcus faecalis* strain N vaccine (serum RS31-6C) was raised in female New Zealand White rabbits. Horse anti-*S. pneumoniae* type 14 capsular polysaccharide serum (bleeding 10, 5/25/39) was obtained from the New York State Department of Health. Quantitative precipitation assay was performed at 0° in a total volume of 0.4 mL as described by Kabat and Mayer[18]. Total nitrogen content in washed antigen–antibody precipitates was estimated by the ninhydrin assay[17]. Sugars used for hapten inhibition were of the highest quality available from either Sigma Chemical Co. or Ptanstiehl Laboratories. *N*-Acetyllactosamine was provided by Dr. Victor Ginsburg, additional samples were purchased from Sigma Chemical Co. Sugars assayed for inhibitory ability by quantitative hapten-inhibition of precipitation were delivered from analytical stock solutions prepared in 0.85% saline solution. Tubes containing inhibitor and antisera were incubated for 60 min at 23° before adding the antigen and adjusting the final volume to 0.4 mL. The total content of nitrogen (μg N) precipitated was determined by the ninhydrin assay[17]. The percent inhibition was calculated from the μg N content precipitated in tubes containing test sugars, as compared with the μg N content precipitated in uninhibited-control tubes.

RESULTS

The interaction of wheat-germ agglutinin (WGA) with isolated, purified, gonococcal LPS samples from strains JW31 and JW31R was examined by quantitative precipitation (Fig. 1). The addition of 150 μg of JW31 LPS, precipitated 2.7 μg

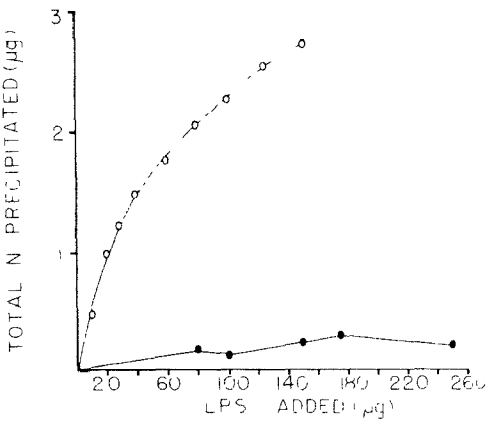

Fig. 1. Quantitative precipitation of WGA by gonococcal lipopolysaccharide from strains JW31 and JW31R. Various amounts of JW31 LPS (○) or JW31R LPS (●) were added to WGA (30 μg) and the final volume of each reaction mixture was adjusted to 0.25 mL

TABLE I

COMPONENTS OF GONOCOCCAL LIPOPOLYSACCHARIDES[a]

Component	*JW31*		*JW31R*	
	LPS	*LF-oligosaccharide*[b]	*LPS*	*LF-oligosaccharide*[b]
KDO	6.7 ± 0.2	11.7 ± 0.2 (1.2 ± 0.05)[d]	6.4 ± 0.1	11.1 ± 0.1 (1.3 ± 0.05)[d]
Heptose	7.0	14.5	6.9	14 6
Phosphate	8.4 ± 1.0	9 4 ± 0.4 (2.1 ± 0.1)[d]	9.3 ± 0.4	8.3 ±0.4 (2.4 ± 0 1)[d]
D-Glucose[e]	7.5 ± 0.5[f]	11.8 ± 0.5[f]	6.3 ± 0.5[f]	11.8 ± 0.5[f]
D-Galactose[e]	10.5 ± 0.5[f]	16.7 ± 0.5[f]	12.4 ± 0.5[f]	20.9 ± 0.5[f]
Hexosamine[e]	11.1 ± 0.1 (13.3 ± 0.3)[g]	9.0 ± 0.1	9.3 ± 0.1 (12.2 ± 0.2)[g]	5.7 ± 0.1

[a]Values are expressed as μg/100 μg of dry weight ± standard deviation unless stated otherwise. [b]LF-Oligosaccharide, lipid-free oligosaccharide, isolated following mild acetic acid hydrolysis. [c]KDO, 3-deoxy-2'-ketooctonic acid. [d]Analysis on the major oligosaccharide peak eluted from Sephadex G-25 (Fig. 2). [e]Samples were hydrolyzed for 8 h at 100° with M hydrochloric acid. [f]D-Glucose and D-galactose values are expressed as μg/100 μg of dry weight ± the maximum range between duplicate samples. [g]Samples were hydrolyzed for 18 h at 100° with M hydrochloric acid

of nitrogen, whereas the addition of up to 250 μg of JW31R LPS precipitated only 0.1–0.3 μg of nitrogen (Fig. 1). Precipitation of WGA by JW31 LPS was completely inhibited by addition of 1.0 mmol of *N,N'*-diacetylchitobiose. In contrast to the results with WGA, both LPS preparations were found to precipitate soybean lectin.

In the composition of JW31 LPS, JW31R LPS, and their lipid A-free oligosaccharides, the constituents listed in Table I account for 53.1 of JW31 LPS, 53.5 of JW31R LPS, 73.1 of JW31 LF-, and 72.4% of JW31R LF-oligosaccharide dry weight. The lipid A content of JW31 and JW31R LPS was estimated from the dry weight of water-insoluble material recovered by centrifugation following dilute acetic acid hydrolysis of LPS. The lipid A content estimated in this way was 46.3 for JW31 LPS and 44.7% for JW31R LPS. After correcting for hexosamine contribution to the weight of lipid A, the constituents reported in Table I, combined with estimates of lipid A content, account for 94.9 and 91.7%, respectively, of the total dry weight for JW31 and JW31R LPS preparations. This is in good agreement with gonococcal LPS composition reported by others[5,19–21].

To separate free 3-deoxy-2-ketooctonic and phosphoric acid from lipid-free oligosaccharide, mild-acid hydrolyzates were fractionated by Sephadex G-25 gel filtration (Fig. 2). Both JW31 and JW31R lipid-free hydrolyzates gave 2 peaks that were recovered and assayed for 3-deoxy-2-ketooctonate and phosphate groups (Table I). The major oligosaccharide peak (Fig. 2, peak I), isolated from JW31 LPS hydrolyzate, contained only 10.3% of the total 3-deoxy-2-ketooctonate and 22.3% of the total phosphate groups present in the whole, unfractionated, mild-acid hydrolyzate. Isolated JW31R LF-oligosaccharide contained 11.7% of the total

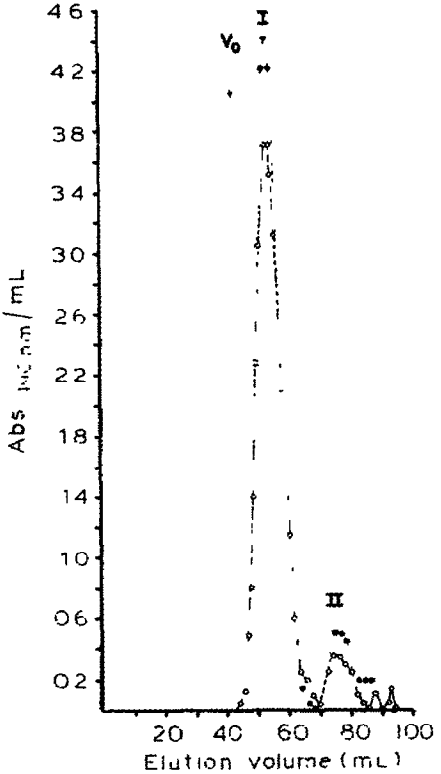

Fig. 2. Gel filtration, on Sephadex G-25, of lipid-free oligosaccharide prepared from JW31 LPS (—○—○—) or JW31R LPS (—●—●—) by mild acetic acid hydrolysis.

3-deoxy-2-ketooctonate and 28.9% of the total phosphate groups present in the unfractionated hydrolyzate. Peak II was not recovered quantitatively for either JW31 or JW31R LF-oligosaccharide but did contain both 3-deoxy-2-ketooctonate and phosphate groups. Within experimental error, no difference was found in the proportions of 3-deoxy-2-ketooctonate, heptose, phosphate, or D-glucose residues between JW31 and JW31R LPS, or between their unfractionated, lipid-free oligosaccharides. However, by weight, intact JW31R lipopolysaccharide contained 1.9% more D-galactose than JW31 LPS. Similarly, lipid A-free JW31R LF-oligosaccharide contained 4.2% more D-galactose than lipid A-free JW31 LF-oligosaccharide. In addition, JW31R LPS and its lipid-free oligosaccharide appeared to contain 1–3% less hexosamine (Table I).

The material of the major oligosaccharide peak (Fig. 2, peak I) of JW31 and JW31R LF-oligosaccharides, eluted from Sephadex G-25, was permethylated, acid hydrolyzed, and reduced with borohydride. G.l.c. of the alditol acetate derivatives[5] (see Fig. 3) indicated the presence of 2-acetamido-2-deoxy-3,4,6-tri-*O*-methyl-D-glucose (R_t 12.93 min) and 3,4,6-tri-*O*-methyl-D-galactose (R_t 14.70 min). Comparison of the area under the curves of these two tri-*O*-methyl deriva-

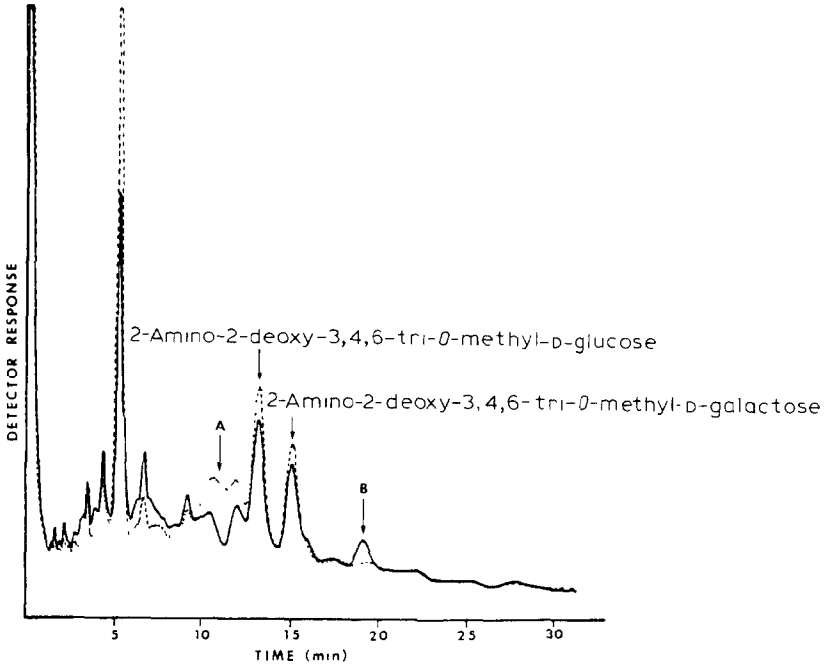

Fig. 3. Isothermal g.l.c. at 200°, on an OV-17 column, of the alditol acetates of the permethylated derivatives of JW31 (——) and JW31R (· · · · · ·) lipid-free oligosaccharides after permethylation, acid hydrolysis, and reduction.

tives suggests that JW31 and JW31R LF-oligosaccharides contain equimolar amounts of D-glucosamine and D-galactosamine as terminal, nonreducing residues. The *O*-methyl derivatives corresponding to regions A and B in Fig. 3 were not identified, but the position of region A suggests the presence of an *O*-methylated neutral sugar derivative from JW31R LF-oligosaccharide not present in JW31 LF-oligosaccharide. An improved resolution of the *O*-methylated neutral sugar derivatives obtained from JW31R LF-oligosaccharide showed a unique peak (R_t 11.17 min) not present for JW31 LF-oligosaccharide (peak C, Fig. 4). The identity of this peak C could not be established, but one *O*-methyl derivative found in both JW31 and JW31R LF-oligosaccharides was identified as 2,3,6-tri-*O*-methyl-D-glucose on the basis of its retention time (11.68 min).

Only trace amounts of 2,3,4,6-tetra-*O*-methyl-D-glucose or 2,3,4,6-tetra-*O*-methyl-D-galactose were identified from either JW31 or JW31R LF-oligosaccharide, and attempts to identify other peaks were unsuccessful. The retention times of these peaks did not correspond to that of 2,3,4- 2,4,6- or 3,4,6-tri-*O*-methyl-D-glucose; or 2,3,4-tri-*O*-methyl-D-galactose.

The molecular weights of lipid-free oligosaccharides isolated from both JW31 and JW31R LPS were estimated by gel filtration to be 1700 with Sephadex G-25 and between 1800 and 2300 with Bio-Gel P-6. As lipopolysaccharides having high molecular-weight O-side chains are eluted in the void volume of Sephadex G-25

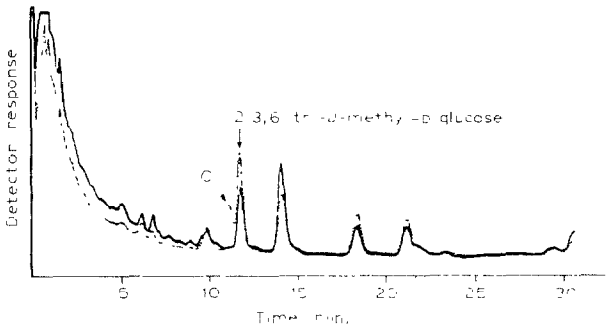

Fig. 4. G l c., on an SP 2340 column, at 190–240°, 4°/min, of the alditol acetates of the permethylated derivatives of JW31 (——) and JW31R (· · · · ·) lipid-free oligosaccharides after permethylation, acid hydrolysis, and reduction

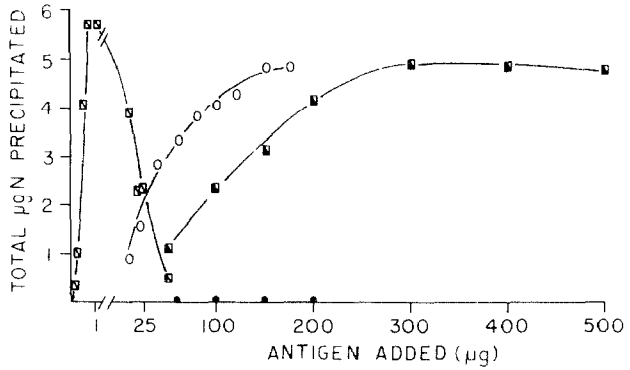

Fig. 5. Quantitative precipitation of antibody from antiserum to *S. faecalis* strain N by various antigens. Increasing amounts of antigen were added to 20-µL volumes of anti-*S. faecalis* serumRS1-6C, the final volume in each tube was adjusted to 0.4 ml. ◪, Homologous lactose polymer. ◨, type 14 polysaccharide. ○, JW31 LPS, and ●, JW31R LPS.

and no high-molecular-weight oligosaccharide was found in the void volume for either JW31 or JW31R lipid-free oligosaccharide (Fig. 2), both JW31 and JW31R possess rough-type LPS, devoid of O-side chains.

Streptococcus faecalis strain N contains an immunogenic, type-specific, cell-wall diheteroglycan composed of D-glucopyranosyl and D-galactopyranosyl residues (lactose polymer), which possesses lactose units as immunodominant groups. Rabbits hyperimmunized with *S. faecalis* strain N vaccine produce large amounts of anti-lactose antibody of restricted heterogeneity[22,23]. The ability of gonococcal LPS from strains JW31 and JW31R, and type 14 pneumococcal capsular polysaccharide to precipitate antibody from anti-*S. faecalis* serum (RS1-6C) was examined by quantitative precipitation (Fig. 5). JW31 LPS precipitated 4.9 µg of nitrogen (µg N), representing 86% of the total nitrogen content precipitable by homologous, lactose-polymer antigen. In contrast, JW31R LPS failed to precipitate the anti-lactose polymer antibody. Type 14 polysaccharide, on a weight basis, was less effective than JW31 LPS in precipitating the anti-lactose polymer; how-

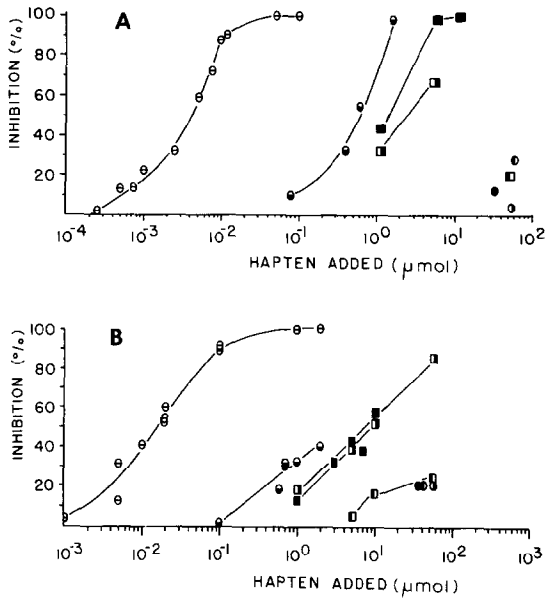

Fig. 6. (A) Quantitative hapten-inhibition of anti-*S. faecalis* precipitation by JW31 LPS. Various amounts of sugars assayed for inhibitory ability were added to 20 μL of serum, incubated for 1 h at 23°, and then 80 μg of JW31 LPS were added and the volume was adjusted to 0.4 mL. Inhibitors: ⊖, lactose; ◒, *N*-acetyllactosamine; ■, 2-acetamido-2-deoxy-D-galactose; ◨, methyl β-D-galactopyranoside; ◧, methyl α-D-galactopyranoside; ◐, methyl α-D-glucopyranoside; ◑, methyl β-D-glucopyranoside; and ●, 2-acetamido-2-deoxy-D-glucose. (B) Quantitative hapten inhibition of anti-*S. faecalis* precipitation by pneumococcal type 14 capsular polysaccharide (200 μg) under the same conditions as in A. Symbols as in A.

ever, at equivalence, type 14 polysaccharide also removed 86% of the total nitrogen content precipitable by the homologous, lactose-polymer antigen. The ability of JW31 LPS and type 14 polysaccharide to each precipitate the same fraction of antibody was shown by absorption experiments. Absorption of anti-*S. faecalis* serum with JW31 LPS removed all the antibody precipitable by type 14 polysaccharide. Similarly, absorption with type 14 polysaccharide abolished the reactivity with JW31 LPS. The combining-site specificity of antibodies cross-precipitated by JW31 LPS and type 14 polysaccharide was examined by quantitative hapten-inhibition (Fig. 6 and Table II). Fifteen sugars were assayed for their ability to inhibit anti-*S. faecalis* (RS1-6C) precipitation by JW31 LPS. Of the sugars tested, lactose was the best inhibitor, requiring only 4 nmol to give a 50% inhibition of precipitation (Fig. 6A). On a molar basis, lactose was 150 times more effective than 2-acetamido-2-deoxy-4-*O*-β-D-galactosyl-D-glucose (*N*-acetyllactosamine). 2-Acetamido-2-deoxy-D-galactose and methyl β-D-galactopyranoside also gave a 50% inhibition of precipitation by JW31 LPS, but were, respectively, 350 and 600 times less effective than lactose. Table II summarizes the results of sugars assayed but found to be poor inhibitors of JW31 LPS precipitation. Lactose was also the best inhibitor of anti-*S. faecalis* precipitation by type 14 polysaccharide, requiring only

TABLE II

HAPTEN INHIBITION OF ANTI-LACTOSE ANTIBODY PRECIPITATION BY JW31 LPS

Hapten	*Amount added (μmol)*	*Inhibition (%)*
Lactose	0.004	50.0
D-Mannose	64.4	40.3
L-Rhamnose	52.3	11.4
L-Fucose	53.8	16.7
3-Deoxy-2-ketooctonic acid	3.3	0.6
D-*glycero*-D-*gluco*-Heptose	9.9	10.0
D-*glycero*-L-*manno*-Heptose	2.2	11.0
Methyl α-D-glucopyranoside	50.5	28.3
Methyl β-D-glucopyranoside	54.3	8.0
Methyl α-D-galactopyranoside	49.2	20.3
2-Acetamido-2-deoxy-D mannose	66.6	24.3
2-Acetamido-2-deoxy-D-glucose	32.1	13.9

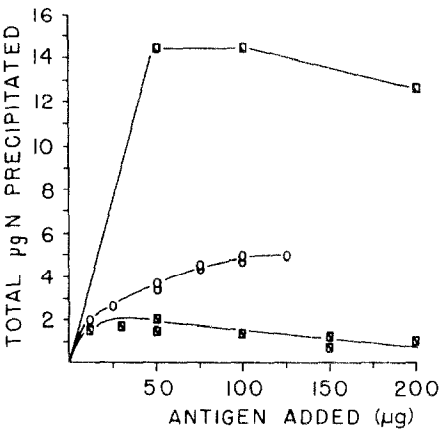

Fig. 7. Quantitative precipitation of horse 635 anti-type 14 pneumococcal serum. Various amounts of antigen were added to 20-μL volumes of anti-type 14 serum, and the final volume in each tube was adjusted to 0.4 mL: ■, Homologous type 14 polysaccharide; ○, lactose polymer; and ●, JW31 LPS.

17 nmol for a 50% inhibition (Fig. 6B). The highest amount of *N*-acetyllactosamine tested (2.0 μmol) gave only a 40% inhibition of precipitation, 180 times the amount of lactose required to give a comparable inhibition. Methyl β-D-galactopyranoside and 2-acetamido-2-deoxy-D-galactose were indistinguishable from each other in their molar inhibitory potency, and were 500 times less effective than lactose. JW31 LPS and lactose polymer were both examined for their ability to precipitate antibody from type 14 horse anti-pneumococcal serum (Fig. 7). Absorption of anti-type 14 serum with homologous type 14 polysaccharide antigen removed all the antibody precipitable by either JW31 LPS or lactose polymer, indicating that the antibodies cross-precipitated by JW31 LPS and lactose polymer were type 14 capsular-polysaccharide specific. Gonococcal LPS from strain JW31 maximally

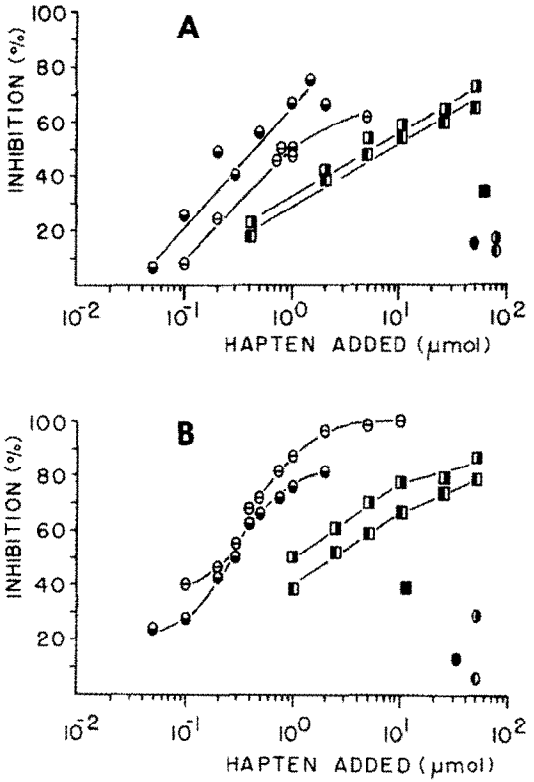

Fig. 8. (A) Quantitative hapten-inhibition of anti-type 14 precipitation by JW31 LPS. Various amounts of the sugar to be tested were added to 20 μL of serum, incubated for 1 h at 23°, and then 74 μg of JW31 LPS were added and the volume in each tube was adjusted to 0.4 mL. Inhibitors used: ⊖, lactose; ◒, *N*-acetyllactosamine; ■, 2-acetamido-2-deoxy-D-galactose; ◨, methyl β-D-galactopyranoside; ◧, methyl α-D-galactopyranoside; ◐, methyl α-D-glucopyranoside; ◑, methyl β-D-glucopyranoside; and ●, 2-acetamido-2-deoxy-D-glucose (B) Quantitative hapten inhibition of anti-pneumococcal type 14 (60 μL of serum per reaction) precipitation by lactose polymer (10 μg) under the same conditions as in A. Symbols as in A.

precipitated 4.9 μg N from horse 635 anti-type 14 serum, representing 34.4% of the total type 14 specific antibody, whereas lactose polymer precipitated only 1.9 μg N, representing 13.2% of the type 14 specific antibody (Fig. 7). JW31R LPS failed to precipitate anti-type 14 antibody. The difference in the μg N precipitated from horse anti-type 14 serum by JW31 LPS and lactose polymer suggests that these antigens removed different populations of equine antibody. To establish this, anti-type 14 serum was absorbed with lactose polymer and the amount of JW31 LPS precipitable-antibody nitrogen content remaining in absorbed serum estimated. JW31 LPS precipitated only 57.9% as much nitrogen from lactose polymer absorbed anti-type 14 serum as from unabsorbed serum. The reciprocal absorption was also done. After absorption of horse 635 anti-type 14 serum with JW31 LPS, lactose polymer precipitated only 45.4% as much nitrogen as it did from unabsorbed anti-type 14 serum. Therefore, on the basis of cross-reactivity, these anti-

gens define four separate populations of type 14 capsular polysaccharide-specific antibody in horse 635 anti-serum. One population is exclusively reactive with homologous type 14 capsular polysaccharide; another population reacts with a determinant common to JW31 LPS, streptococcal lactose polymer, and type 14 capsular polysaccharide; and two other fractions of antibody are uniquely cross-reactive with either JW31 LPS or lactose polymer but not both antigens. Of eight sugars assayed for their ability to inhibit anti-type 14 precipitation by JW31 LPS (Fig. 8A), *N*-acetyllactosamine was the best inhibitor, requiring only 0.37 μmol for a 50% inhibition. Lactose was found to be 2.7 times less effective as an inhibitor than *N*-acetyllactosamine. Methyl α- and β-D-galactopyranoside were comparable in molar inhibitory ability, but less effective than *N*-acetyllactosamine (Fig. 8A). At the concentrations tested, none of the sugars gave a 100% inhibition of JW31 LPS precipitation. Hapten inhibition of anti-type 14 precipitation by lactose polymer gave qualitatively and quantitatively different results from those obtained with JW31 LPS (Fig. 8B). Lactose and *N*-acetyllactosamine were equally effective (0.27 μmol each) at the 50% level of inhibition. At higher hapten concentrations, however, lactose appeared to surpass *N*-acetyllactosamine in molar inhibitory potency, giving a 100% inhibition when ~5 μmol were added (Fig. 8B).

DISCUSSION

The lipopolysaccharides isolated from pyocin 103-sensitive gonococcal strain JW31 and its isogenic pyocin-resistant variant JW31R are known to differ in their ability to function as a receptor for pyocin[2] 103, antigenic specificity[24], and mobility in $NaDodSO_4$–polyacrylamide gel electrophoresis[24]. In the present study, the chemical and immunochemical properties of purified LPS samples isolated from gonococcal strains JW31 and JW31R were investigated, and marked differences found in their immunochemical behavior. Whereas wheat-germ agglutinin, rabbit antibodies to a streptococcal lactose polymer, and horse antibodies to pneumococcal type 14 capsular polysaccharide were readily precipitated by JW31 LPS (Figs. 1, 5, and 7), the lipopolysaccharide from pyocin-resistant strain JW31R showed little or no reactivity with these reagents.

Although markedly different in immunochemical reactivity, these lipopolysaccharides exhibit only small differences in overall chemical composition (Table I). In addition, 2-acetamido-2-deoxy-D-glucose and -D-galactose were the only sugars shown by methylation analysis to occupy terminal, nonreducing positions in both JW31 and JW31R LPS (Fig. 3). That terminal, nonreducing 2-acetamido-2-deoxy-D-galactose residues are a structural feature common to both gonococcal antigens is supported by the finding that both preparations of LPS precipitate soybean lectin. Another structural unit identified in both gonococcal lipopolysaccharides by methylation analysis were the (1→4)-linked D-glucosyl residues (Fig. 4), which provides chemical support for the immunochemical data indicating that lactosyl units are a structural feature of JW31R LPS.

The presence of lactosyl residues as a part of JW31 LPS structure was inferred from the ability of this LPS to precipitate lactose-specific antibody from *S. faecalis* antiserum (Fig. 5). An immunochemical specificity involving lactose is supported by hapten inhibition data (Fig. 6A) which show lactose to be the best inhibitor of the JW31 LPS cross-reaction. The conclusion that lactose, rather than β-linked D-galactose units, play an immunodominant role in mediating this cross reaction is evident from hapten-inhibition data (Fig. 6A). Since, on a molar basis, methyl β-D-galactopyranoside and *N*-acetyllactosamine are, respectively, 600 and 150 times less effective than lactose at inhibiting precipitation, the 4-*O*-substituted D-glucosyl residue must contribute significantly to the interaction with antibody-combining sites.

Antisera raised in response to *S. faecalis* vaccine and a synthetic lactosyl conjugate have been shown by Bundle[25] to agglutinate red cells coated with gonococcal LPS. Fractionation of antisera from a lactose-immunoadsorbent by sequential elution with D-galactose and lactose yielded "anti-D-galactose" and "anti-lactose" fractions. Although only a small fraction of the total agglutinating activity of whole antiserum was recovered, agglutinating activity of anti-streptococcal sera was confined only to the "anti-D-galactose" fraction. Data obtained in the present study by hapten inhibition of LPS cross-precipitation from unfractionated serum and data obtained by Bundle[25] from passive hemagglutination by fractionated antibody are in agreement in that both studies demonstrate the participation of β-linked D-galactopyranosyl residues in cross-reaction. Whether the failure of isolated "anti-lactose" fractions to hemagglutinate may be attributable to strain differences in the gonococcal LPS antigens employed, differences in antisera, or a low recovery of the total "anti-lactose" present in whole serum is not clear.

By absorption experiments, pneumococcal type 14 capsular polysaccharide, which also cross-precipitates anti-*S. faecalis* antibody (Fig. 5), was found to remove the same population of anti-lactose antibodies as did JW31 LPS. Hapten-inhibition analysis confirmed that the combining site specificity of the antibody precipitated by type 14 capsular polysaccharide was the same as that of the antibody precipitated by gonococcal LPS.

From recent structural studies[26], pneumococcal type 14 capsular polysaccharide is known to possess terminal β-D-galactopyranosyl, 6-*O*-substituted 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucopyranosyl, and 3-*O*-substituted 4-*O*-β-D-galactopyranosyl-D-glucopyranosyl residues as structural components. It appears unlikely that β-D-galactopyranosyl end-groups and 6-*O*-substituted 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-D-glucopyranosyl residues contribute significantly to cross-precipitation by type 14 polysaccharide. Since methyl β-D-galactopyranoside and *N*-acetyllactosamine show only 1/200 and 1/500th the molar inhibitory ability of lactose (Fig. 6B), recurrent, internal 3-*O*-substituted 4-*O*-β-D-galactopyranosyl-D-glucopyranosyl residues are the most likely feature of type 14 capsular polysaccharide structure to mediate anti-lactose cross-precipitation.

From an earlier study of cross-reactions with blood-group substances[27], type 14 anti-pneumococcal horse serum (H635) is known to exhibit a heterogeneity with respect to antibody combining-site specificity. In the present study, gonococcal JW31 LPS and streptococcal lactose polymer were found to cross-react with horse anti-14 (Fig. 7) and to precipitate separate, distinct fractions of antibody. The conclusion that these antigens cross-react with separate populations of equine antibody, which differ somewhat in combining-site specificity, is indicated by a comparison of the hapten inhibition data given in Figs. 8A and B. Whereas lactose and *N*-acetyllactosamine are equally effective at the 50% level in inhibiting precipitation by streptococcal lactose polymer, N-acetyllactosamine was 2.7 times more effective than lactose in inhibiting precipitation by JW31 LPS. Whether N-acetyllactosamine, in addition to lactose units, also occur as structural moieties of gonococcal lipopolysaccharide is not known. The limited number of appropriate haptens tested precludes any definitive elucidation of combining-site specificity and identification of gonococcal LPS structure mediating anti-type 14 cross-reaction.

The ability of pyocin-sensitive and -resistant gonococcal lipopolysaccharides to precipitate WGA was also investigated. The WGA-combining site is complementary to a sequence of three β-D-(1→4)-linked 2-acetamido-2-deoxy-D-glucose residues[3]. The lectin has also been shown to bind terminal, nonreducing β-linked 2-acetamido-2-deoxy-D-glucosyl, or internal β-(1→4)- or β-(1→6)-substituted 2-acetamido-2-deoxy-D-glucosyl residues[3,4]. The marked contrast in the ability of isolated, purified JW31 and JW31R LPS to precipitate WGA (Fig. 1) is consistent with the lectin agglutination[28] and binding properties of these strains reported by Connelly *et al.*[7].

The chemical composition of the gonococcal lipopolysaccharides used in this study agrees with the compositions reported by others[5,19–21]. Perry *et al.*[5] previously showed the occurrence, in *N. gonorrhoeae* strain GC6(T4) LPS, of terminal, nonreducing and of internal (1→6)-linked 2-acetamido-2-deoxy-D-glucose residues. The presence of these residues in terminal, nonreducing positions in JW31 and JW31R LPS is consistent with the observation of Perry *et al.*[5]. Whether peak B of Fig. 3 represents an internal amino sugar not found in JW31R lipopolysaccharide is not yet known. Analysis of *O*-methyl neutral sugars by g.l.c. showed the presence of a component unique to JW31R LPS (Fig. 4, peak C). The presence of a new, unidentified sugar in the LPS from a pyocin-resistant strain has also been reported recently by Guymon *et al.*[29].

The specificity of a monclonal antibody (3F11), obtained by fusion of spleen cells from mice immunized with heat-killed gonococcal strain 4505, was investigated by Apicella *et al.*[30]. Based upon complete inhibition of ELISA by D-galactosamine and partial inhibition by lactose, a specificity directed against *O*-(2-acetamido-2-deoxy-D-galactosyl)-→*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose units was inferred for the combining site of monoclonal antibody 3F11. LPS-derived polysaccharide produced by alkali digestion of JW31 LPS was recently reported by Morse and Apicella[31] to react with monoclonal antibody 3F11, whereas

a polysaccharide derived from JW31R LPS failed to react. On the basis of the specificity assigned to monoclonal antibody 3F11, it was proposed that gonococcal strain JW31R LPS lacks an *O*-(2-acetamido-2-deoxy-D-galactosyl)→*O*-β-D-galactopyranosyl-(1→4)-D-glucopyranose unit[31]. However, the compositions obtained for JW31 and JW31R LPS in the present study (Table I), which show an increase in the total D-galactose content of JW31R LPS, do not support the conclusion that JW31R LPS is deficient in this trisaccharide unit.

The structural basis for the marked differences in immunochemical behavior of JW31 and JW31R LPS is not understood at present. Terminal, nonreducing 2-acetamido-2-deoxy-D-glucosyl groups were shown to occur in JW31R lipid-free oligosaccharide by g.l.c. analysis (Fig. 3). This finding rules out the absence of terminal 2-acetamido-2-deoxy-D-glucosyl groups as the basis for the failure of JW31R LPS to precipitate WGA. Since JW31R LPS does not acquire reactivity with WGA after treatment with aqueous acetic anhydride, a failure of the variant strain to *N*-acetylate 2-acetamido-2-deoxy-D-glucosyl residues may also be excluded as a possible explanation. Further chemical studies are required in order to elucidate the alteration(s) in gonococcal lipopolysaccharide fine-structure responsible for the observed differences in pyocin 103-receptor activity[2], precipitation of anti-streptococcal lactose polymer and anti-pneumococcal type 14 antibody, as well as changes in WGA reactivity. A substitution on or adjacent to an antibody- or lectin-binding site which obstructs interaction, or changes in the anomeric configuration of carbohydrate linkages from β to α could bring about the failure of JW31R LPS to react with these reagents.

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