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Immunochemical Studies on a *Mycoplasma pneumoniae* Polysaccharide Fraction: Cross-Reactions With Type 23 and 32 Antipneumococcal Rabbit Sera

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Lipid-free polysaccharide fraction 2 extracted from *Mycoplasma pneumoniae* strain FH by Prescott et al. (J. Bacteriol. **91**:2117-2115, 1966) was examined for its ability to cross-precipitate antibody from type-specific rabbit antipneumococcal sera types 1 to 34 inclusive. Cross-precipitation in type-specific pneumococcal anti-type 23 and anti-type 32 sera was examined in detail and could be attributed to a rhamnose-galactose-rich component of crude *M. pneumoniae* polysaccharide fraction 2 recovered from immunoprecipitates formed with anti-type 23 serum. Immunochemically isolated mycoplasma polysaccharide was found to contain glucose, galactose, rhamnose, and mannose in 1:14:5:4 molar proportions. Comparison of the ability of 6-*O*-α-L-rhamnosyl-D-glucose and free L-rhamnose to inhibit precipitation by homologous pneumococcal and heterologous mycoplasma polysaccharide antigens indicates a combining site specificity for anti-type 23 and anti-type 32 antibodies directed largely against the α-linked L-rhamnosyl determinants and the occurrence of α-L-rhamnosyl units in type 32 and *M. pneumoniae* polysaccharides. Hapten inhibition of the cross-precipitation of pneumococcal type 23 capsular polysaccharide in anti-type 32 serum helps to establish that cross-reactivity can be attributed to interaction of recurrent, α-L-rhamnosyl units of type 23 with anit-α-L-rhamnoside combining sites of anti-type 32 antibodies.

Four polysaccharide fractions free of detectable lipid and nitrogen were chemically extracted from *Mycoplasma pneumoniae* strain FH by Prescott et al. (12), and their serological and immunogenic activities were reported (15, 16). One fraction (polysaccharide fraction 2) showed no interaction with complement-fixing antibodies and was found unable to block the effect of indirect hemagglutinating and growth-inhibiting antimycoplasma antibodies. When examined by gel diffusion, however, polysaccharide fraction 2 gave two lines of precipitation with hyperimmune sera from rabbits injected with a suspension of *M. pneumoniae* (15). More recently, the interaction of *M. pneumoniae* polysaccharide fraction 2 with phytolectins was examined by Schiefer et al. (13). Precipitation of fraction 2 by extracts of *Ricinus communis* and *Canavalia ensiformis* indicated the presence of polysaccharide possessing multiple recurrences of D-galactose and D-glucose or D-mannose.

In the present study, crude *M. pneumoniae* polysaccharide fraction 2 was examined for its ability to precipitate antibody from rabbit antisera specific for pneumococcal capsular polysaccharides types 1 to 34 inclusive. Reactions involving a cross-precipitation of specific antibodies to pneumococcal types 7, 10, 14, 21, 22, 23, 29, and 32 were found to occur. Cross-reactions with antisera to pneumococcal types 23 and 32 were examined in detail and could be attributed to a rhamnose-galactose-rich component of *M. pneumoniae* polysaccharide fraction 2 which was recovered from specific immunoprecipitates formed with type 23 antipneumococcal serum. The sugar composition of the immunochemically isolated cross-reactive polysaccharide was determined and compared with that of the original crude *M. pneumoniae* polysaccharide fraction 2 from which it was obtained as well as with types 23 and 32 pneumococcal capsular polysaccharides. Structural features of homologous pneumococcal and heterologous mycoplasma polysaccharide antigens involved in precipitation of anti-type 23 (anti-23) and anti-type 32 (anti-32) antibodies were examined by quantitative hapten inhibition. In addition, the combining-site specificity of the fraction of pneu-

mococcal type 32 antibodies cross-reactive with type 23 pneumococcal capsular polysaccharide was examined by hapten inhibition.

MATERIALS AND METHODS

Mycoplasma polysaccharide fractions. *M. pneumoniae* strain FH, *M. hyorhinis* strain 7, *M. fermentans* strain PG18, and *M. neurolyticum* type A were grown as described in an earlier study (12) in 5-liter Povitsky bottles containing 1 liter of a broth medium consisting of seven parts PPLO broth (Difco), two parts unheated horse serum, and one part yeast extract. The medium was supplemented with 1% glucose, 0.002% phenol red, 1,000 U of penicillin, and thallium acetate at a final dilution of 1:2,000. *M. hominis* type 1 (strain DC 63) was similarly cultivated except that glucose was omitted from the growth medium. Procedures used in cultivation, harvesting, washing, and sonification of organisms have already been described (12, 16). The chemical extraction procedure used to obtain polysaccharide fraction 2 from *M. pneumoniae* strain FH sonic extracts has been described in detail (12, 16). Briefly, polysaccharide fraction 2 was obtained from the sediment of sonically treated cells by a procedure employing extraction with acetone, ethanol, and trichloroacetic acid followed by cold ethanol-ether precipitation. *M. pneumoniae* polysaccharide fraction 2, used as antigen in the present study, was found (12, 16) to consist entirely of carbohydrate and showed no detectable protein; lipid was not found in hydrolyzed samples of the material. Essentially the same extraction procedure was employed to prepare polysaccharide fractions from *M. hominis* type 1 (strain DC 63), *M. hyorhinis* strain 7, *M. fermentans* strain PG 18, and *M. neurolyticum* type A, except that these organisms were first extracted with chloroform-methanol (2:1, vol/vol).

Medium controls. Portions of the same batch of yeast extract, pooled normal horse serum, and PPLO broth used as growth medium supplements were lyophilized and dissolved in saline, and solutions containing up to 10 mg/ml were tested by immunodiffusion for reactivity with rabbit antipneumococcal sera. Polysaccharide fractions were also prepared from portions of the same lots of these materials by the identical chemical procedure used to isolate polysaccharide fraction 2 from *M. pneumoniae* (12). Saline-dissolved powders and polysaccharide fractions chemically isolated from yeast extract, pooled normal horse serum, and PPLO broth served as antigen controls and were examined for reactivity with rabbit antipneumococcal sera. Apart from the polysaccharide fraction isolated from PPLO broth which cross-precipitated with rabbit anti-14 serum, medium controls showed no cross-reactivity with rabbit antisera to pneumococcal types 7, 10, 21, 22, 23, 29, and 32.

Antisera. Rabbit antisera to pneumococcal types 1 to 34 inclusive were prepared by the Division of Laboratories and Research, New York State Department of Health. Rabbit anti-23 (R23-28; 6/22/45) and rabbit anti-32 (R32-17; 1/1/45) used for quantitative studies were absorbed with rough-phase type 1 pneumococci to remove anti-C carbohydrate. Antibody nitrogen (Ab N) precipitated from undiluted serum by homologous antigen was estimated by the quantitative precipitin method employing micro-Kjeldahl nitrogen analysis (8). Maximum Ab N removed from anti-23 serum was 99 μg of Ab N per 100 μl, whereas 108 μg of Ab N per 100 μl was removed from anti-32 serum. These sera showed weak, reciprocal cross-reactions; type 23 polysaccharide precipitated a maximum of 23 μg of Ab N per 2.0 ml of anti-32 serum, whereas type 32 polysaccharide removed only 4 μg of Ab N per 2.0 ml of anti-23 serum.

Initial precipitation of anti-23 and anti-32 sera with homologous antigen leaves no antibody behind in supernatants precipitable by a subsequent addition of *M. pneumoniae* polysaccharide fraction 2. Initial precipitation with the mycoplasma antigen removed 13 μg of N from anti-23 serum, leaving only 88 μg of Ab N recoverable from the supernatant by the addition of type 23 polysaccharide. Similarly, the mycoplasma polysaccharide removed 12 μg of N from anti-32 serum, leaving 93 μg of Ab N precipitable from the supernatant by the addition of type 32 polysaccharide. Absorption data establish that the precipitation reactions given by mycoplasma polysaccharide are cross-reactions involving the cross-precipitation of a small portion of antipneumococcal antibody.

Quantitative precipitin and hapten inhibition assays. Quantitative precipitin determinations and hapten inhibitions assays were analyzed by the scaled-down Folin-Ciocalteau procedure described by Kabat and Schiffman (9). Assays with rabbit anti-23 were carried out with 75 μl of a 1/5 dilution of serum containing 8.5 μg of Ab N precipitated by 5 μg of type 23 polysaccharide or 75 μl of neat serum containing 7.9 μg of Ab N precipitated by 100 μg of *M. pneumoniae* polysaccharide fraction 2. Assays with rabbit anti-32 were carried out with 50 μl of a 1/4 dilution of serum containing 7.6 μg of Ab N precipitated by 25 μg of type 32 polysaccharide or 100 μl of neat serum containing 6.8 μg of Ab N precipitated by 100 μg *M. pneumoniae* polysaccharide fraction 2. All reactions were carried out in a final total volume of 0.50 ml. Quantitative precipitin determinations were done in duplicate or triplicate, and averaged values were plotted in constructing quantitative precipitin curves. Quantitative hapten inhibition assays were carried out by employing four to six separate determinations for each amount of inhibitor tested. Each set of inhibition values was averaged, and a standard deviation was calculated for the set; the standard deviation did not exceed ±2.8%.

Inhibitors of the cross-precipitation of rabbit anti-32 serum by type 23 pneumococcal capsular polysaccharide were assayed in a test system consisting of 2.0 ml of anti-32 serum containing 23 μg of Ab N and 75 μg of type 23 polysaccharide. The final total volume of this test system was 2.5 ml.

Qualitative precipitin tests. Qualitative screening of *Mycoplasma* and control polysaccharide preparations for precipitation with rabbit antipneumococcal sera was carried out by a tube method. Solutions of polysaccharide (200 μl containing 200 μg of antigen) were added to 200 μl of serum in test tubes (6 by 50 mm). Tubes were capped, kept at room temperature for 1 h, placed in a refrigerator overnight, and then centrifuged before reading. Tubes were gently tapped

to dislodge any precipitate, the bulk of which was estimated on a scale of + to ++++.

Immunodiffusion was also used in screening for cross-reactivity with antipneumococcal sera. Gel diffusion in two dimensions was carried out in 1% agar in 0.02 M phosphate (pH 7.2)-buffered saline containing 2% polyethylene glycol (average molecular weight 6×10^3 to 7.7×10^3) to enhance precipitation of soluble complexes.

Immunochemical fractionation. A rhamnose-galactose-enriched, cross-reactive component was isolated from *M. pneumoniae* polysaccharide fraction 2 by immunoprecipitation with excess antibody. Crude *M. pneumoniae* fraction 2 (30 mg in 2 ml of saline) was added to 30 ml of rabbit anti-23 serum, and the reaction mixture was kept at 4°C for 5 days. The antigen-antibody precipitate which formed was sedimented by centrifugation, washed four times with 5-ml portions of cold saline, suspended in 5 ml of 1 M L-rhamnose in 0.85% NaCl, and gently agitated at 37°C till completely dissolved. An equal volume of 20% trichloroacetic acid was then added, and the precipitated antibody globulin was removed by centrifugation. After exhaustive dialysis against water, polysaccharide was recovered from the dialyzed trichloroacetic acid extract by lyophilization. An alternate isolation procedure, permitting recovery of antibody as well as antigen, employed the addition of an equal volume of solution saturated with both L-rhamnose and $(NH_4)_2SO_4$ to rhamnose-solubilized immunoprecipitates. This separation procedure permitted recovery of antibody from the salt-precipitated globulins while soluble polysaccharide antigen could be recovered by dialysis of the supernatant solution. Both procedures gave yields of immunochemically purified cross-reactive polysaccharide amounting to 7% of the dry weight of initial crude *M. pneumoniae* polysaccharide fraction 2.

Pneumococcal polysaccharides. Pneumococcal types 23 and 32 capsular polysaccharides were prepared from 24-h broth cultures of encapsulated pneumococci grown in Todd-Hewitt medium. Type-specific polysaccharides were isolated from cell-free culture filtrates by the calcium phosphate method described by Felton et al. (3). Cultures of pneumococci used to prepare capsular polysaccharide were subtyped in the laboratory of Robert Austrian and found to correspond to types 23F and 32F in the Danish scheme of classification.

Sugar analysis. Polysaccharides (10 mg) were hydrolyzed in 2 N H_2SO_4 (2.5 ml) by heating in sealed tubes for 2 h at 105°C. Qualitative and quantitative determination of monosaccharides was carried out on hydrolysates by the paper chromatographic method of Colombo (2) as described by Caldes and Prescott (1), except that aniline malonate was used in place of aniline phthalate for color development. Hydrolysates were passed through a column (1 by 50 cm) of Amberlite IRA 410 in the acetate form before chromatography on paper.

Inhibitors. Lactose, D-mannose, L-rhamnose, methyl-α- and methyl-β-D-galactopyranosides, methyl-α- and methyl-β-D-glucopyranosides, melibiose, cellobiose, methyl-α-D-mannopyranoside, D-galactose, and D-glucose were purchased from Pfanstiehl Laboratories Inc. Rutinose (6-*O*-α-L-rhamnosyl-D-glucose) (4) was prepared by hydrolysis of rutin in 10% acetic acid as described by Zemplen and Gerecs (20). The hydrolysate was applied to a Darco G 50 charcoal column and eluted with water followed by 1%, then 25%, ethanol. Crude rutinose isolated from the 25% ethanol fraction by vacuum distillation was subjected to descending chromatography on sheets (46 by 57 cm) of Whatman 3-mm paper by employing multiple descents of the upper phase of *n*-butanol/glacial acetic acid/water (4:1:5, vol/vol/vol) solvent A. Papers were oven dried between each descent of solvent. Rutinose, the main component, located by guide strips sprayed with silver (17) was eluted with water and re-chromatographed until free of contaminating glucose and rhamnose. The final product was filtered through a calibrated Bio-Gel P2 column, eluted with water, and isolated by lyophilizing fractions with an elution volume corresponding to that of lactose. On paper chromatograms, purified rutinose gave a single spot with an $R_{glucose} = 0.59$ in solvent A and one spot with an $R_{glucose} = 0.83$ in ethyl acetate/pyridine/water (10:4:3, vol/vol/vol). Sugars used in hapten inhibition assays were dried to constant weight at 60°C in vacuo over P_2O_5.

RESULTS

Qualitative precipitin tests. Crude *M. pneumoniae* polysaccharide fraction 2 was tested for its ability to precipitate with a panel of rabbit antisera to pneumococcal types 1 to 34 inclusive. Crude fraction 2 cross-precipitated with antisera specific for pneumococcal types 7, 10, 14, 21, 22, 23, 29, and 32; no precipitation was obtained with the remaining sera of the panel (Table 1). However, the rhamnose-galactose-enriched polysaccharide isolated from immunoprecipitates formed by crude fraction 2 with anti-23 serum reacted only with antisera specific for pneumococcal types 22, 23, and 32.

Apart from a control polysaccharide fraction isolated from PPLO broth which precipitated with anti-14 serum, growth medium control preparations showed no cross-precipitation with the panel of rabbit antisera employed (Table 1). Similarly, polysaccharide preparations obtained from *M. hominis, M. neurolyticum, M. hyorhinis,* and *M. fermentans* gave no precipitation with rabbit antipneumococcal sera reactive with *M. pneumoniae* polysaccharide fraction 2.

Immunodiffusion. When diffused against anti-22, anti-23, or anti-32 sera in agar diffusion, both crude and immunochemically purified *M. pneumoniae* fraction 2 polysaccharides produced a single band of precipitation which showed complete fusion but gave partial fusion with the band produced by homologous types 22, 23, or 32 pneumococcal polysaccharides.

Quantitative precipitation. Quantitative precipitation of Ab N from rabbit anti-23 and anti-32 sera by crude and immunochemically

purified *M. pneumoniae* fraction 2 polysaccharides is shown in Fig. 1. Purified polysaccharide is more effective per unit weight in precipitating antibody than crude fraction 2. Whereas only 20 to 25 μg of purified polysaccharide gave maximum precipitation of Ab N, 75 to 100 μg of crude fraction 2 was required. Based upon the relative ability per unit weight of the two polysaccharide

TABLE 1. *Precipitin reactions of various mycoplasma polysaccharide fractions with rabbit antipneumococcal sera*

Rabbit antipneumococcal serum	*M. pneumoniae*		*M. hominis*	*M. neurolyticum*	*M. hyorhinis*	*M. fermentans*	Culture medium control polysaccharides isolated from:		
	Original[a] fraction 2	Purified[b] fraction 2					Yeast extract	Horse serum	PPLO broth
Anti-7	+	−	−	−	−	−	−	−	−
Anti-10	+	−	−	−	−	−	−	−	−
Anti-14	++	−	−	−	−	−	−	−	+
Anti-21	+	−	−	−	−	−	−	−	−
Anti-22	++	++	−	−	−	−	−	−	−
Anti-23	+++	+++	−	−	−	−	−	−	−
Anti-29	+	−	−	−	−	−	−	−	−
Anti-32	+++	+++	−	−	−	−	−	−	−

[a] Tested with antisera against types 1 to 34 inclusive; however, only the positive reactions obtained are listed.
[b] Recovered from immunoprecipitates formed with excess type-specific pneumococcal anti-23 serum.

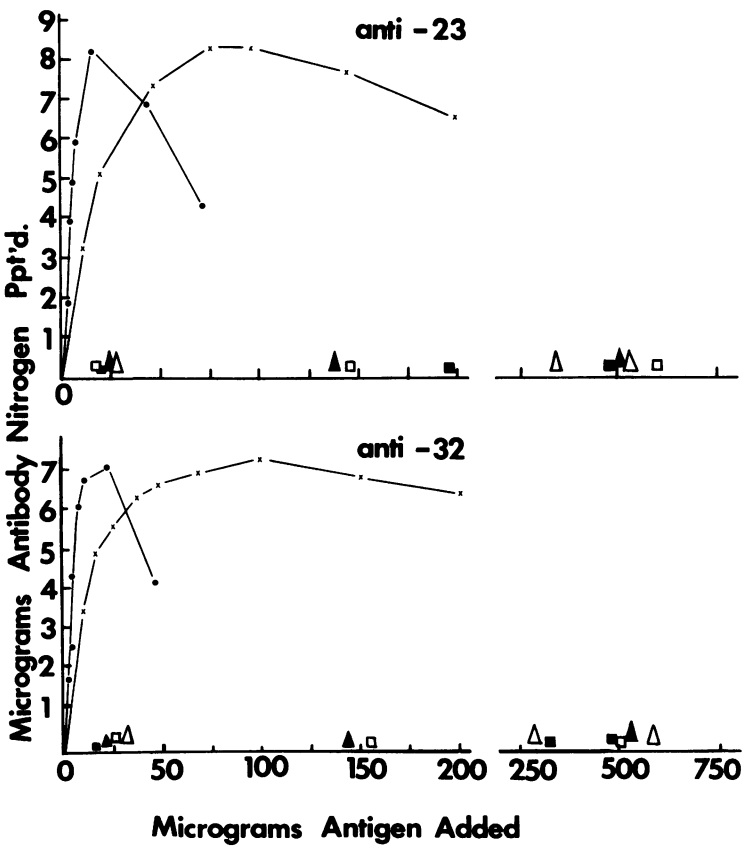

FIG. 1. *Quantitative precipitin curves obtained with rabbit antisera to pneumococcal types 23 and 32. Antigens: ×, M. pneumoniae, crude fraction 2 polysaccharide; ●, M. pneumoniae immunochemically purified fraction 2 polysaccharide; and polysaccharide obtained from △, M. hominis; ▲, M. fermentans; □, M. neurolyticum; and ■, M. hyorhinis.*

preparations to precipitate comparable amounts of Ab N in the presence of excess antibody, purified antigen constitutes only 20 to 23% of the weight of crude fraction 2.

Preparations of polysaccharide isolated from *M. hominis, M. neurolyticum, M. hyorhinis,* and *M. fermentans* showed no ability to precipitate Ab N from anti-23 or anti-32 serum when tested quantitatively in amounts ranging from 15 to 600 μg (Fig. 1).

Sugar analysis. The sugar compositions of crude and of immunochemically isolated *M. pneumoniae* fraction 2 polysaccharides are given in Table 2. Analyses for types 23 and 32 pneumococcal capsular polysaccharides are also included. The composition of pneumococcal type 23 polysaccharide has been previously determined by J. K. N. Jones and M. Perry, who identified glucose, galactose, and rhamnose as its constituent sugars (5). As indicated in Table 2, *M. pneumoniae* fraction 2 polysaccharides contain glucose, galactose, and rhamnose, which are found also as constituents of both type 23 and 32 pneumococcal capsular polysaccharides. However, mycoplasma preparations contain an additional sugar, mannose, not found in pneumococcal types 23 and 32 polysaccharides. Data presented in Table 2 provide approximate molar ratios of glucose/galactose/rhamnose/mannose (1:14:5:4) for the sugar constituents of the immunochemically isolated *M. pneumoniae* fraction 2 component. Compared to the original fraction 2 starting material, polysaccharide recovered from immunoprecipitates formed by crude fraction 2 with anti-23 serum showed an enrichment in galactose and rhamnose content and a reduction in relative glucose content. Arabinose present in crude fraction 2 was not detected in the polysaccharide isolated from fraction 2 by specific immunoprecipitation. Data summarized in Table 2 also provide approximate molar ratios of 1:1.2:2 for the glucose/galactose/rhamnose components of type 23 and approximate ratios of 1.1:0.5:1:1.2 for the glucose/galactose/rhamnose/uronic acid constituents of type 32 pneumococcal capsular polysaccharide.

Inhibition of anti-23 precipitation. The ability of various sugars to inhibit the cross-precipitation of rabbit anti-23 antibody by *M. pneumoniae* polysaccharide fraction 2 is shown in Fig. 2. L-Rhamnose required only 5×10^{-1} μM to give 50% inhibition. Greater than 80 μM of methyl-α- and methyl-β-D-glucopyranoside was required to obtain 52% inhibition; 80 μM mannose gave only 20% inhibition. Melibiose and lactose possessing terminal, nonreducing α- and β-linked galactosyl residues required 15 and 30 μM, respectively, to give 60% inhibition. Of the sugars tested, 6-*O*-α-D-rhamnosyl-D-glucose (rutinose) was found to be the most potent inhibitor of anti-23 precipitation by *M. pneumoniae* fraction 2 polysaccharide, 1.2×10^{-1} μM giving 50% inhibition.

Inhibition by various sugars of rabbit anti-23 precipitation by type 23 pneumococcal capsular polysaccharide is shown in Fig. 3. Methyl-α- and methyl-β-D-glucopyranoside, methyl-α- and methyl-β-D-galactopyranoside, D-mannose, and melibiose assayed in amounts ranging from 5×10^1 to 2.5×10^2 μM did not produce greater than 22% inhibition. Whereas 3.9×10^1 μM L-rhamnose gave 50% inhibition, 6-*O*-α-L-rhamnosyl-D-glucose was 3.5-fold more effective than L-rhamnose because 1.1×10^1 μM of the α-L-rhamnoside was required to obtain 50% inhibition.

Inhibition of anti-32 precipitation. Quantitative hapten inhibition of the cross-precipitation of antipneumococcal antibody from rabbit anti-32 serum by *M. pneumoniae* polysaccharide fraction 2 is given in Fig. 4. When assayed in amounts ranging from 25 to 100 μM, methyl-

TABLE 2. *Sugar composition of M. pnuemoniae fraction 2 polysaccharides and types 23 and 32 pneumococcal capsular polysaccharide*

Polysaccharide	Sugar composition (mol %)[a]					
	Glucose	Galactose	Rhamnose	Mannose	Arabinose	Uronic acid[b]
M. pneumoniae						
Crude fraction 2	22	38	6	27	7	ND[c]
Isolated from fraction 2 by anti-23 precipitation	4	56	23	17	ND	ND
Pneumococcal capsular						
Type 23	24 25[d]	28 25[d]	48 50[d]	ND		ND
Type 32	28	16	25	ND		31

[a] Expressed as moles of sugar residue per 100 mol of sugar in hydrolysate.
[b] Determined as glucuronic acid.
[c] ND, None detected.
[d] Unpublished data provided by Malcolm Perry.

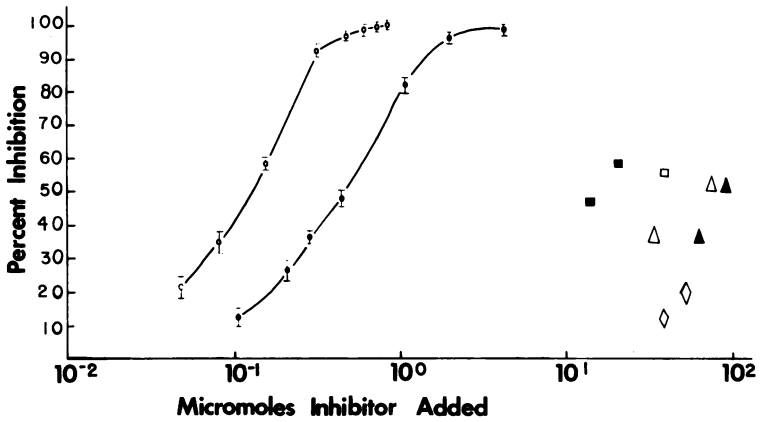

FIG. 2. *Inhibition by various sugars of antipneumococcal antibody cross-precipitation from rabbit anti-23 serum by M. pneumoniae polysaccharide fraction 2. Inhibitors: ○, 6-O-α-L-rhamnose; ▲, methyl-α-D-glucopyranoside; △, methyl-β-D-glucopyranoside; ◇, D-mannose; □, lactose; ■, melibiose.*

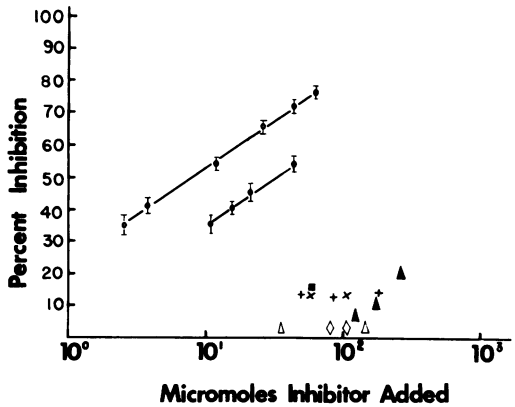

FIG. 3. *Inhibition by various sugars of antipneumococcal antibody precipitation from rabbit anti-23 serum by homologous type 23 pneumococcal capsular polysaccharide. Inhibitors: ○, 6-O-α-L-rhamnosyl-D-glucose; ●, L-rhamnose; ▲, methyl-α-D-glucopyranoside; △, methyl-β-D-glucopyranoside; +, methyl-α-D-galactopyranoside; ×, methyl-β-D-galactopyranoside; ◇, D-mannose; ■, melibiose.*

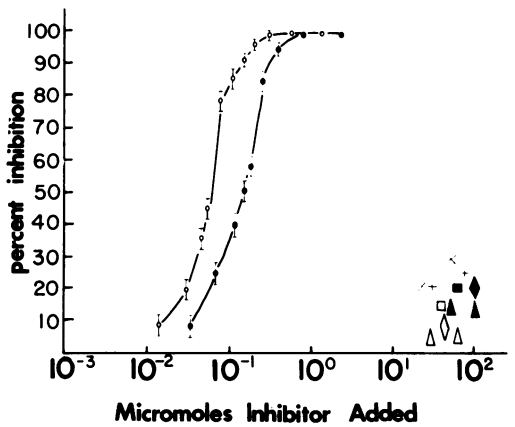

FIG. 4. *Inhibition by various sugars of the cross precipitation of antipneumococcal antibody from rabbit anti-32 serum by M. pneumoniae polysaccharide fraction 2. Inhibitors: ○, 6-O-α-L-rhamnosyl-D-glucose; ●, L-rhamnose; ▲, methyl-α-D-glucopyranoside; △, methyl-β-D-glucopyranoside; +, methyl-α-D-galactopyranoside; ×, methyl-β-D-galactopyranoside; ◇, D-mannose; ◆, methyl-α-D-mannopyranoside; □, lactose; ■, melibiose.*

α- and methyl-β-D-glucopyranoside, methyl-α- and methyl-β-D-galactopyranoside, mannose, methyl-α-D-mannopyranoside, lactose, and melibiose did not produce greater than 30% inhibition. L-Rhamnose required 1.2×10^{-1} μM to give 50% inhibition. Amounts of L-rhamnose greater than 6 μM gave complete inhibition of cross-precipitation. Of the sugars examined, 6-O-α-L-rhamnosyl-D-glucose was the most effective inhibitor of cross-precipitation, 5.8×10^{-2} μM giving 50% inhibition. Total inhibition of cross-precipitation could be obtained with 2.8×10^{-1} μM of 6-O-α-L-rhamnosyl-D-glucose.

Inhibition by various sugars of rabbit anti-32 precipitation with homologous type 32 pneumococcal capsular polysaccharide is shown in Fig. 5. Similar to inhibition data obtained with the *M. pneumoniae* polysaccharide fraction 2 anti-32 cross-reaction, methyl-α- and methyl-β-D-glucopyranoside, methyl-α- and methyl-β-D-galactopyranoside, D-mannose, methyl-α-D-mannopyranoside, lactose, and melibiose did not yield greater than 25% inhibition when tested in amounts ranging from 30 to 100 μM. Whereas 4.0 μM D-rhamnose gave 50% inhibition, 6-O-α-L-rhamnosyl-D-glucose was 2.7-fold more effective because 1.45 μM produced a comparable percent inhibition. As shown in Fig. 5, essentially

complete inhibition of type 32-anti-32 precipitation can be obtained with 20 μM 6-*O*-α-D-rhamnosyl-D-glucose (98% inhibition) or 25 μM L-rhamnose (92% inhibition).

Inhibition by various sugars of the cross-precipitation of rabbit anti-32 by type 23 pneumococcal capsular polysaccharide is shown in Fig. 6. Like inhibition data obtained with the homologous system, cross-precipitation was also best inhibited by L-rhamnose and its α-linked rhamnoside. Whereas 2.5×10^{-1} μM L-rhamnose gave

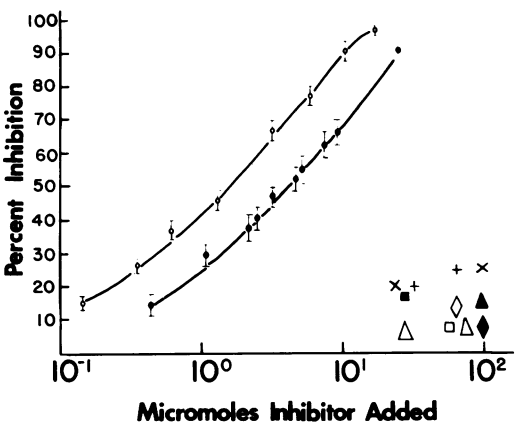

FIG. 5. *Inhibition by various sugars of antipneumococcal antibody precipitation from rabbit anti-32 serum by homologous type 32 pneumococcal capsular polysaccharide. Inhibitors:* ○, *6-O-α-L-rhamnosyl-D-glucose;* ●, *L-rhamnose* ▲, *methyl-α-D-glucopyranoside;* △, *methyl-β-glucopyranoside;* +, *methyl-α-D-galactopyranoside;* ×, *methyl-β-D-galactopyranoside;* ◇, *D-mannose;* ◆, *methyl-α-mannoside;* □, *lactose;* ■, *melibiose.*

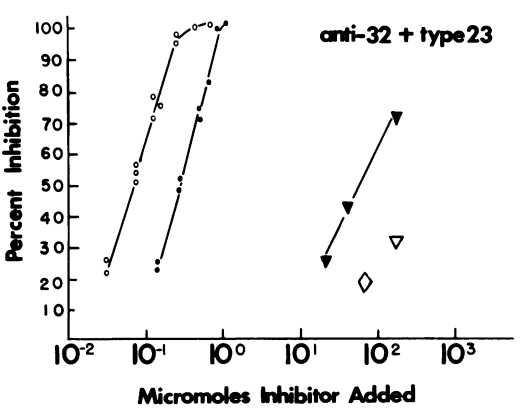

FIG. 6. *Inhibition by various sugars of the cross-precipitation of antipneumococcal antibody from rabbit anti-32 serum by heterologous type 23 pneumococcal capsular polysaccharide. Each point represents a single determination with inhibitors:* ○, *6-O-α-L-rhamnosyl-D-glucose;* ●, *L-rhamnose;* ▽, *D-glucose;* ▼, *D-galactose;* ◇, *D-mannose.*

50% inhibition, 6-*O*-α-L-rhamnosyl-D-glucose was 3.9-fold more effective, because 6.4×10^{-2} μM gave comparable inhibition. D-Galactose required 55 μM to produce 50% inhibition, whereas 66 μM D-mannose and 160 μM D-glucose gave only 15 and 32% inhibition, respectively.

DISCUSSION

Cross-precipitation with type-specific antipneumococcal sera was employed in the present study to obtain information about the heterogeneity and fine structure of components of lipid-free polysaccharide fraction 2 extracted from *M. pneumoniae*. Apart from a control polysaccharide isolated from PPLO broth, which precipitated with rabbit anti-14 serum, polysaccharides isolated from culture medium components showed no reaction with the antipneumococcal sera employed (Table 1). Similarly, preparations of polysaccharide obtained from cultures of *M. hominis, M. neurolyticum, M. hyorhinis,* and *M. fermentans* grown in the same medium used to cultivate *M. pneumoniae* failed to show cross-reactivity with antipneumococcal sera. Thus, cross-reactions of *M. pneumoniae* polysaccharide fraction 2 with types 23 and 32 antipneumococcal sera examined in the present study cannot be attributed to polysaccharide components present in the medium.

That *M. pneumoniae* polysaccharide fraction 2 is heterogeneous is indicated by its ability to cross-react with antisera to several antigenically unrelated pneumococcal capsular polysaccharides (e.g., types 7, 10, 21, and 22). Cross-precipitation with anti-22, anti-23, and anti-32 sera, however, can be attributed to the same component, because in immunodiffusion these antisera produce only one band of precipitation with crude fraction 2 which shows complete fusion. Moreover, purified mycoplasma polysaccharide isolated from specific precipitates formed by crude fraction 2 with anti-23 serum retains reactivity with both anti-22 and anti-32 serum (Table 1). In addition, initial reaction of anti-32 serum with purified mycoplasma antigen recovered from anti-23 immunoprecipitate leaves no antibody behind precipitable by the subsequent addition of crude polysaccharide fraction 2. As estimated from quantitative precipitin curves (Fig. 1), only 20 to 23% (dry weight) of crude *M. pneumoniae* polysaccharide fraction 2 is immunochemically reactive with anti-23 and anti-32 sera. Heterogeneity in the composition of fraction 2 polysaccharide components is also apparent from sugar analyses (Table 2). Cross-reaction with anti-23 serum precipitates from fraction 2 a polysaccharide selectively enriched in both rhamnose and galactose but reduced in glucose and mannose content.

Although a reciprocal cross-reaction between pneumococcal types 23 and 32 has been known for a long time (10, 18), the structural basis for this antigenic relationship has never been elucidated. Pneumococcal type 23 polysaccharide consists of D-glucose, D-galactose, and L-rhamnose (data from Jones and Perry, [5]) present in 1:1:2 molar proportions (Table 2). Structural studies in progress by Malcolm B. Perry show that half of the L-rhamnose present occurs as nonreducing α-linked L-rhamnosyl end groups. Precipitation in anti-23 serum by homologous polysaccharide, by the cross-reactive group-specific polysaccharides of streptococcal groups B and G, and by *Klebsiella* capsular polysaccharides was studied by Heidelberger et al. (5-7) and shown to be mediated by L-rhamnose end groups. Moreover, L-rhamnose was found to be the major determinant of antigenic specificity, and the free sugar gave extensive inhibition of precipitation by homologous as well as by heterologous antigens (5). Glucose and rhamnose have been previously identified among the products of hydrolysis of pneumococcal type 32 polysaccharide (14). As shown in Table 2, galactose and uronic acid also occur as constituents of type 32 polysaccharide. Thus, pneumococcal type 23 and 32 capsular polysaccharides and the anti-23 and anti-32 cross-reactive component of *M. pneumoniae* polysaccharide fraction 2 share the common occurrence of rhamnose, galactose, and glucose as structural components.

Hapten inhibition data (Fig. 2 through 6) show that, of the sugars occurring in common, L-rhamnose is the most potent monosaccharide inhibitor of anti-23 and anti-32 precipitation by homologous and cross-reactive polysaccharides. The finding that 6-*O*-α-L-rhamnosyl-D-glucose is two to fourfold more effective as an inhibitor of precipitation than free L-rhamnose indicates that an α configuration for the L-rhamnosyl unit contributes to interaction with antibody and is involved in the combining site specificity of both anti-23 and anti-32 sera.

The increased ability of the α-linked L-rhamnoside to inhibit completely anti-32 precipitation by homologous antigen shows that at least part of the L-rhamnose present in type 32 polysaccharide must occur as α-L-rhamnosyl units and that the α-L-rhamnosyl unit plays an immunodominant role in the structure of antigenic determinants of type 32 pneumococcal capsular polysaccharide. Hapten inhibition data (Fig. 6) help to elucidate the structural basis for the cross-precipitation of type 23 polysaccharide in anti-32 serum which can be attributed largely to the interaction of recurrent α-L-rhamnosyl units of type 23 polysaccharide with complementary anti-32 combining sites.

Cross-precipitation of the rhamnose-galactose-rich component of *M. pneumoniae* polysaccharide fraction 2 in anti-23 and anti-32 sera and its inhibition by 6-*O*-α-L-rhamnosyl-D-glucose indicate that at least a portion of the L-rhamnose present in the mycoplasma polysaccharide must occur as α-linked L-rhamnosyl units, probably as nonreducing end units. Whether the rhamnose-galactose-rich polymer isolated from *M. pneumoniae* polysaccharide fraction 2 in the present study has any structural or immunochemical relationship to group F and type III cell wall antigens of *Streptococcus* MG, which also contain rhamnose, glucose, and/or galactose (11, 19), is not known and requires further study.

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