Immunochemical Analysis of Streptococcal Group A, B, and C Carbohydrates, with Emphasis on Group A†

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Streptococcal group A, B, and C carbohydrates were analyzed by counterimmunoelectrophoresis, immunoelectrophoresis, and inhibition of immunoprecipitation. Extracts of streptococci group A or C were shown by counterimmunoelectrophoresis to contain both anodic and cathodic migrating components. In immunoelectrophoresis, group A and C substances formed a continuous precipitation line stretching from the anode to the cathode, suggesting a heterogeneous population of molecules with immunochemical identity. This identity was confirmed by inhibition of immunoprecipitation, in which both anodic and cathodic immunoprecipitates were inhibited by the same constituent sugars: group A-anti-A was inhibited by N-acetylglucosamine, and group C-anti-C was inhibited by N-acetylgalactosamine. Extracts of group B showed only anodic migration in counterimmunoelectrophoresis and a narrow, anodic arc in immunoelectrophoresis. The group B-anti-B reaction was inhibited by rhamnose. Carbohydrates of variant strains of group A streptococci were also analyzed by the same methods. The results suggest that the heterogeneity of group A carbohydrate may have resulted from attachment of various amounts of N-acetylglucosamine to the polyrhamnose backbone.

The structure of group A streptococcal carbohydrate has been proposed (5, 17) to consist of a polyrhamnose (polyRha) core in alternating 1,2 and 1,3 linkages with N-acetylglucosamine (GlcNAc) residues β-1,2-linked to available 3-positions of the core (Fig. 1). This structure has been elucidated from serological and chemical analysis of group A and A-variant (Av) streptococcal carbohydrates (5, 10, 20).

The Av strain was obtained by serial passage of group A streptococci in mice (25). The group carbohydrate of this strain consists primarily of a polyRha backbone (Fig. 1), and antisera with Av specificity appears to be dependent on the Rha-Rha linkages. The Av carbohydrate does not react with antiserum made to group A carbohydrate, since group A specificity is conferred by the GlcNAc moiety.

The structure of group A carbohydrate, however, may be more heterogeneous than that described by Coligan et al. (5). The first evidence of such heterogeneity was the observation (21) of an intermediate (I) strain of group A streptococci whose group carbohydrate reacts equally well with antiserum to both A and Av carbohydrates (anti-A and anti-Av, respectively). The I strain was also obtained by mouse passage of group A streptococci. Its I carbohydrate has a glucosamine/Rha ratio midway between those of the group A and Av strains. Isolation of the I and Av strains indicates that synthesis of group A carbohydrate may proceed with various amounts of GlcNAc attached to the polyRha backbone.

\[ \text{GROUP} \quad \text{PROPOSED STRUCTURE} \]

\[ \begin{array}{c}
\text{A} \\
\left[ \begin{array}{c}
\text{Rha} \\
\text{Rha} \\
\text{Rha} \\
\text{Rha} \\
\end{array} \right] \text{A} \\
\text{β1→3} \\
\text{β1→3} \\
\text{GlcNAc} \\
\text{GlcNAc} \\
\end{array} \]

\[ \begin{array}{c}
\text{AV} \\
\left[ \begin{array}{c}
\text{Rha} \\
\text{Rha} \\
\text{Rha} \\
\text{Rha} \\
\end{array} \right] \text{AV} \\
\text{β1→3} \\
\text{β1→3} \\
\text{GlcNAc} \\
\text{GlcNAc} \\
\end{array} \]

\[ \begin{array}{c}
\text{C} \\
\left[ \begin{array}{c}
\text{Rha} \\
\text{Rha} \\
\text{Rha} \\
\text{Rha} \\
\end{array} \right] \text{C} \\
\text{β1→3} \\
\text{β1→3} \\
\text{GlcNAc} \\
\text{GlcNAc} \\
\end{array} \]

\[ \begin{array}{c}
\text{I} \\
\left[ \begin{array}{c}
\text{Rha} \\
\text{Rha} \\
\text{Rha} \\
\text{Rha} \\
\end{array} \right] \text{I} \\
\text{β1→3} \\
\text{β1→3} \\
\text{GlcNAc} \\
\text{GlcNAc} \\
\end{array} \]

FIG. 1. Proposed structures of the group-specific carbohydrates of streptococcal groups A, Av, and C (17). Reprinted by permission.

† Part of the study was done at the Division of Clinical Microbiology and Immunology, Erie County Laboratory, Buffalo, New York.
The second line of evidence suggesting heterogeneity is the electrophoretic migration of group A carbohydrate. Durfee et al. (9) observed that extracts of group A streptococci migrate both anodically and cathodically in counterimmunoelectrophoresis (CIE) and suggested that the cathodic migrating substance represents nonspecific precipitation. We have also observed (J. C. Fung, J. Wilson, and K. Wicher, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, C136, p. 285) anodic and cathodic migrating material in group A streptococcal extracts. In this communication we present evidence suggesting that both migrating components are group specific. Our results indicate that the heterogeneity in migration may result from attachment of various amounts of GlcNAc to the polyRha backbone of group A carbohydrate.

MATERIALS AND METHODS

Bacterial strains. Streptococci of groups A, B, and C were obtained from clinical specimens. Streptococci of group A strain T27A and the temperature-dependent Av strain A486-Var were from a bacterial stock collection at Rockefeller University. Strain T27A demonstrated Av antigenic specificity regardless of the incubation temperature (22 or 37°C). Strain A486-Var demonstrated Av antigenicity when grown at 37°C but A antigenicity at 22°C (3).

Extracts of streptococci. Streptococci were cultured in Todd-Hewitt broth or, for some purposes, in chemically defined medium (23) under aerobic conditions at 37 or 22°C. Most streptococcal extracts were prepared by the autoclave method of Rantz and Randall (22). A group A extract was also prepared by the hot-HCl method of Lancefield (19).

The time course of the conversion of strain A486-Var from Av specificity (37°C) to A specificity (22°C) was followed by taking 100 ml of broth culture grown overnight at 37°C, centrifuging it at 5,000 × g for 15 min, resuspending the pellet in fresh Todd-Hewitt broth to an initial McFarland standard turbidity of 0.5, and incubating the culture at 22°C. Aliquots (30 ml) were removed after 0, 3, 6, and 9 h of incubation for extract preparation by the autoclave method.

Reagents. Group-specific antisera to group A, B, and C streptococci were obtained from Wellcome Research Laboratories (Research Triangle Park, N.C.). Anti-Av was prepared as previously described (21). N-Acetylgalactosamine (GalNAc) and GlcNAc were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Rha from Difco Laboratories (Detroit, Mich.).

A preparation of A-enzyme was used to remove 50 to 70% of the total GlcNAc residues from group A carbohydrate. This decreases the ability of the carbohydrate to react with anti-A while increasing its ability to react with anti-Av (20).

Electrophoresis. (i) CIE was done according to the method of Coonrod and Rytel (7) with 1% agarose (Seakem, Marine Colloid Division, Rockland, Maine) in barbital buffer (pH 8.2, ionic strength 0.05). Depending on the experimental protocol, antigen was electrophoresed alone or simultaneously with antibodies for 30 min at 250 V. To detect cathodically migrating components of streptococcal extracts, a parallel row of antibody wells was cut on the cathode side of the antigen wells after the electrophoresis, and antisera were allowed to diffuse from the antibody wells into the agarose. The plate was examined for the presence of immunoprecipitates after 5 h and again after overnight incubation. (ii) Immunelectrophoresis (IEP) was done by using Immuno-Agaroslide (Millipore Corp., Bedford, Mass.) with barbital buffer (pH 8.6, ionic strength 0.1). Electrophoresis was performed for 30 min at 100 V. When so stated, IEP was also performed under the exact conditions described for CIE.

IEP. Inhibition of immunoprecipitation (IEP) (Fig. 2) is a modification of the CIE technique. Extracts of autoclaved streptococci were electrophoresed for 30 min at 250 V. A set of troughs cut above and below the antigen well was filled with a solution of 25% GlcNAc-

![Figure 2](http://iai.asm.org/)

**FIG. 2.** IIP reaction with constituent sugars. Ag, Streptococcal group A, B, or C extract; Ab, antiserum specific for group A, B, or C antigen. After electrophoresis of the antigen, the constituent sugar was applied, and 3 h later the specific antiserum (shaded wells) was applied.
25% GalNAc–50% Rha or with phosphate-buffered saline. After 3 h of diffusion, antibody wells were cut anodically, cathodically, or both to the antigen well and filled with group-specific antisera. Plates were examined for the presence of immunoprecipitates 5 and 24 h later.

A-enzyme activity on group A carbohydrate. A-enzyme in a 70% saturated solution of ammonium sulfate (0.4 ml total) was centrifuged at 3,000 × g for 10 min. The sediment was suspended in 0.8 ml of group A streptococcal extract in saline and incubated at 35°C. Aliquots (0.1 ml) were removed at 0, 1, 2, 3, 4, 6, 9, and 24 h of incubation, boiled for 5 min, and subjected to electrophoresis. Control samples of group A carbohydrate were processed in parallel, with A-enzyme omitted.

RESULTS

Anodic and cathodic migration of streptococcal group carbohydrate. Simultaneous electrophoresis of streptococcal extracts and group-specific antisera in CIE resulted in the formation of an immunoprecipitin line between the antigen and antibody wells on the anode side of the antigen (Fig. 3). However, results with a second parallel row of antibody wells added cathodically to the antigen wells indicated that extracts of group A or C but not of group B streptococci contained a second component, which migrated cathodically in the electric field. This phenomenon was observed for group A streptococci cultured in Todd-Hewitt broth or in chemically defined medium and with extracts prepared by the autoclave or hot-HCl method. Semiquantitation by CIE of serial twofold dilutions of the group A and C extracts demonstrated that the oppositely migrating components were present in comparable quantities.

IEP of streptococcal group carbohydrate. The dual anodic and cathodic migrations of group A and C antigens were also evident by IEP. The precipitation reaction between group A or C extract and its homologous antiserum demonstrated one continuous precipitation line from the anode to the cathode (Fig. 4). In contrast, the reaction between the group B extract and its specific antiserum resulted in a narrow, anodal precipitation line. The IEP patterns of group A, B, and C extracts were the same with the commercially prepared Immuno-Agaroslide as with IEP performed under the exact conditions used for CIE.

Immunochromical identity of anodic and cathodic migrating components. The uninterrupted IEP line of group A and C carbohydrates suggested that the anodic and cathodic migrating components may be immunochromically identical. This possibility was further investigated by IEP. Previous studies (8, 18, 20) on the structure of group A, B, and C carbohydrates indicated that their immunodominant sugars are GlcNAc, Rha, and

![FIG. 3. CIE. Electrophoretic migration of extracts of group A, B, and C streptococci. Column Ag. Extracts of streptococcal groups A, B, and C; column Ab, antisera to streptococcal groups A, B, and C. Open wells contained reagents which were electrophoresed simultaneously, and shaded wells contained antisera subjected to diffusion after electrophoresis.](image1)

![FIG. 4. IEP. Migration patterns of extracts of group A, B, and C streptococci. Wells contained extracts of group A, B, and C streptococci. Troughs contained antiserum to group A, B, or C antigen.](image2)
GalNAc, respectively. If the oppositely migrating components are immunochemically identical, formation of the anodic and cathodic immunoprecipitates should be inhibited by the same constituent sugar. This was indeed the case. Both cathodic and anodic immunoprecipitates of each group were inhibited by a single sugar: group A by GlcNAc, group B by Rha, and group C by GalNAc (example in Fig. 5).

Electrophoretic mobility of Av extracts. By CIE both the anodic and the cathodic components of group A streptococci reacted strongly with anti-A (Table 1). With anti-Av, however, only the cathodic components reacted and then very weakly. Extracts of Av T27A did not react with anti-A; but with anti-Av the cathodic components reacted strongly, and their anodic counterparts reacted weakly. Extracts prepared from the Av A486-Var demonstrated temperature-dependent specificity. Extracts from organisms grown at 37°C reacted only with anti-Av; the cathodically migrating components reacted strongly, and the anodically migrating components reacted very weakly. Extracts of organisms grown at 22°C reacted with anti-A; this reaction was much stronger with the anodic substances than with the cathodic substances. The cathodically migrating components also reacted very weakly with anti-Av.

IEP analysis (Fig. 6) of the Av extracts confirmed the CIE results. The extract of group A streptococci reacted with anti-A, demonstrating cathodic and anodic migration (Fig. 6a). However, only the cathodic portion of the group A streptococcal antigen reacted with anti-Av (Fig. 6b). Extracts of Av T27A (Fig. 6c) and of Av A486-Var grown at 37°C failed to react with anti-A. When extracts of Av T27A (Fig. 6d) and Av A486-Var grown at 22°C were reacted with anti-Av, most immunoprecipitation occurred in the cathodic region.

Time course of conversion of Av A486-Var from Av to A specificity. Extracts prepared from Av A486-Var taken at various times after the temperature shift from 37 to 22°C were examined by IEP and CIE. Both methods showed that with increased incubation at 22°C the extracts reacted more strongly with anti-A and less so with anti-Av (Table 2). The components precipitating with anti-A were predominantly anodic, whereas those precipitating with anti-Av were predominantly cathodic. The switch from cathodic to anodic reactivity and from anti-Av to anti-A reactivity suggests that the addition of GlcNAc to the polyRha backbone of group A carbohydrate alters its mobility in the electric field.

A-enzyme conversion of group A streptococcal extract from A to Av specificity. If the electrophoretic property of group A carbohydrate depends on the amount of GlcNAc present, removal of GlcNAc from the group A antigen should alter its electrophoretic mobility and reactivity with anti-A. This was tested by treating group A streptococcal extract for various times with A-enzyme, which can remove 50 to 70% of the total GlcNAc from group A substance. When the kinetics of A-enzyme activity was followed by CIE and IEP, the reactivity of the enzyme-treated group A extract with anti-A decreased, whereas its reactivity with anti-Av increased (Table 3). The loss of reactivity with anti-A appeared first with the cathodic components and then with the anodic components of group A substance. This suggests that the cathodic components may have less GlcNAc attached to the polyRha backbone than the anodic components; thus, complete removal of GlcNAc occurs more rapidly from the cathodic than from the anodic components. The components of the enzyme-treated extract which reacted with anti-Av migrated cathodically.

**DISCUSSION**

The present study demonstrated that the group A streptococcal substance comprises a heterogeneous population of molecules which can be separated electrophoretically by CIE or IEP. The heterogeneity appears to be related to the amount of GlcNAc attached to the polyRha backbone of the carbohydrate; as the GlcNAc in...
the polyRha backbone increases, the molecules migrate more anodically. The evidence supporting this hypothesis is that: (i) the cathodically migrating components of Av T27A reacted more strongly with anti-Av than did the anodically migrating components, and Av T27A did not react with anti-A; (ii) in the temperature-dependent variant strain A486-Var, a shift from 37 to 22°C resulted in expression of the group A specificity and a more anodic migration of the extract; (iii) cleavage of GlcNAc residues from group A substance altered its migration to a more cathodic position and caused loss of reactivity with anti-A; and (iv) the reactivity of group A extract with anti-Av was limited to the cathodal elements of group A.

TABLE 1. Electrophoretic mobility and specificity of streptococcal extracts examined by CIE against anti-A and anti-Av

<table>
<thead>
<tr>
<th>Strain</th>
<th>Incubation temp (°C)</th>
<th>Intensity of immunoprecipitation* with</th>
<th>Group specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A streptococcus</td>
<td>37 and 22</td>
<td>+++</td>
<td>A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>−</td>
<td></td>
</tr>
<tr>
<td>Group A streptococcus</td>
<td>37 and 22</td>
<td>−</td>
<td>Av</td>
</tr>
<tr>
<td>variant T27A</td>
<td></td>
<td>−</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>++++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Group A streptococcus</td>
<td>37</td>
<td>−</td>
<td>Av</td>
</tr>
<tr>
<td>variant A486-Var</td>
<td>22</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(+)</td>
<td></td>
</tr>
</tbody>
</table>

* The intensity of the immunoprecipitation reaction was graded from + to ++++. (+), Barely visible precipitation line; −, no reaction.

FIG. 6. IEP of streptococcal group A and Av carbohydrate. The group-specific antisera to group A and Av streptococci are anti-A and anti-Av, respectively.

The oppositely migrating components of group A streptococcal extracts, precipitable with anti-A, were present in comparable quantities (Fig. 3). The immunochemical identity of these components is demonstrated by the absence of spurs in the IEP precipitation line and by the ability of GlcNAc to inhibit both anodic and cathodic immunoprecipitation reactions. Similar inhibition experiments using GalNAc and Rha failed to inhibit immunoprecipitate formation between anti-Av and either Av T27A extract or group A streptococcal extract. The ability of Rha to inhibit group B-anti-B reaction but not group A-anti-Av or group Av-anti-Av reaction suggests that the Rha monosaccharide is important in group B specificity but not in Av specificity. Consistent with these findings is our observation that group B carbohydrate does not precipitate with anti-Av.

The antigenic specificity of Av substance is based on its Rha-Rha linkages (10, 20). The group B carbohydrate consists of Rha and glucosamine residues, although the structure of its repeating unit has not yet been elucidated (8). The Rha residues in group B substance may not be linked in multiple groupings comparable to those in group A carbohydrate. The ability of the cationic components of group A carbohydrate to react with anti-Av suggests that the incomplete addition of GlcNAc to the polyRha backbone may expose sites available for anti-Av reactivity.

The group A streptococcal components can be separated electrophoretically; the anodal component has A specificity, and the cathodal component has both A and Av specificities. Neutral polysaccharides, such as the group A carbohydrate, migrate in an electric field with a charged carrier, such as borate or barbital ions (6). Extensive studies of anodic complex formation between borate ions and the hydroxyl groups of carbohydrates have shown that two hydroxyl groups within an approachable distance of 0.24 nm are required (24). Factors influencing the
TABLE 2. Electrophoretic mobility and specificity of extracts of the temperature-dependent group A streptococcus variant A486-Var examined by CIE against anti-A and anti-Av

<table>
<thead>
<tr>
<th>Time after shift to 22°C (h)</th>
<th>Intensity of immunoprecipitation with Anti-A</th>
<th>Intensity of immunoprecipitation with Anti-Av</th>
<th>Group specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cathode Anode</td>
<td>Cathode Anode</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>- -</td>
<td>++++</td>
<td>(+)</td>
</tr>
<tr>
<td>3</td>
<td>- +</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>(+) +</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>(+) +</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* See Table 1 for explanation of notations.

The antigenic properties of group A carbohydrate are clinically significant because the carbohydrate cross-reacts with the structural glycoproteins of human heart valves (12, 13). This cross-reactivity appears to involve GlcNAc. The role of anti-A in rheumatic fever remains uncer-
tain. Ayoub and Shulman (4) reported its persistence in chronic rheumatic valvular disease. However, its presence in patients with other group A streptococcal diseases, including pyoderma, leaves uncertain its etiological role in rheumatic heart disease (15).

The heterogeneity of the streptococcal antigen should be taken into consideration in antiserum production and in immunological analysis. The cationic component of group A substance reacts very weakly with anti-Av. This Av specificity is a result of Rha-Rha linkages which enable anti-Av to react also with group C-variant streptococcal extract, which has a specificity similar to that of the Av preparation (2). Most of the cross-reactivity observed between group A and C streptococci may result from antibodies to Rha-Rha linkages. It is conceivable that the specificity of anti-A reflects the homogeneity of the carbohydrate in the strain used for antiserum production. Strains with carbohydrates of Av or I specificity may give rise to antisera which will cross-react with group A streptococci. On the other hand, group A strains with no Av specificity might give rise to antisera which are group A specific and will not react with group C streptococci.

Av strains are rarely encountered in clinical material. McCarty and Lancefield (21) did not observe Av specificity in any of 1,815 group A strains or 33 nongroupable streptococci isolated from human sources. From well over 14,000

TABLE 3. Electrophoretic mobility and specificity of group A streptococcal extract treated with A-enzyme and examined by CIE against anti-A and anti-Av

<table>
<thead>
<tr>
<th>Duration of enzyme treatment (h)</th>
<th>Intensity of immunoprecipitation with Anti-A</th>
<th>Intensity of immunoprecipitation with Anti-Av</th>
<th>Group specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cathode Anode</td>
<td>Cathode Anode</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>+++</td>
<td>++++</td>
<td>(+)</td>
</tr>
<tr>
<td>1</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
</tr>
<tr>
<td>4*</td>
<td>-</td>
<td>-</td>
<td>++++</td>
</tr>
</tbody>
</table>

* See Table 1 for explanation of notations.

* Results obtained after 6, 9, and 24 h of treatment were identical to those obtained after 4 h.
beta-hemolytic streptococcal specimens of human origin submitted to the Centers for Disease Control during a 7-year period, only one A\text{-}IV and one A-I strain were isolated (11).

It is noteworthy that the group C streptococcal carbohydrate behaves similarly to the group A carbohydrate in its heterogeneity of migration. The group C heterogeneity may result as varying degrees of GalNAC disaccharide are added to the polyRha backbone. Group B streptococcal antigen appears to be more homogeneous in structure, since it migrates as a narrow arc in IEP.

In our study, cathodically migrating components of the streptococal extracts were detected by adding a second row of wells cathodic to the antigen wells after the electrophoretic step (Fig. 2). This modification of CIE may enable the detection of all capsular polysaccharides of pneumococci with one CIE system (16). A different buffer system (1) for cathodic detection of type VII and XIV pneumococcal antigens may not be necessary.

The IIP technique designed in this study for the demonstration of antigenic specificity of streptococcal carbohydrate could be applied to other antigen systems, but its use may be limited. The technique is based on the diffusability of the competing immunodominant constituents into the surrounding agarose. For example, in cases where antigenicity might be dependent on the interaction of several molecular components of the macromolecule or on a configurational structure of the antigen, the use of IIP to elucidate the immunodominant constituent may not be applicable.

LITERATURE CITED


