THE DETERMINATION OF ANTIBODY TO GROUP A STREPTOCOCCAL POLYSACCHARIDE IN HUMAN SERA BY HEMAGGLUTINATION*, ‡

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Extensive investigations of the immunological response to streptococcal infection in man have been conducted to obtain information necessary to define streptococcal immunity and explain the pathogenesis of streptococcal infection, rheumatic fever, and glomerulonephritis. Most of this work has been concerned with the host response to soluble enzymes and toxins of Group A streptococci (1, 2). Studies of the host response to streptococcal cellular components have been limited because of the difficulty in obtaining purified preparations of these constituents (1). Recently cell wall (3, 4) and cell membrane (5) antigens of defined chemical and immunological specificity have been obtained. A cell wall polysaccharide fraction described in the preceding paper (6) was utilized in the present investigation as hemagglutination antigen to measure antibody to Group A carbohydrate in human sera. Experiments demonstrating the specificity of this serologic reaction and data describing the antibody response to streptococcal A polysaccharide in man are presented in this report.

Since Lancefield described the C carbohydrate antigen of hemolytic streptococci (7), a number of investigators have reported finding precipitating antibody to A polysaccharide in sera from normal persons, patients convalescent from streptococcal infection, and patients with rheumatoid arthritis, rheumatic fever, and glomerulonephritis (8–18). In all but two of these investigations, crude acid extracts of Group A streptococci were used as antigen and it is likely that the precipitating antibody detected was a composite, representative of multiple streptococcal cellular antigens. Antibody precipitated by purified A

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carbohydrate was found by Wilson and Wiley in the serum of a patient with streptococcal parotitis, otitis, and septicemia (9). Halbert described immunodiffusion reactions with human gamma globulin and *Streptomyces albus* enzyme–prepared A polysaccharide and an A polysaccharide–protein complex (8). In these studies, A carbohydrate antibody was not quantified nor were surveys of sera from different sources reported.

Materials and Methods

Polysaccharide Antigens.—Ethanol and acetone-precipitated fractions of A polysaccharide were prepared from diisopropyl fluorophosphate (DFP)-treated *Streptomyces albus* enzyme lysates of streptococcal cell walls as described in the preceding paper (6). Similar polysaccharide fractions were precipitated from lysates obtained with *S. albus* enzyme that was not treated with DFP. Ethanol and acetone fractions of A-variant polysaccharide (19) were isolated from lysates of cell walls of the T27A strain of streptococci according to the methods employed for A polysaccharide. Group A carbohydrate was also obtained from cell walls by extraction with formamide and purified with ion exchange resins (20). Specific enzymatic hydrolysis of preparations of A polysaccharide with A enzyme, which selectively destroys A serologic activity, was performed at pH 6, $\Gamma/2$ 0.2, at 25° and 37° C for 20 to 24 hours as described by McCarty (21). Dr. M. McCarty generously provided the A enzyme.

Column Chromatography.—Chromatography of polysaccharide preparations on CM cellulose was performed according to Peterson and Sober (22). Columns 0.9 x 25 cm were used. Gradient elution was performed with phosphate buffer, pH 6.4, $\Gamma/2$ 0.01; the ionic strength was gradually increased to 0.5 with NaCl.

Preparation of Serum Specimens.—Hyperimmune rabbit sera containing antibody to A and A-variant polysaccharides were prepared according to methods described by McCarty and Lancefield (19). Human serum specimens were collected in the usual manner and stored at −20°C, or at refrigerator temperature. All sera used in hemagglutination tests were centrifuged and passed through a Millipore filter (porosity 0.45 μ) for clarification and inactivated by heating to 60°C for 15 minutes. They were then absorbed with human O erythrocytes by mixing 1 volume of serum with 1 volume of packed erythrocytes (RBC). The suspension was kept at room temperature for 10 minutes with occasional mixing and then centrifuged at 2500 RPM for 20 minutes at 2°C. The serum was carefully removed from the cells and stored at −20° or at 4°C. All serum dilutions were made with buffered saline, pH 7.2 (23) containing 1 per cent inactivated normal rabbit serum (NRS) that had been absorbed with human O RBC.

Preparation of Reagents for Hemagglutination and Hemagglutination Inhibition.—

Human O erythrocytes: Several type O donors were used. Cells from one donor produced more compact, clear-cut agglutination of RBC; therefore, cells from this donor were almost always used for comparing various sera or antigens. RBC were collected in ACD (24) solution and centrifuged at 2000 RPM at 2°C (all centrifugations were performed at this temperature) for 30 minutes. The supernatant fluid and the buffy coat were removed and the RBC resuspended in ACD solution and centrifuged 20 minutes. This was repeated twice and the last centrifugation was run for 30 minutes to measure the packed RBC volume. RBC were stored as a 50 per cent suspension in ACD solution of 2°C for no longer than 4 days.

To prepare a 2.5 per cent suspension of RBC for treatment with tannic acid, 1 ml of 50 per cent RBC was added to 20 ml of pH 7.2 buffer, mixed and centrifuged at 1500 RPM for 10 minutes. The supernatant fluid was removed and the RBC resuspended in 19.5 ml of pH 7.2 buffer.

Preparation of Tanned RBC.—A 1:100 stock solution of reagent grade tannic acid was made in 0.85 per cent saline weekly and kept at 2°C. Each day, a 1:20,000 solution of tannic acid

in pH 7.2 buffer was prepared. Then 2 volumes of 2.5 per cent suspension of RBC were mixed with 1 volume of 1:20,000 tannic acid and 1 volume of pH 7.2 buffer and incubated at 37°C for 10 minutes. The suspension was centrifuged at 1500 RPM for 10 minutes and the supernate removed. The RBC were dispersed in the same volume of pH 7.2 buffer and centrifugation repeated. The RBC were then added to sufficient 0.85 per cent saline to make a 2.5 per cent suspension.

Sensitization of Tanned RBC.—One volume of 2.5 per cent tanned RBC was mixed with 0.9 to 0.4 volume of pH 6.4 buffered saline (23) and 0.1 to 0.6 volume of antigen in distilled water or pH 6.4 buffered saline. The suspension was incubated at 37°C for 30 minutes with occasional mixing; it was then centrifuged at 1500 RPM for 10 minutes and the supernate removed. To wash the sensitized RBC, they were resuspended in pH 7.2 buffer containing 2 per cent heat-inactivated bovine serum albumin (BSA) to a final concentration of RBC of 1.25 per cent and centrifuged at 1500 RPM for 10 minutes. This was repeated once; then the RBC were added to pH 7.2 buffer containing 2 per cent BSA to make a 2.5 per cent suspension of sensitized RBC. An unsensitized 2.5 per cent RBC suspension was prepared in the same manner without added antigen. Sensitized RBC were prepared fresh daily. Because agglutination of RBC was less compact in the presence of merthiolate, it was not put in any of the reagents except the original undiluted serum specimen to be tested. Bacterial contamination also interfered with hemagglutination so that all reagents were autoclaved or sterilized by filtration, and sterile technique was used in all procedures from the drawing of blood through the preparation of sensitized cells.

Hemagglutination.—Dilutions of sera to be tested were pipetted in 0.5 ml amounts into carefully cleaned 10 x 75 mm test tubes and 0.05 ml of sensitized RBC suspension was added. The tubes were shaken to mix sensitized cells and serum thoroughly and then kept at 4°C overnight. The reactions were graded from 0 to 4+ according to criteria slightly modified from those described by Stavitsky (23). Serum controls consisted of one or more of the lower serum dilutions reacted with non-sensitized, tanned RBC. For antigen controls, sensitized, tanned RBC were reacted with dilutions of normal rabbit or normal human sera, rabbit sera containing heterologous antibody, and with saline. A standard serum producing positive hemagglutination of a known titer was included with each group of sera tested.

Hemagglutination Inhibition.—Solutions of antigens for inhibition were diluted in pH 7.2 buffer containing 1 per cent NRS. The dilutions of inhibiting antigens (usually a 100- to 1000-fold range) were added in 0.1 ml amounts to 0.4 ml of one or more selected dilutions of a serum of known hemagglutination titer. The tubes were mixed and allowed to stand at room temperature for 30 minutes. Then 0.05 ml of sensitized RBC was added and the tubes were shaken again and refrigerated overnight. Reactions were graded as in hemagglutination. A titration of the hemagglutinating system without inhibitor was made concurrently.

RESULTS

After it was found in preliminary experiments that certain preparations of A carbohydrate would attach to tanned human O erythrocytes and produce agglutination in the presence of specific antibody, other experiments were conducted to determine optimum conditions for the reaction. The conditions described under Materials and Methods for concentration of RBC, tannic acid, pH, temperature, and time for tanning and sensitizing RBC represent optima. Treatment of RBC with tannic acid was essential. Hemagglutination in the A polysaccharide system was much better at 4° than at 25° or 37°C. Reactions could be read as early as 4 hours but were maximal after 16 hours. Time and

temperature had little if any effect on the reaction of antibody with inhibiting antigen.

Preparations of Polysaccharide Effective as Hemagglutinating Antigens.—A survey of a number of preparations of A polysaccharide revealed marked differences in their ability to sensitize tanned RBC for hemagglutination.

To compare the various forms of polysaccharide, a volume of tanned O RBC was mixed with an equal volume of polysaccharide of the concentration listed in Table I for sensitization. After washing, the sensitized cells were added to serial dilutions of a Group A antistreptococcal serum and to two dilutions of a Group B serum.

TABLE I

Hemagglutination Reactions of Preparations of A Polysaccharide

Sensitizing antigen		Reciprocal of serum dilution							
Preparation	Conncentration	Group A						Group B	
		100	200	400	800	1600	3200	50	100
	μg rhamnose/ml								
Formamide (No. 5)	45	−*	−	−	−	−	−	−	−
S. albus (No. 10)	45	3+	3+	2+	+	±	−	−	−
S. albus (No. 14)	49	−	−	−	−	−	−	−	−
DFP-*S. albus* (No. 13)	5	4+	4+	4+	4+	4+	3+	−	−
DFP-*S. albus* (No. 12) ethanol fraction	5	4+	4+	4+	4+	4+	3+	−	−
DFP-*S. albus* (No. 12) ethanol fraction	2.6	4+	4+	4+	4+	3+	+	−	−
DFP-*S. albus* (No. 12) acetone fraction	47	−	−	−	−	−	−	−	−
DFP-*S. albus* (No. 12) ethanol fraction + trypsin	130	−	−	−	−	−	−	−	−

* Reactions as described in reference 23 with the exception that the more compact reaction read as ± there is read as − here.

The relative efficacy of the different preparations of A carbohydrate as hemagglutinating antigens was estimated from the extent of agglutination produced per microgram of polysaccharide rhamnose employed for sensitization. The results are presented in Table I. Sensitization of tanned RBC with formamide-extracted A carbohydrate could not be accomplished. Of several preparations tested, only one lot (No. 10) of polysaccharide prepared with *S. albus* enzyme without DFP sensitized RBC for hemagglutination at relatively high antigen concentrations. A-carbohydrate purified from DFP-treated *S. albus* enzyme lysates produced uniformly good sensitization of RBC at concentrations less than 5 μg of polysaccharide rhamnose per ml. After fractionation of the 0.6 saturated $(NH_4)_2SO_4$ supernate of DFP-*S. albus* enzyme lysates with ethanol and acetone (6), hemagglutinating antigen was found only in the ethanol precipitate.

Treatment of ethanol-precipitated A polysaccharide with crystalline trypsin (10 μg trypsin per ml at pH 8, 37°C for 5 hours; reaction stopped with DFP) abolished the ability to sensitize tanned RBC for hemagglutination. Since the mucopeptide is the only portion of enzymatically prepared A carbohydrate that can be hydrolyzed by trypsin, it may be inferred from the trypsin effect that the attachment of A polysaccharide to tanned O erythrocytes is effected by way of the mucopeptide.

Further fractionation of ethanol-precipitated polysaccharide by chromatography on CM cellulose has also provided evidence of the dependence of sensitization of RBC on the mucopeptide conjugated to A polysaccharide. The major portion of ethanol-precipitated A carbohydrate was not adsorbed to CM cellulose equilibrated with pH 6.4, Γ/2 0.01 phosphate buffer and emerged from

TABLE II

Specificity of A Polysaccharide Hemagglutination

Sensitizing antigen		Reciprocal of serum dilution									
Preparation	Concentration	Group A				Group B		Group C		Group G	
		100	400	800	1600	50	100	50	100	50	100
	μg rhamnose/ml										
A polysaccharide DFP–*S. albus*–ethanol	10	4+	4+	3+	+	±	−	−	−	−	±
A polysaccharide DFP–*S. albus*–ethanol + A enzyme	128	−	−	−	−	−	−				
A-variant polysaccharide DFP–*S. albus*–ethanol	50	+	−	−	−	−	−				

the column with the initial solvent. This fraction contained relatively less sensitizing antigen than a small portion of the A polysaccharide eluted from the column between ionic strength 0.05 and 0.1. The latter fraction contained from 2.5 to 3 times as much mucopeptide as the major unadsorbed polysaccharide fraction.

Specificity of A Polysaccharide Hemagglutination.—Reactions demonstrating the specificity of A polysaccharide hemagglutination are presented in Table II. Agglutination of tanned RBC sensitized with A polysaccharide was demonstrable with Group A antistreptococcal rabbit sera but not with Groups B, C, or G antisera.

Sensitization of human O RBC with the ethanol-precipitated fraction of A carbohydrate was performed as detailed in the Materials and Methods section. Similar conditions were employed in treating tanned O RBC with several preparations and concentrations of A-variant polysaccharide. To sensitize RBC with A enzyme–treated A carbohydrate, several milligrams

of ethanol polysaccharide were hydrolyzed with A enzyme. The hydrolysate was then diluted, and a number of concentrations were reacted with tanned O RBC in the usual manner.

Tanned RBC sensitized with A polysaccharide that had been treated with A enzyme which hydrolyzes the immunologically specific A determinant (21) were not agglutinated by Group A or other antistreptococcal rabbit sera. Attempts to sensitize tanned RBC with A-variant polysaccharide produced variable results. Clear-cut hemagglutination patterns were not obtained, and when agglutination did occur it was of low titer. Reactions with A as well as A-variant sera were usual; often the titer with A antiserum was greater than that of the A-variant serum. It is likely that the positive reactions were due to

TABLE III

Inhibition of A Polysaccharide Hemagglutination

Inhibitor	Hemagglutination in presence of inhibitor, concentration of inhibitor in μg. of rhamnose					
	5.0	1.0	0.5	0.05	0.005	0
A polysaccharide (formamide)	−	−	±	+	2+	3+
A polysaccharide (*S. albus*)	−	−	−	+	3+	3+
A polysaccharide (DFP–*S. albus*–ethanol)	−	−	−	±	3+	3+
A polysaccharide + trypsin	−	−	−	−	2+	3+
A polysaccharide + A enzyme	+	2+	2+	2+	2+	3+
A-variant polysaccharide	+	2+	3+	3+	3+	3+
β-phenyl-*N*-acetylglucosaminide*‡	2+	2+	2+			3+

* Concentration expressed in μg of phenylacetylglucosaminide.

‡ Generously supplied by Dr. M. McCarty.

small amounts of A polysaccharide present in the preparations of A-variant carbohydrate.

Hemagglutination Inhibition Reactions.—Inhibition of A polysaccharide hemagglutination provided further evidence of the specificity of this serological reaction. Concentrations of A carbohydrate as small as 0.05 μg of polysaccharide rhamnose produced inhibition of agglutination of A carbohydrate–sensitized tanned RBC. All preparations of A polysaccharide had approximately the same inhibition effect as illustrated in Table III. A-variant polysaccharide had no inhibiting effect. The evidence that trypsin-treated A polysaccharide was a good inhibitor provided further indication that the action of trypsin on the RBC-sensitizing property of the ethanol fraction of A polysaccharide previously described (Table I) was not on the immunologic determinant but on the site of attachment of the polysaccharide molecules to the tanned red cell. Exposure of A carbohydrate to A enzyme, which removes the β-*N*-acetylglucosamine serologic determinant (21), destroyed its hemagglutination-

inhibiting ability. McCarty, in establishing the identity of the A polysaccharide determinant as β-linked *N*-acetylglucosamine, demonstrated significant inhibition of the precipitin reaction of A polysaccharide and A antibody by *N*-acetylglucosamine and phenyl-β-*N*-acetylglucosaminide (25). Experiments employing these reagents in concentrations of 0.02 to 2000 μg per ml as inhibitors of A polysaccharide hemagglutination were unsuccessful. An example of the inability of several concentrations of β-phenyl-*N*-acetylglucosaminide to inhibit A polysaccharide hemagglutination is illustrated in Table III.

Hemagglutination and Hemagglutination Inhibition with Human Sera.—To determine the specificity of the A polysaccharide hemagglutination reaction with human antisera, sera from two patients with Group A streptococcal

TABLE IV

A Carbohydrate Hemagglutination and Hemagglutination Inhibition with Human Serum Dra

Sensitizing antigen	Reciprocal of serum dilution				
	200	400	800	1600	3200
A polysaccharide DFP–*S. albus*–EtOH	2+	2+	2+	2+	3+
A polysaccharide DFP–*S. albus*–EtOH + A enzyme	±	–	–	–	–

Inhibiting antigen	Concentration of inhibitor in μg rhamnose/ml					
	5.0	2.5	1.0	0.5	0.05	0
A polysaccharide DFP–*S. albus*–EtOH	–	–	–	–	–	4+
A polysaccharide DFP–*S. albus*–EtOH + A enzyme	4+	4+	4+	4+		4+

sepsis, known to contain precipitating antibody to A carbohydrate, were compared with Group A rabbit antisera. Serum from one of these patients, Sch., was kindly provided by Dr. A. T. Wilson who previously described capillary precipitation and immunoelectrophoretic reactions of this serum with purified A carbohydrate (9). The second patient, Dra., was one of several with group A streptococcal sepsis, observed and treated at Cleveland Metropolitan General Hospital, who were found to have A carbohydrate precipitating antibody in their sera. Hemagglutination and hemagglutination inhibition reactions of sera from Sch. and Dra. with preparations of A polysaccharide and enzyme-treated A polysaccharide were qualitatively identical with the reactions of these materials with antistreptococcal rabbit sera described above and illustrated in Tables I to III. Experiments illustrating reactions of the ethanol-precipitated fraction of A carbohydrate, before and after treatment with A enzyme, as sensitizing and inhibiting antigen with Dra. serum are presented in

Table IV and in Fig. 1. This serum agglutinated A polysaccharide-sensitized, tanned RBC to a dilution of 1/3200. No hemagglutination was obtained with the same antigen after enzymatic removal of the specific immunologic determinant with A enzyme. A-carbohydrate inhibited the reaction of A polysaccharide-sensitized RBC with Dra. serum, and loss of inhibitory activity accompanied loss of the A polysaccharide determinant.

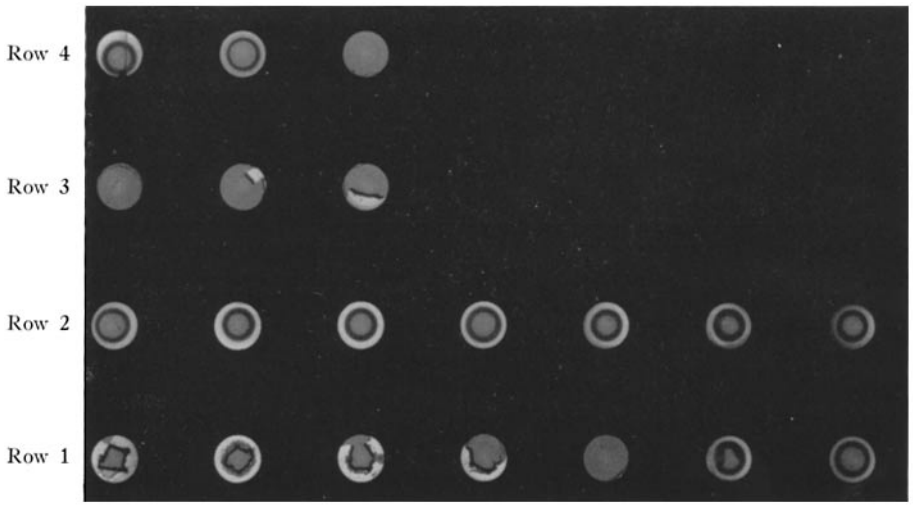

FIG. 1. A carbohydrate antibody in human convalescent serum demonstrated by hemagglutination and hemagglutination inhibition with A polysaccharide and A polysaccharide treated with A enzyme.

Row 1. Hemagglutination: tanned cells sensitized with A carbohydrate; Dra. serum dilutions, left to right: 1/200 to 1/3200, group B rabbit 1/50, NRS. *Row. 2.* Cells sensitized with A carbohydrate treated with A enzyme; same serum dilutions, no hemagglutination. *Row 4.* Hemagglutination inhibition by A polysaccharide: concentration of inhibitor in μg rhamnose, left to right, 0.5, 0.05, 0.005. *Row 3.* Abolition of A polysaccharide inhibition; same concentration of A polysaccharide inhibitor used after treatment with A enzyme.

After demonstration of the specificity of A polysaccharide hemagglutination with human sera, serum specimens from patients with various forms of streptococcal infection, non-suppurative streptococcal sequelae, and from normal children and adults were tested to obtain information about the nature of the A carbohydrate antibody response in man. Most of the sera examined contained A carbohydrate antibody, and considerable variation in the titers of this antibody was evident. Quantitative comparisons of sera from various categories of streptococcal disease were made after experiments were performed to determine the accuracy of the titration of A polysaccharide antibody by hemagglutination. Repeated titrations of several human and rabbit sera were made; titrations of

the same serum did not usually result in a variation of more than one dilution increment in the end point (reaction of 1+ or more). The hemagglutination titers of A antibody in a series of rabbit sera were roughly proportional to amounts of A antibody in these sera determined by quantitative precipitation. Therefore, estimation of A polysaccharide antibody by hemagglutination is

TABLE V

A Polysaccharide Hemagglutinating Antibody in Human Sera

Serum	Reciprocal of serum dilution								
	20	50	100	200	400	800	1600	3200	6400
Dra. 1*, ‡		3+	3+	3+	2+	+	±	−	−
Dra. 2‡		4+	4+	4+	4+	+	−	−	−
Dra. 3‡		4+	4+	4+	4+	4+	2+	2+	±
Sch. 2‡	4+	4+	4+	4+	3+	3+	+	±	−
Car.§	4+	4+	2+	−	−	−	−		
Dav.§	4+	4+	2+	+	−	−	−		
Fra.§	4+	3+	+	+	−	−	−		
Sev. 1‖	4+	4+	2+	±	−	−	−		
Sev. 2§	4+	3+	3+	2+	+	±	−		
Ruc.¶	4+	3+	2+	2+	+	+	−	−	
Bar.¶	4+	4+	4+	4+	−	−	−	−	
Jon.¶	4+	4+	4+	4+	4+	+	±	−	
Cor.‖	−	−	−	−					
Cru.**	3+	3+	−	−					
Har.**	3+	2+	−	−					
Gur.‡‡	3+	3+	−	−	−	−	−		
Gin.‡‡	−	−	−	−	−	−	−		
Nid.‡‡	4+	4+	4+	+	+	−	−		

* Numbers indicate the sequence of specimens obtained from the same patient.
‡ Streptococcal sepsis.
§ Convalescent untreated streptococcal infection.
‖ Acute phase streptococcal infection.
¶ Acute rheumatic fever.
** "Normal" child.
‡‡ "Normal" adult.

probably comparable to titrations of other streptococcal antibodies which are similarly subject to the limitations of the serum dilution technique.

A typical titration of A carbohydrate antibody in sera from normal children, adults, and patients during several phases of streptococcal infection and rheumatic fever is presented in Table V. Serum Dra. 1 was obtained during the acute phase of streptococcal sepsis; serum Dra. 2 drawn 3 weeks later had the same titer of A antibody: 800. A rise in antibody to 3200 was detected in a third serum obtained 8 weeks after the second. Serum Sch. was from the

patient with sepsis studied by Wilson. Sera from Car., Dav., Fra., and Sev. (2) were collected during the convalescent stage of untreated streptococcal pharyngitis, 5 to 7 weeks after the onset of symptoms. In the second serum from Sev., which was obtained 5 weeks after the first specimen, a rise in the A antibody titer from 100 to 400 was found. A-polysaccharide hemagglutinating antibody titers of 200 to 800 units were found in the three serum specimens from patients with acute rheumatic fever. Sixty to 70 per cent of normal children and adults were found to have demonstrable A carbohydrate antibody in their sera; the titers were usually less than 100. Children were less likely to have detectable A antibody, depending upon age and epidemiological circumstances. In a small collection of sera from young children with viral disease, 75 per cent did not contain A-hemagglutinating antibody, whereas 8 of 9 children in an orphanage population where many streptococcal carriers were present had measurable

TABLE VI

A Polysaccharide Hemagglutinating Antibody

Category	Reciprocal of serum dilution	
	Range	Median
Normal child	0–800	50
Normal adult	0–400	50
Streptococcal infection	50–800	200
Rheumatic fever or nephritis	50–1600	400
Streptococcal sepsis	800–3200	1600

antibody titers. In several sera from infants collected during the acute phase of streptococcal infection, A carbohydrate antibody was not present nor did it appear in specimens collected 2 to 3 weeks later. In adults who had A antibody initially, a rise in antibody did not occur until 4 weeks or more after streptococcal infection. A antibody titers decreased minimally or persisted for at least several months.

If the A polysaccharide antibody titers of normal persons and patients with localized streptococcal infections, sepsis, rheumatic fever, and glomerulonephritis are collectively examined, a variation in the intensity of antibody response similar to that of several other streptococcal antibodies (1, 2) is found. A summary of the antibody determinations of 86 sera showing the range and the median titers in these categories, is presented in Table VI. Normal persons were found to have a median titer of A hemagglutinating antibody of 50. Patients convalescent from Group A streptococcal infections demonstrated a fourfold greater median value. The median response in patients with rheumatic fever and glomerulonephritis is somewhat higher than that following uncomplicated

infection. Although few patients with sepsis were studied, the greatest antibody response was evident in this group experiencing intensive antigenic stimulus.

DISCUSSION

Previous experience with the measurement of streptococcal antibodies in man has demonstrated that highly sensitive techniques are necessary to detect the small amounts of a specific antibody formed after natural infection (26). In this investigation, hemagglutination was selected as a method for measuring A polysaccharide antibody because of its sensitivity (23) and because the specificity of the hemagglutination reaction could be critically tested with the specific β-acetylglucosaminidase and the β-acetylglucosaminide inhibitor employed by McCarty in the lucid demonstration of the chemical nature of the A carbohydrate determinant (21, 25).

Recent evidence that mucopeptide components of the cell wall remain conjugated to enzymically released A polysaccharide (6, 27) provided another reason for attempting hemagglutination with the A carbohydrate; certain mucopeptide fragments might contain reactive groups more likely to bind polysaccharide to tannic acid–treated erythrocytes. Substantiation for this hypothesis was found in the observation that only a particular, mucopeptide-containing fraction of A polysaccharide solubilized by DFP-treated *S. albus* enzyme, namely the ethanol precipitate and chromatographic fractions of this precipitate, attached to RBC; whereas the acetone precipitate of DFP-treated *S. albus* lysates, *S. albus* lysates (with one exception), and formamide-extracted A carbohydrate would not sensitize RBC. It was also found that trypsin destroyed the capacity of the ethanol A carbohydrate fraction to attach to tanned RBC without altering its activity in hemagglutination inhibition.

A series of experiments was presented to identify the hemagglutinating reactant in human sera as A carbohydrate antibody. First, it was demonstrated that A polysaccharide-sensitized RBC reacted with Group A rabbit antisera but not with antisera of Groups B, C, and G. This reaction was inhibited by A carbohydrate and abolished by treatment of the RBC-sensitizing antigen with A enzyme which removes the immunologically specific A carbohydrate determinant. Then, it was shown that A polysaccharide inhibited hemagglutination of the same antigen-coated RBC by human sera known to contain A carbohydrate antibody. This reaction was eliminated by treatment of the inhibiting antigen with A enzyme and by A enzyme hydrolysis of the sensitizing antigen before it was reacted with tanned RBC.

No ready explanation is forthcoming to account for the failure to demonstrate inhibition of A polysaccharide hemagglutination by *N*-acetylglucosamine or phenyl-*N*-acetylglucosaminide which McCarty found effective in producing partial inhibition in the A carbohydrate–A antibody precipitating system (25). It has been suggested that incomplete inhibition in this precipitating

system and lack of cross-reactivity between A polysaccharide and azophenyl-*N*-acetylglucosaminide antisera (25) occur because A antibody-combining sites are mainly complementary to A carbohydrate oligosaccharides with terminal β-*N*-acetylglucosaminide units (28). It is possible that the determinants of the A carbohydrate fixed to tanned RBC are by chance those that react only with A antibody with combining sites complementary to oligosaccharides which monomeric inhibitors will not satisfy. It is also possible that inhibition of hemagglutination was not demonstrated because the glucosaminide inhibitor was employed in too low a concentration.

The studies of A polysaccharide antibody in human sera that have been conducted do not as yet permit accurate description of the natural history of the A carbohydrate antibody response following streptococcal infection, but several tentative features emerge. Most persons except infants and some young children have circulating A carbohydrate antibody. The A antibody response appears to be relatively slow following streptococcal infection and is more persistent, resembling M antibody in contrast to streptolysin O antibody (2, 26, 29). Patients with the non-suppurative sequelae seem to have a somewhat greater A antibody response than those experiencing uncomplicated streptococcal infection.

SUMMARY

A hemagglutination method employing tanned human O erythrocytes sensitized with purified Group A streptococcal polysaccharide has been developed to measure A polysaccharide antibody in antistreptococcal rabbit and human sera.

The reaction of sensitized RBC with known Group A streptococcal rabbit antisera, inhibition of hemagglutination with A polysaccharide, and abolition of hemagglutination and hemagglutination-inhibition by exposure of polysaccharide antigen to A enzyme, which destroys the specific immunologic determinant of A carbohydrate, were utilized to demonstrate the specificity of this hemagglutinating system.

The results of A polysaccharide antibody determinations on sera from normal persons, patients convalescent from streptococcal infection, and patients with non-suppurative streptococcal sequelae are presented.

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