DETECTION OF STREPTOCOCCAL GROUP-SPECIFIC ANTIBODIES IN HUMAN SERA*

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Human antibodies directed against the specific carbohydrate of Group A streptococci are not readily detected. Although Rosendal (1) has demonstrated the agglutination of Group A streptococci by human sera, which may be dependent upon anti-Group A agglutinins, these results are difficult to interpret because of frequent spontaneous agglutination of the cell suspension, and because agglutination may be dependent upon components of the cell wall other than the group carbohydrate. Group A antibodies have been detected in an occasional human serum by precipitin methods (2, 3) but it has not proved feasible to devise a quantitative estimate of antibodies with these techniques. This report describes the detection of human anti-Group A agglutinins by a modification of the Coombs indirect agglutination test which employs purified Group A cell walls. In addition, the direct agglutination of Group A variant cell walls, but not those of Group A, suggests that human sera contain antibodies with specificity directed against the rhamnose moiety of the Group A carbohydrate.

Materials and Methods

Strains of Streptococci.—Streptococcal strains were obtained from Dr. R. C. Lancefield, The Rockefeller Institute.

Chemical Analyses.—Rhamnose was determined by the anthrone method of Helbert and Brown (4) and the method of Dische and Shettles (5). Glucosamine was estimated by a modification of the Elson and Morgan procedure (6).

Preparation of Carbohydrate.—Group-specific carbohydrates were prepared from Groups A (S43/100), A-intermediate (C121), A-variant (K43 var.), B (090R), C-variant (C31), C (H46A), D (H46D5), and G (B549) streptococci. Organisms were grown in Todd-Hewitt broth, collected by centrifugation, and the cell walls were prepared from the washed cells

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according to the method of Bleiweis *et al.* (7). Group carbohydrates were extracted and prepared from the cell walls by the hot formamide method of Fuller (8) as described by Krause and McCarty (9). Analyses of the carbohydrates revealed typical chemical compositions as described by Krause (10).

Preparation of Cell Walls for Agglutination.—Cell walls, devoid of other cellular elements, were prepared by the method previously described (7). These cell walls were further treated with crystalline pepsin (0.1 mg/ml) for 4 hours pH 2.0, at 37°C to remove trypsin resistant proteins. After 3 washings with sterile saline, the cell walls were resuspended in 0.2 per cent formalinized saline (pH 7.0). The cell wall suspension employed in the agglutination test had an optical density of 0.30 at 580 mμ measured in a 7 mm cuvette in a Coleman Jr. Spectrophotometer.

Antisera Preparation.—The procedure employed in the preparation of the vaccines and the immunization of rabbits were those of McCarty and Lancefield (11).

Antibody Determinations.—Streptococcal precipitin antibodies were detected by the capillary method of Swift, Wilson, and Lancefield (12).

Anti–Group A agglutinins were detected by a modified Coombs technique (13) which employs Group A cell walls. The procedure was as follows: twofold dilutions of the test serum were made so that the final volume of dilutions was 0.5 ml. To each serial dilution of serum, 0.5 ml of standard cell wall suspension was added. The mixtures were allowed to incubate for 2 hours at 37°C and then overnight at 4°C. After this fixation of the antibodies, the cell walls were centrifuged in the cold for 15 minutes at 2000 *g*. The supernatants were discarded and the packed cell walls were washed 3 times with cold saline. After washing, the coated cell walls were resuspended in 0.5 ml of buffered saline and gently mixed to obtain an even cell suspension. A drop of cell wall suspension from each dilution was delivered into the wells of a Boerner microscopic slide. To each dilution of coated cell wall suspension was added a drop of a 1:20 dilution of anti-human gamma globulin, prepared in rabbits. The mixtures were rotated gently to allow sufficient mixing and read during 15 minutes. Results were recorded as follows: 4 plus, immediate agglutination; 3 plus, agglutination after 2 minutes; 2 plus, agglutination after 10 minutes; 1 plus, weak agglutination after 15 minutes.

The direct slide agglutination of cell walls was performed in a similar manner as the indirect agglutination. Twofold dilutions were made of the test serum and single drops of each dilution were placed in the wells of a Boerner slide. To each well was added 1 drop of a standard cell wall suspension of Group A or A-variant streptococci.

Human Sera.—Sera were obtained from the Central Laboratories of Barnes Hospital, St. Louis, and the St. Louis Children's Hospital.

Separation of 7S and 19S Immunoglobulins.—Immunoglobulins were separated from serum by gel-filtration using a column of sephadex G-200 (Pharmacia, Upsala, Sweden).

Antistreptolysin O Determination.—The ASO titers of sera were performed by the method of Robinson, Crawford, and Rohalt (14).

EXPERIMENTAL

The agglutination of intact streptococci with human sera has been frequently reported (1). Because the whole cell contains a number of surface antigens, the agglutination may be dependent upon antibodies directed against one or several of these substances. In an effort to devise an agglutination technique which would detect Group A carbohydrate antibodies, the streptococcal suspensions were treated with trypsin and pepsin to remove protein antigens. Although streptococci treated in this fashion could be employed in agglutination tests for several days after preparation, spontaneous agglutination was noted

following continued storage in the cold or the frozen state. For these reasons streptococcal cell walls, which had been treated with proteolytic enzymes and RNAse and DNAse, were employed in the agglutination test. The two major components of these cell wall preparations are the group-specific carbohydrate and the mucopeptide matrix.

The specificity of the agglutination procedure was determined with rabbit grouping antisera prior to the efforts to detect group-specific carbohydrate agglutinins in human sera. Presented in Table I are direct agglutinin titers for Groups A, A-variant, C, and B antisera, and homologous and heterologous cell walls. In each instance the antisera exhibited a higher titer with homologous than with heterologous cell walls, a result comparable to the findings of

TABLE I

Direct Cell Wall Agglutination Titers with Homologous and Heterologous Rabbit Grouping Antisera

Group antiserum	Direct agglutination titer*			
	Cell wall suspension			
	A	A-variant	C	B
A	*640*	320	40	<10
A-variant	160	*2560*	160	<10
C	40	160	*640*	<10
B	<10	<10	10	*160*

* Titer is the reciprocal of the highest serum dilution which gave a 3+ or 4+ agglutionation reaction. This convention was employed for the remainder of the tables.

Cummins and Slade (15). The limited but definite cross-reactivity between Groups A, A-variant, and C, and the absence of cross-reactivity with Group B, is in agreement with previous studies on the antigenic relationships among these group-specific carbohydrates. Although Groups A, A-variant, and C carbohydrates have certain antigenic similarities, there is a fundamental difference from an immunochemical point of view between these carbohydrates and the carbohydrate of Group B.

Direct Anti-A-variant Agglutinins.—The results obtained for 32 human sera by the direct agglutination of streptococcal cell walls are depicted in Table II. Although an appreciable level of direct agglutinins was not demonstrable when Group A cell walls were employed in the test procedure, 16 of the 32 sera had agglutination titers of 20 to 320 when Group A-variant cell walls were employed. These data suggest that the direct agglutinins have a specificity which is directed against the masked rhamnose A-variant moiety of the A carbohydrate but not directed against the terminal *N*-acetylglucosaminide residues upon

which the Group A specificity is dependent. Certain additional evidence supports this hypothesis. For instance, the adsorption of human serum with Group A-variant cell walls removed the direct agglutinins, whereas adsorption with Groups A or B cell walls was ineffective. This point will be amplified in a later section.

TABLE II

Direct Groups A and A-variant Agglutination Titers of 32 Human Sera

Titer	No. of sera	
	Group A agglutination	Group A variant agglutination
<20	32	16
20	0	5
40	0	5
80	0	1
160	0	4
320	0	1

TABLE III

Groups A and A-variant Indirect Agglutination Titers of 32 Human Sera

Titer	No. of sera	
	Group A indirect agglutination	Group A variant indirect agglutination
<20	3	4
20	2	2
40	6	6
80	7	13
160	7	6
320	4	1
640	2	0
1280	1	0

Indirect Anti-Group A Agglutinins.—Although these findings suggest that human sera contain direct antibodies with specificity for the A-variant or rhamnose moiety of the carbohydrate, indirect anti-Group A agglutinins were detected by the addition of Coombs reagent to the sensitized cell walls. Tabulated in Table III are the Coombs-type indirect agglutinin titers of the previous 32 sera when tested with Groups A and A-variant cell walls. Twenty-nine sera, which gave negative direct Group A cell wall agglutination, exhibited titers from 20 to 1280 when tested by the indirect agglutination technique. While not recorded in the Table, no appreciable agglutination was detected with Groups B and G cell walls.

The specificity of the indirect Group A cell wall agglutination was substantiated by additional experiments in which the homologous reaction was inhibited by Group A carbohydrate. These data are tabulated in Table IV. Because of the antigenic similarity between the A and C carbohydrates it is to be expected that A-variant, C-variant, and C carbohydrates would have some inhibitory effect, although is is clear that the inhibitory activity of the Group A carbohydrate is more pronounced.

The gamma globulin nature of the agglutinins was confirmed by physiochemical means in the case of several representative sera. The agglutinins were

TABLE IV

Inhibition of the Indirect Agglutination between Group A Cell Walls and Human Serum by Group-Specific Carbohydrates

Carbohydrate inhibitor	Titer of indirect Group A agglutination	
	10 mg/ml	5 mg/ml
None	320	320
Group A	<10	<10
Group A-intermediate	<10	<10
Group A-variant	40	160
Group C-variant	80	320
Group C	160	320
Group B	320	320
Group G	320	320
Group D (type 1)	320	320

The serum employed in this experiment had a direct A-variant agglutinin titer of <10.

detected in the 7S fraction of serum eluted from a sephadex column, and were identified in the γ_2-region by immunoelectrophoresis.

Separation of A and A-variant Antibodies by Cell Wall Adsorption.—The separate status of the direct A-variant and the indirect Group A agglutinins was clarified by adsorption studies with Group C-variant cell walls. In these experiments the direct A-variant agglutinins were adsorbed onto Group C-variant cell walls, and were recovered by elution. The Group A indirect agglutinins remained in the serum fraction after adsorption. Group C-variant cell walls were employed because they adsorbed only Group A-variant antibodies from a serum. Group A-variant cell walls, on the other hand, contain sufficient Group A determinants to adsorb a portion of the Group A antibodies. Thus, the antibodies recovered by elution from A-variant cell walls are a mixture of A and A-variant antibodies.

The adsorption and elution procedure was as follows. One ml of human serum was added to 0.3 ml of packed C-variant cell walls. The suspension, with con-

stant mixing, was incubated for 1 hour at 37°C and overnight in the cold. The suspension was centrifuged at 2000 *g* for 15 minutes at 4°C, and the supernatant, which contains the indirect Group A antibodies, devoid of A-variant antibodies, was collected and stored in the cold. The packed coated cell walls were washed 3 times with cold saline and the bound Group A-variant antibodies were eluted from the cell walls with glycine-HCl saline buffer, pH 3.0 (16). The dissociated cell wall–antibody complexes were separated by centrifugation at 2000 *g* at 4°C. The supernatant which contained the eluted Group A-variant antibodies was neutralized with 0.1 N NaOH.

Recorded in Table V are agglutination studies on serum before and after adsorption with C-variant cell walls, and on the antibodies recovered by dissocia-

TABLE V

Agglutination Tests on Serum before and after Adsorption with C-variant Cell Walls, and on the Antibodies Recovered from the C-variant Cell Walls by Dissociation

Serum	Agglutination titer with A and A-variant cell walls			
	Direct		Indirect	
	A	A-variant	A	A-variant
Unadsorbed	<10	160	1280	320
Adsorbed with C-variant cell walls	<10	<10	320	20
Eluate from C-variant cell walls	<4	64	20	80

tion from the antibody-coated cell walls. It is obvious that the direct A-variant agglutinins were adsorbed out of the serum by the C-variant cell walls and were subsequently recovered from the cell walls by dissociation and elution. On the other hand, indirect Group A agglutinins were not removed by adsorption but remained in the serum fraction.

The specificity of the A-variant antibodies adsorbed onto C-variant cell walls, and that of the unadsorbed Group A antibodies was indicated by agglutination-inhibition tests. In these tests homologous and heterologous carbohydrates were added to the cell wall-serum mixture. The results of these experiments are depicted in Tables VI and VII. Group C-variant carbohydrate inhibited the direct agglutination between the purified A-variant antibodies and A-variant cell walls, but Groups A and G carbohydrates did not inhibit this reaction. The Group A carbohydrate inhibited the indirect agglutination between Group A antibodies and homologous cell walls, but Groups C-variant and G carbohydrates did not inhibit this reaction.

Relationship of Group-Specific Antibodies to Antistreptolysin O Antibodies.—

Although it is probable that the group-specific antibodies in human sera represent an antibody response to a streptococcal infection, it is not feasible to describe the nature of this antibody response because none of the sera studied here were collected in series on a single patient after an untreated illness. It is to be anticipated, however, that if the anti-group carbohydrate agglutinins are indic-

TABLE VI

Direct Agglutination-Inhibition of A-variant Antibodies with Formamide-Extracted Groups A, C-variant and G Carbohydrates

Cell walls	Carbohydrate inhibitor	Direct agglutination reactions					
		Concentration of inhibitor (mg/ml)					
		10.0	5.0	2.5	1.25	0.62	0.31
A-variant (T27A)	Saline	4+	4+	4+	4+	4+	4+
	Group A	2+	4+	4+	4+	4+	4+
	Group C-variant	0	0	0	0	2+	4+
	Group G	4+	4+	4+	4+	4+	4+

TABLE VII

Indirect Agglutination-Inhibition of Group A Antibodies with Formamide-Extracted A, C-variant, and G Carbohydrate

Cell walls	Carbohydrate inhibitor	Indirect agglutination with A cell walls					
		Concentration of Inhibitor (mg/ml)					
		10.0	5.0	2.5	1.25	0.62	0.31
A (S43/100)	Saline	4+	4+	4+	4+	4+	4+
	Group A	0	0	0	2+	3+	4+
	Group C-variant	4+	4+	4+	4+	4+	4+
	Group G	4+	4+	4+	4+	4+	4+

ative of a poststreptococcal antibody response, the magnitude of the titer should bear a direct relationship to that of the antistreptolysin O titer. Direct A-variant agglutinins and antistreptolysin O determinations, performed on 84 sera, and indirect Group A agglutinins and antistreptolysin O determinations, performed on 47 sera, are presented in Figs. 1 and 2 respectively. There is an obvious correlation between the magnitude of the antistreptolysin O titers and that of the direct A-variant and indirect Group A agglutinin titers. These findings are consistent with the view that anticarbohydrate antibodies are a feature of the immune response which develops after streptococcal infections.

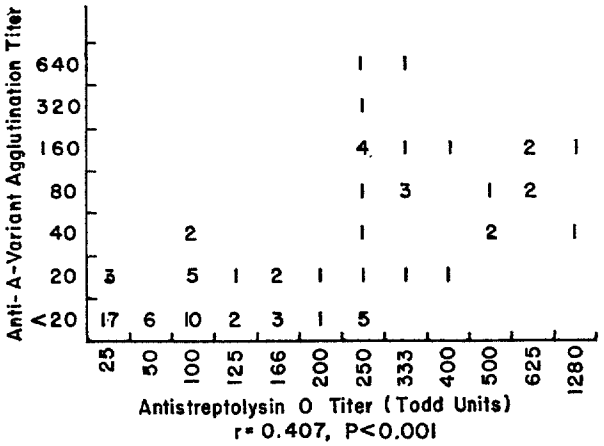

FIG. 1. The direct anti–Group A-variant agglutination titers and the antistreptolysin O titers on 84 human sera.

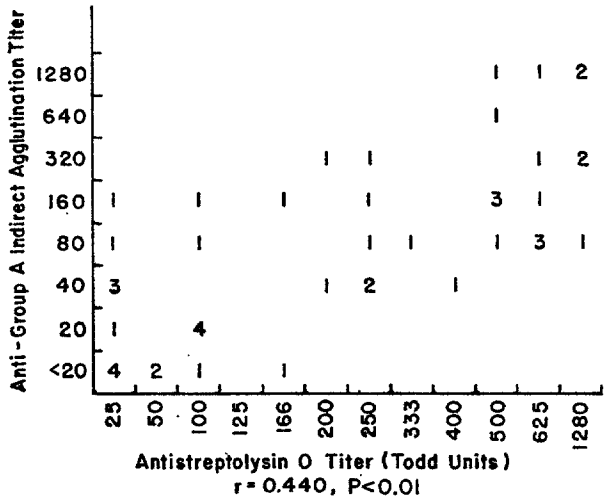

FIG. 2. The indirect anti–Group A agglutination titers and the antistreptolysin O titers on 47 human sera.

DISCUSSION

Although an antibody response following a streptococcal infection has been described for a majority of the known streptococcal antigens, little attention has been devoted to the anti-group-specific carbohydrate antibodies. Group-specific antibodies have been detected in human sera in certain cases by double diffusion in agar gel, but in general this method is not sufficiently sensitive to

detect the minimal concentrations of antibodies present in the majority of human sera (3). Recently several procedures, other than a precipitin technique, have been employed which detect anti-group-specific carbohydrate antibody. Cummins and Slade (15) described the agglutination of cell walls by homologous rabbit group-specific antisera. Schmidt (17) has detected anti-group-specific antibodies in poststreptococcal human sera with a hemagglutination technique which employs tanned red blood cells coated with group-specific carbohydrate.

The current studies were undertaken to clarify the nature of streptococcal agglutinins in poststreptococcal human sera. Rosendal (1) has directed attention to the streptococcal agglutinins in human sera, but in view of the fact that the group specific carbohydrate is only one of several surface antigens, agglutination of the whole organisms may be dependent upon agglutinins other than those with anticarbohydrate specificity. Cell walls, enzymatically treated to remove all antigens except the carbohydrate, have proved to be a useful reagent for the agglutination tests which detect agglutinin antibodies reactive against group-specific carbohydrates.

While the direct agglutination of purified Group A cell walls by human sera is not a sufficiently sensitive method to consistently detect anti-Group A agglutinins, the sensitivity of the agglutination procedure is enhanced if the cell walls, after exposure to the test serum, are treated with Coombs reagent. The inhibition of this indirect agglutination reaction with soluble Group A carbohydrate is indicative of the fact that the specificity of the agglutinins are directed against the antigenic determinant of the Group A antigen.

The frequently undetectable, or at most minimal, direct agglutination of Group A cell walls by human sera stands in contrast to the appreciable agglutination observed with Group A-variant cell walls. It will be recalled that the major difference between Groups A and A-variant carbohydrates is that in the case of Group A, *N*-acetylglucosaminide residues occupy a terminal position on rhamnose side chains, whereas in the case of Group A-variant, the rhamnose side chains are devoid of the hexosamine residues. The terminal residues confer Group A specificity onto the Group A carbohydrate, and at the same time mask the Group A-variant antigenic reactivity of the rhamnose moiety (18). The data reported here suggest that human sera contain direct agglutinins with anti-Group A-variant specificity. In this connection, it is important to emphasize that for a number of human sera the magnitude of the titers for both types of antibodies was directly related to the height of the antistreptolysin O titer. Further studies are in progress to follow the development of the agglutinins in man during the post-streptococcal period and in rabbits following immunization with Group A cell walls.

In view of the fact that the immunological reactivity of the A-variant rhamnose moiety of the Group A carbohydrate is masked by the terminal β-*N*-acetylglucosaminide residues, the stimulus for the production of A-variant antibody

must be dependent upon an *in vivo* alteration of the Group A carbohydrate. During the degradation of the streptococcus after phagocytosis it is conceivable that a β-*N*-acetylglucosaminidase removes a portion of the terminal residues, thus exposing the A-variant antigenic feature of the Group A carbohydrate (19).

SUMMARY

Two serologically distinct antibodies directed against different antigenic sites on the Group A carbohydrate were detected in human sera by agglutination techniques. Agglutinins with A-variant specificity were demonstrable by the direct agglutination of purified A-variant cell walls, whereas indirect Coombs-type agglutinins with Group A specificity were demonstrable with Group A cell walls. Selective adsorption with homologous cell walls as well as inhibition studies with the soluble carbohydrates confirmed the specificity of the two types of agglutinins.

There was an obvious correlation between the magnitude of the antistreptolysin O titer, and the direct Group A-variant and the indirect Group titers. These data suggest that indirect anti–Group A agglutinins and direct anti–Group A-variant agglutinins are produced following a Group A streptococcal infection.

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