INFECTION AND IMMUNITY, Oct. 1971, p. 441-445

Vol. 4, No. 4
Printed in U.S.A.

Micro Complement Fixation Assay for Type-Specific Group A Streptococcal Antibody

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Received for publication 13 May 1971

A micro complement fixation assay was devised to measure the type-specificity of anti-M antibody. Unabsorbed sera were from rabbits hyperimmunized with heat-killed streptococci and from humans with naturally occurring antibody or immunized with purified M protein vaccines. These sera fixed complement only in the presence of homologous M proteins (serotypes 1, 3, 6, 12, and 14). The complement fixation reaction paralleled the results obtained with the Lancefield bactericidal (opsonic) assay and did not exhibit the cross-reactions frequently seen with type-specific anti-M sera assayed by passive hemagglutination. All serological activity of the antisera resided in the fraction corresponding to 7S globulin eluted from a Sephadex G-200 gel filtration column. Because of the close correlation between micro complement fixation and the bactericidal assay for type-specific antibody, it is proposed that the micro complement fixation procedure, as we have described it, merits further evaluation as a substitute for the bactericidal test of immunity to group A streptococcal infection.

Type-specific immunity to group A streptococcal infection in man and experimental animals is directly related to levels of opsonic antibody (15). Naturally occurring or induced anti-M antibody may be assayed directly for bactericidal (opsonic) activity by passive mouse protection or in vitro by Lancefield's phagocytic bactericidal test (13). The long-chain test of Stollerman and Ekstedt (19), in which type-specific antibody stimulates long-chain growth of homologous streptococci, correlates well with high-titer bactericidal antibodies, but results with low-titer type-specific antisera often require statistical analysis for interpretation. Passive hemagglutination (HA) of tanned human erythrocytes coated with purified M proteins has been used to monitor antibody responses to M protein immunization (9, 11). However, the latter procedure reveals extensive cross-reactions among serotypes, and titers frequently do not coincide with results obtained in the bactericidal assay. Data are presented here demonstrating a close correlation between type-specific complement-fixing and bactericidal antibodies. Results indicate that complement fixation may be of value as an index of protective antibody in group A streptococcal immunity.

MATERIALS AND METHODS

Antigens. Purified group A streptococcal M proteins were prepared as previously described (7). Protein concentrations were determined by the method of Lowry et al. (17). Preparation of alum-precipitated M protein (APM) vaccines for human use was as previously described (8).

Antisera. Hyperimmune anti-M sera were prepared in rabbits by foot-pad injection of heat-killed whole cells emulsified in complete Freund's adjuvant (6). These sera were not absorbed with heterologous serotypes of streptococci. All rabbit antisera exhibited strong precipitating bands in immunodiffusion analysis with homologous purified M protein. Human sera were obtained from volunteers (laboratory personnel), and plasma was from infants recently immunized with APM vaccines (11). The plasma samples were from peripheral blood collected in preservative-free heparin. All sera and plasma samples were heated at 56 C for 30 min and then stored at −70 C.

Complement fixation assay. The method of Levine (16) was used with slight modification. The reaction mixture contained 0.5 ml of diluted M protein, 0.5 ml of diluted antiserum, 0.5 ml of complement, and 2.0 ml of diluent buffer. The diluent buffer contained 0.1% bovine serum albumin (Fraction V, Nutritional Biochemical Corp., Cleveland, Ohio), 0.01 M tris(hydroxymethyl)aminomethane (*p*H 7.4), 0.15 M sodium chloride, 5×10^{-4} M magnesium sulfate, and 1.5×10^{-4} M calcium chloride. Serum and antigen dilutions were made in this buffer. Guinea pig complement (lyophilized) and sheep hemolysin were purchased from Markham Laboratories, Chicago, Ill. The complement fixation by the M-anti-M system was carried out at 5 C for 16 to 18 hr, after which time 0.5 ml of sensitized sheep erythrocytes (5×10^6 red blood cells per ml) was added, and the mixture was incubated at 37 C for 60 min. The tubes were then centrifuged at

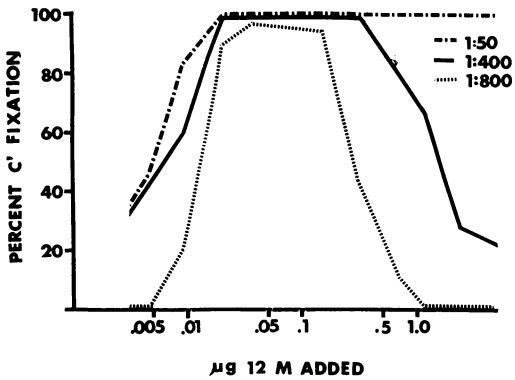

FIG. 1. *Quantitative complement fixation assay of three dilutions of rabbit anti-12M serum. Percentage of complement fixed is plotted against micrograms of M protein per 0.5 ml of reaction mixture; abscissa is log scale.*

1,000 rev/min for 10 min at 5 C, and the supernatant liquid was assayed for soluble hemoglobin in a Beckman DU spectrophotometer at an absorption of 413 nm. Controls for anticomplementary activity of antigens and antisera were included. M proteins in the absence of antibody did not inactivate complement.

Other serological assays. The bactericidal test for opsonic activity was a slight modification of the method of Lancefield (13); the procedure and scoring of the results have been described (9, 10). Passive HA was carried out by the Microtiter system with human group O erythrocytes tanned and coated with purified M proteins (10).

RESULTS

Anti-M sera were prepared in rabbits by hyperimmunization with heat-killed whole streptococci. The unabsorbed sera were reacted in the presence of complement with purified M proteins. Figure 1 shows the percentage of complement fixed with three dilutions of anti-12M serum and homologous M protein. Most hyperimmune sera diluted between 1:50 and 1:400 exhibited maximum complement fixation with M protein at concentrations ranging from 0.02 to 0.20 μg/0.5 ml of reaction mixture. For routine antiserum assays, sera were diluted 1:50 and 1:100, and antigen concentrations were selected at 0.02 and 0.05 μg/0.5 ml.

Table 1 summarizes results obtained with hyperimmune rabbit sera specific for five serotypes of group A streptococci: 1, 3, 6, 12, and 14. Homologous and heterologous reactions are compared by the three serological techniques of complement fixation, passive HA, and the bactericidal test. Considerable cross-reaction between serotypes is seen in the passive HA assay, but the bactericidal activity and complement fixation with homologous M protein are type-specific. The data in Table 1 were obtained with five individual rabbit sera; comparable results were obtained with type-specific sera from all rabbits (two or three for each serotype) immunized.

A survey of 12 normal adults (students and laboratory personnel), some of whom were known to possess naturally occurring type 3 or type 12

TABLE 1. *Type-specificity of rabbit anti-M antisera compared by microcomplement fixation, passive hemagglutination, and bactericidal assays*

M protein serotype[a]	Antiserum serotype[b]	Complement fixed (%)[c] with M protein			Passive hemagglutination (reciprocal titer)	Bactericidal index[d]
		0.12 μg[e]	0.06 μg[e]	0.03 μg[e]		
1 M	1	99	98	98	102,400	3
	3	0	0	0	1,600	0
	6	<5	0	0	6,400	0
	12	0	0	0	100	0
	14	0	0	0	6,400	0
3 M	1	5	<5	<5	12,800	0
	3	97	90	88	12,800	4
	6	0	0	0	12,800	0
	12	0	0	0	800	0
	14	0	0	0	1,600	0
6 M	1	0	0	0	25,600	0
	3	0	0	0	6,400	0
	6	52	64	0	25,600	2
	12	0	0	0	12,800	0
	14	6	10	0	1,600	0
12 M	1	0	0	0	25,600	0
	3	0	0	0	6,400	0
	6	0	0	0	25,600	0
	12	99	100	96	12,800	4
	14	0	0	0	1,600	0
14 M	1	0	0	0	25,600	0
	3	0	0	0	800	0
	6	0	8	0	3,200	0
	12	<5	0	0	200	0
	14	98	99	99	102,400	4

[a] Antigen used for complement fixation and red blood cell sensitization in passive hemagglutination.

[b] Unabsorbed rabbit serum after hyperimmunization with heat-killed group A streptococci.

[c] Antiserum diluted 1:50.

[d] Extent of in vitro opsonization in Lancefield's bactericidal test with live streptococci and anti-M rabbit sera. See reference 10 for explanation of index scale: 4 = complete phagocytosis and 0 = no significant phagocytosis when compared with nonimmune serum.

[e] Indicates micrograms of M protein per 0.5 ml.

TABLE 2. *Assay of anti-3 and -12 antibody in sera of normal adult humans; comparison of serological techniques*[a]

Subject	Type 12			Type 3		
	Passive hemagglutination (reciprocal titer)	Complement fixed (%)	Bactericidal index	Passive hemagglutination titer	Complement fixed (%)	Bactericidal index
MW	800	90	4	1,600	65	3
SF	100	0	0	400	0	0
PW	100	27	1	800	54	3
JQ	100	<5	2	800	<5	0
VT	200	<5	0	800	0	0
CP	200	0	0			
FW	800	93	4			
CB	100	<5	0			
SL	400	18	3			
TB	800	73	1			
RB	800	<5	0			
JO	400	89	1			

[a] See Table 1 for explanation of techniques.

TABLE 3. *Infant response to alum-precipitated M protein vaccines, types 3 and 6; comparison of serological data*

Vaccine serotype[a]	Subject	Serum sample[b]	Passive hemagglutination (reciprocal titer)		Complement fixed (%)		Bactericidal index	
			Type 3	Type 6	Type 3	Type 6	Type 3	Type 6
3	LA	Pre	100	400	11	0	0	0
		Post	1,600	1,600	33	0	1	0
3	SP	Pre	100	800	0	0	0	0
		Post	12,800	6,400	11	0	1	0
3	HH	Pre	100	400	0	0	0	0
		Post	51,200	25,600	59	0	2	0
3	MB	Pre	100	3,200	0	0	0	0
		Post	200	3,200	0	0	2	0
3	NEP	Pre	100	3,200	0	0	0	0
		Post	400	3,200	0	0	±	0
3	NRP	Pre	200	1,600	0	0	0	0
		Post	400	3,200	0	0	±	0
6	DL	Pre	1,600	800	0	23	0	±
		Post	12,800	3,200	0	31	0	1
6	PW	Pre	400	1,600	0	0	0	0
		Post	3,200	6,400	0	12	0	0
6	MH	Pre	100	800	0	0	0	0
		Post	3,200	12,800	0	0	0	0

[a] Three monthly injections of AMP vaccine: type 3, 35 μg of M protein combined with 2.2 mg of aluminum hydroxide; type 6, 50 μg of M protein combined with 2.2 mg of aluminum hydroxide, administered subcutaneously.

[b] Pre, preimmunization serum; post, serum sample taken 1 month after final injection.

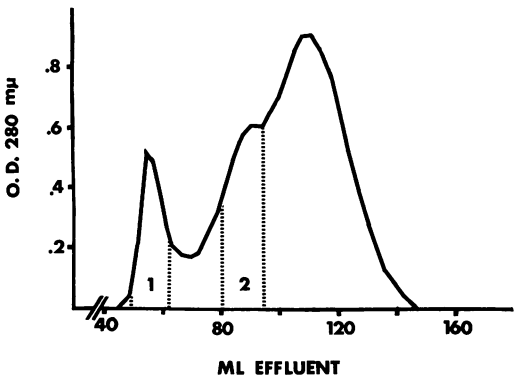

FIG. 2. *Sephadex G-200 elution pattern of human anti-12M serum from a column (2.6 by 40 cm) at 5 C. Ultraviolet absorption at 280 nm is plotted against milliliters effluent of which 4-ml fractions were collected. Areas 1 and 2 are pooled fractions containing primarily 19 and 7S immunoglobulin, respectively.*

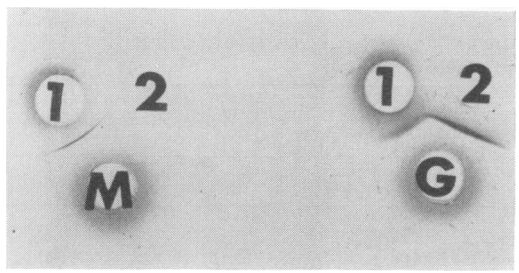

FIG. 3. *Immunodiffusion pattern of fractions 1 and 2 (latter diluted 1:10 from original serum volume) reacted with rabbit anti-human IgM and IgG, respectively. Antiglobulins supplied by Hyland Laboratories, Los Angeles, Calif.*

bactericidal antibodies, was carried out to compare the type-specific complement-fixing activity with the bactericidal test. Antisera capable of fixing approximately 20% or more of the complement were bactericidal (Table 2). One subject (JQ) exhibited some bactericidal activity for type 12 in the absence of antibody detectable in any appreciable amount by complement fixation or passive HA. This is an unusual situation for which we have no explanation. Owing to the exhaustion of our supply of purified type 3M protein, we were unable to complete the data for type 3 antibodies in seven subjects.

The serological response of nine infants between the ages of 6 and 36 months, immunized with APM vaccines types 3 or 6, are shown in Table 3. Each infant received three subcutaneous injections of vaccine at monthly intervals. The antibody levels at time zero and 30 days after the final injection are shown. It may be seen that those subjects who responded to the immunization with detectable amounts of bactericidal antibody also exhibited type-specific complement-fixing antibody. Although most of the subjects exhibited significant anti-M antibody responses to the vaccine as measured by passive HA, a high degree of cross-reaction between types 3 and 6 was seen in this test.

In view of the disparity of results found when comparing passive HA and complement fixation assays of anti-M antibody, we attempted to determine whether immunoglobulin classes IgG or IgM contained the antibodies primarily responsible for the various serological reactions. Sera from two human donors and two rabbits with high anti-12 bactericidal antibody titers were fractionated on Sephadex G-200 (12). The elution pattern for one of the human sera is shown in Fig. 2. Effluent fractions 1 and 2, containing primarily 19*S* and 7*S* immunoglobulin, respectively, were each concentrated to the original serum volume. Immunodiffusion analysis (Fig. 3) with rabbit anti-human IgG and IgM showed the extent of separation of the IgG and IgM components. Fraction 2 appeared to be pure IgG, but fraction 1, the IgM component, contained a small amount of IgG. Anti-12 antibody assay of the IgG and IgM components of four sera fractionated on Sephadex G-200 is shown in Table 4. In each case, two human sera and two rabbit hyperimmune sera exhibited type-specific complement-fixing activity only in the 7*S* fraction. Our previous studies showed that both the bactericidal and passive HA activity of rabbit anti-M immune sera also resided in IgG antibodies (8).

DISCUSSION

It was demonstrated by Bone et al. (1) that complement-fixing antibodies to M proteins could be found in the sera of glomerulonephritic patients. The antigenic reagents were crude acid extracts of M proteins from isolated epidemic strains of group A streptococci. They demonstrated that complement-fixing antibodies generally were seen in those sera exhibiting type-specific bacteriostatic

TABLE 4. *Percentage of complement fixed by Sephadex G-200 fractions of four anti-12M sera*[a]

Sample	Human serum (MW)	Human serum (FW)	Rabbit anti-12 (no. 020)	Rabbit anti-12 (no. 96)
Whole.....	75	74	85	98
Fraction 1...	0	0	0	0
Fraction 2...	79	98	99	100

[a] Whole human sera diluted 1:50 and rabbit sera diluted 1:200. Type 12M protein concentration at 0.1 μg/0.5 ml.

activity and capable of passive mouse protection. Although their antigens were unpurified and the complement-fixation test was a "macro" adaptation, their observations are consistent with the data in the present report.

The micro complement fixation test utilizing purified M proteins to assay type-specific antibody appears to reflect the bactericidal activity of anti-M antisera. In view of the acceptance of the in vitro bactericidal assay as the most convincing criterion for immunity to group A streptococcal disease (18), we propose that, if confirmed by further testing, titers of type-specific complement-fixing antibodies may also be adopted for evaluating the parameters of the immune response to group A streptococci. The long-chain test of Stollerman and Ekstedt (19) likewise has been used in various laboratories as an assay of protective antistreptococcal antibodies (2).

Micro complement fixation in the M protein system is optimal with fresh heat-inactivated serum. Sera stored for long periods of time or repeatedly frozen and thawed exhibit considerable anticomplementary activity. We extrapolate from the work of Ishizaka and Ishizaka (12) that ag-aggregated gamma globulin in old sera may be responsible for binding complement in the absence of M protein.

We have demonstrated by a variety of techniques such as immunodiffusion and cross-absorption that certain serotypes of M proteins share antigenic determinants. Cross-reactions seen in the passive HA assay probably result from these shared antigens rather than the presence of nonspecific contaminants in the purified M proteins (10). The close correlation of the opsonic and complement-fixing capacity of immune anti-M sera may reflect the ability of these assays to utilize primarily high-affinity IgG antibody directed toward the major (type-specific) determinants of M proteins. Streptococcal bactericidal antibodies are generally obtained in rabbits only after hyperimmunization (6) and in humans after infection (14) or multiple doses of M protein vaccine (11). These conditions are consistent with results obtained in other systems in which prolonged antigenic stimulation produces high-affinity antibody directed toward immunodominant determinants of antigens (3, 5). We therefore assume that these latter antigenic determinants, rather than the minor ones shared between M proteins, are mainly responsible for inducing measurable amounts of opsonic and complement-fixing antibodies.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI 04342 and a contract (PH 43-68-83) from the Vaccine Development Branch, National Institute of Allergy and Infectious Diseases.

We thank L. M. Pachman and A. Dorfman for their participation in the clinical vaccine program and Peggy Dunbar for her skillful technical assistance.

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