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Microtiter Indirect Hemagglutination Procedure for Identification of Streptococcal M-Protein Antibodies

ROBERT A. ZIMMERMAN, JAMES MATHEWS, AND ELIZABETH WILSON

Streptococcal Disease Section, National Communicable Disease Center, Public Health Service, Fort Collins, Colorado 80521

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A number of investigators have attempted to utilize the hemagglutination system for detection of streptococcal type-specific antibody in human sera. Cross-reactions have made the procedure unreliable without cumbersome and time-consuming manipulation of the test sera. A method is described in which a microtiter indirect hemagglutination technique, using sensitized sheep erythrocytes, is sensitive, specific, and reliable for titration of type-specific antibody after naturally acquired or induced streptococcal infection.

The role of group A streptococcus M antigens in virulence and the production of protective antibodies in man is well documented (6, 11). Several types of procedures have been reported for the identification of the type-specific antibody, including the mouse protection (2, 8), bactericidal (5, 10, 13), long chain (14), and hemagglutination tests (1, 3, 12, 16). The bactericidal reaction has been considered the most reliable test for determining type-specific antibodies for group A streptococci in human sera (6). However, the bactericidal and mouse protection tests have the disadvantages of being time-consuming and cumbersome, whereas the long chain procedure is less sensitive than other methods. The hemagglutination procedure is particularly sensitive, relatively easy to perform, and lends itself better than the others to the examination of large numbers of specimens. However, the hemagglutination techniques described to date have had the disadvantage of extensive cross-reactions. Although these cross-reactions have been removed by adsorption of the test sera, this added manipulation has limited the practicability of the method.

This report describes a microtiter indirect hemagglutination technique which is characterized by a high degree of sensitivity, the requirement of a small volume of patients' serum and reagents, and minimal nonspecific reactions. The procedure has been valuable in measuring host immunological response following known streptococcal infections by various group A serotypes.

MATERIALS AND METHODS

Organisms. Specific serotypes of group A streptococci isolated from individuals participating in our epidemiological studies were used for preparation of M antigen. These were chosen for their ability to produce large quantities of M protein as determined by capillary precipitin reaction (15). M types 1, 4, 5, 6, 9, and 12 are the subject of this report. A 5-ml amount of a rapidly growing 4- to 6-hr seed culture was inoculated into 1-liter portions of Todd-Hewitt broth (Consolidated Laboratories, Chicago Heights, Ill.). The flasks were incubated for 18 hr at 35 to 37 C, and cells were harvested by centrifugation with a Szent-Gyorgyi continuous-flow head.

Preparation of partially purified type-specific antigen. The antigen was extracted from the cells by the acid-heat method described by Lancefield (4). The cells were extracted twice and, when both fractions showed activity, they were pooled before the purification procedure.

Semipurified M antigens were prepared from the crude extract by a modification of the method described by Lancefield and Perlmann (7). After ribonuclease treatment, protein was precipitated by increasing concentrations of ammonium sulfate. The most reactive material was precipitated at between 30 and 70% saturated $(NH_4)_2SO_4$, depending on the M type.

Each fraction was dialyzed against borate buffer (*p*H 7.8 to 8.0) until all $(NH_4)_2SO_4$ had been removed. Total nitrogen was then determined colorimetrically by a modified micro-Kjeldahl procedure (9). Each fraction was tested for presence of M protein by the hemagglutination method. The optimal range of antigen concentration which gives maximal sensitivity was determined by titration against adsorbed homologous rabbit antiserum (obtained from the National Communicable Disease Center, Atlanta Ga.). Once this range is selected, further adjustment of antigen concentration will result in optimal specificity as determined by titration against a battery of heterologous rabbit antisera. Those fractions not yielding the desired level of sensitivity and specificity are discarded.

Sera. All sera, human, chimpanzee, and rabbit, are inactivated and adsorbed with sheep erythrocytes (RBC). Inactivation was done in a water bath at 56 C for 30 min. Adsorption was carried out in a ratio of one part of washed, packed RBC to nine parts of serum at room temperature for 20 min. All sera were diluted 1:5 in phosphate-buffered saline (PBS) before use in the test. Merthiolate was added to the diluted sera to a concentration of 1:10,000.

Buffers. The borate-buffered saline, *p*H 7.8 to 8.0, used in the salt fractionation procedure contained: boric acid, 10 g; $Na_2B_4O_7 \cdot 10H_2O$, 5.7 g; NaCl, 4.4 g, deionized water to 1 liter.

The PBS, *p*H 7.55 to 7.6, used in the hemagglutination test contained: Na_2HPO_4, 12.7 g; $NaH_2PO_4 \cdot H_2O$, 1.5 g; NaCl, 85 g, deionized water to 1 liter. The buffer was diluted 1:10 for a working solution; it must be made up fresh on the day of tests.

The quality of the deionized water used in the buffers and glassware preparation is critical. In our laboratory, we used water treated with apparatus from the Continental Water Conditioning Corp., El Paso, Tex., which yields a high quality water demonstrated by resistance of 14 to 16 million ohms.

Glassware and lucite plate preparation. All glassware used in the hemagglutination procedure was submerged in a concentrated solution of Haemosol, heated in an autoclave at 121 C for 20 min, washed in a fresh solution of Haemosol, rinsed in five changes of running tap water, rinsed in five changes of running deionized water, and then air-dried.

Lucite microtiter plates were soaked in a 5% concentration of chlorine cleaning solution for at least 1 hr, rinsed individually in running tap water, washed in a Haemosol solution, rinsed individually 10 times each in tap and deionized water, and air-dried. Absolutely clean glassware and microtiter plates are essential.

Hemagglutination technique. The RBC was collected 3 to 5 days before the test in an equal volume of Alsever's solution and stored at 4 C. RBC used before 3 days or after 5 days may give unreliable results. A 20-ml amount of the Alsever's-RBC suspension was centrifuged at 2,000 rev/min for 5 min. The packed cells were suspended in 80 ml of PBS (*p*H 7.55) and washed four times. A 1.5-ml amount of washed, packed RBC was resuspended to a 5% concentration in PBS. The suspension was warmed in a water bath at 37 C prior to tanning.

An equal volume of prewarmed, fresh tannic acid-PBS (1:10,000) solution was added to the 5% RBC suspension. This mixture was shaken in a rotary shaker-water bath at 37 C for 15 min. The suspension was centrifuged for 10 min at 1,500 rev/min, and the packed cells were washed once in warm (37 C) PBS. The RBC was resuspended to 5% in warm PBS.

A 5-ml amount of the tanned cell suspension was added to an equal volume of PBS solution containing an optimal concentration of the appropriate M antigen. This suspension was placed on a rotary shaker-water bath (37 C) for 45 min at 100 rev/min. Sensitized cells were centrifuged at 1,500 rev/min for 5 min and washed twice in warm adsorbed normal rabbit serum (1:150) in PBS for 10 min at 1,500 rev/min. The packed, sensitized cells were resuspended in warm normal rabbit serum (1:150) to approximately a 0.5% concentration. This suspension was adjusted so that a sample diluted 1:10 had an optical density of 0.40 at 490 μm at a sensitivity setting of 3 on a Beckman model B spectrophotometer. This yields a dropping solution of approximately an 0.5% concentration of RBC.

Dropping pipettes, diluting loops, and U-bottom transparent Lucite plates used in this test are manufactured by Cooke Engineering Co., San Mateo, Calif.

To each well was added 0.025 ml of 1:100 adsorbed normal rabbit serum. To the first well was added prepared serum to be tested, and twofold serial dilutions were made with the 0.025-ml diluting loop. This yields a dilution range of 1:20 to 1:40,960. Finally, 0.025 ml of the sensitized RBC dropping suspension was added to each well. The plate was gently agitated, covered with transparent tape, and refrigerated overnight at 4 C.

The control for nonspecific agglutination of unsensitized cells, run with each serum test, was done by using tanned RBC in place of the sensitized RBC.

The titer was read as the reciprocal of the dilution in the last well showing complete agglutination. There should be no agglutination in the tanned cell control.

Controls. Before the hemagglutination procedure was set up with unknown sera, the sensitized cells were tested with sera of known hemagglutination titers. Evidence that the system is working satisfactorily can be determined after 1 hr of incubation at room temperature.

The testing of unknown sera was accompanied by two sets of controls with known sera. One control was set up immediately before the unknown sera were titrated and one immediately after, to check the performance of the reagents throughout the entire procedure. The results in the two sets of controls must agree within one well of the known titer for the tests on the unknown sera to be regarded as reliable.

Hemagglutination inhibition test. The inhbitors used in the hemagglutination inhibition tests were the same semipurified M antigens used for sensitizing RBC. The inhibiting dropping solution (0.025 ml per well) contained approximately 30 μg of antigen per ml to insure maximal inhibition of high-titer homologous rabbit antiserum. After addition of the inhibitor to the test serum, the plates were agitated gently and incubated at room temperature for 30 min before the sensitized RBC were added.

Bactericidal test. The method used in this test was similar to that previously described by Lancefield (5). We employed four dilutions of *Streptococcus pyogenes* culture with controls containing type-specific rabbit antiserum or normal rabbit serum. A value of 0 to 4 was given according to the growth on each dilution plate, which ranged from no growth to more than 500 colonies with confluent hemolysis. Thus, a bactericidal index of 0 to 16 is possible with the amount of growth inhibition inversely proportional to the magnitude of the index value.

RESULTS

The sensitivity and specificity of our procedure for measurement of type-specific streptococcal antibody (TSA, type-specific antibody, as determined by the hemagglutination procedure described in this report) was maintained at a high level by selecting as antigen only those fractions containing the partially purified M protein that exhibited high homologous titer and minimal heterologous reactions with adsorbed type-specific rabbit antisera.

TABLE 1. *Titration of type 12 antigen for sensitivity against homologous hyperimmune rabbit serum*

Antigen concn[a] (μg/ml)	$(NH_4)_2SO_4$ fraction				
	30%	40%	50%	60%	70%
0.5–3.0	20 5,120 40,960	5,120	81,920	5,120	
3.1–5.0	10,240 40,960 40,960		20,480	20,480	40,960
5.1–11.0		40,960 81,920	20,480		
20.0–40.0		20,480		5,120	20,480 20,480

[a] Total nitrogen.

The first step was to determine the antigen concentration which provides maximal homologous reaction. Table 1 shows titers obtained with various $(NH_4)_2SO_4$ fractions. There was activity in all fractions, with apparent peaks related to antigen concentration as estimated by micrograms of total nitrogen. The selection of useful fractions was then based upon specificity (Table 2). There were differences in heterologous cross-reactions with two fractions of type 12. With the exception of types 31 and 39, cross-reactions were minimal at most antigen concentrations in this battery of 22 heterologous sera. With this particular type 12 antigen, titrations demonstrated that the 30, 40, and 50% fractions could be used, whereas the 60 and 70% fractions were unacceptable owing to the number of cross-reactions that could not be removed by dilution.

Specificity of the TSA was further demonstrated by the hemagglutination inhibition test. Adsorp-

TABLE 2. *Titration of type 12 antigen for specificity against heterologous hyperimmune rabbit sera*

Antiserum type	Antigen concn (μg/ml)[a]							
	40% fraction				50% fraction			
	3.3[b]	4.4	6.6	11.0	0.35	0.7	1.4	2.8[b]
1	—[c]	—	—	—	—	—	—	—
3	20	20	20	80	—	—	—	—
4	—	—	—	—	—	—	—	—
5	—	20	40	80	—	—	—	—
6	—	—	—	—	—	—	—	—
12	20,480	20,480	20,480	81,920	320	5,120	2,560	81,920
14	—	20	20	80	—	—	20	40
17	—	—	20	40	—	—	—	—
18	40	40	20	160	—	—	—	—
19	—	—	—	20	—	—	—	—
23	—	20	320	2,560	—	—	—	—
24	20	—	20	80	—	—	—	—
26	20	20	20	40	—	—	—	—
29	—	20	80	320	—	—	—	—
30	—	—	—	—	—	—	—	—
31	10,240	40,960	10,240	40,960	—	2,560	10,240	20,480
36	—	—	—	40	—	—	—	—
37	—	—	20	320	—	—	—	—
38	—	20	40	40	20	20	—	20
39	5,120	1,280	2,560	1,280	—	1,280	2,560	10,240
41	—	—	40	80	—	—	—	—
46	—	—	—	—	—	—	—	—
47	—	—	—	40	—	—	—	—

[a] Total nitrogen.
[b] Optimal antigen concentration.
[c] No detectable titer.

TABLE 3. *Effect of inhibitors on homologous type-specific antibody titers in rabbit antisera*

Type-specific rabbit antiserum[a]	Sensitizing antigen	Inhibitor				
		None	Type 1	Type 5	Type 6	Type 12
Type 1	Type 1	40,960	—[b]	40,960	40,960	40,960
Type 5	Type 5	40,960	40,960	40	40,960	40,960
Type 6	Type 6	5,120	5,120	5,120	40	10,240
Type 12	Type 12	2,560	2,560	2,560	2,560	—

[a] These rabbits had been specifically immunized with appropriate antigen for production of typing antisera supplied by National Communicable Disease Center, Atlanta, Ga.

[b] No detectable titer.

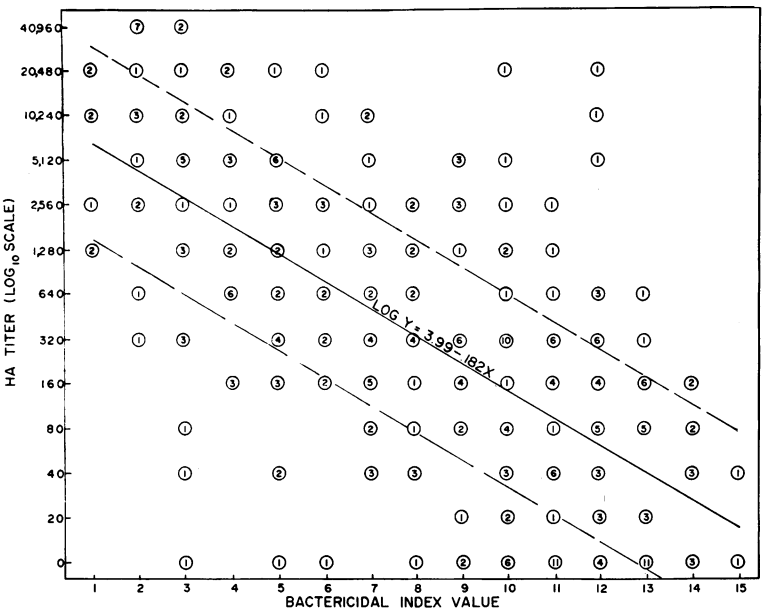

FIG. 1. *Correlation of hemagglutination titer with bactericidal activity.*

tion of type-specific rabbit antiserum with the homologous semipurified antigen preparation blocked the specific antigen-antibody reaction (Table 3).

Since the bactericidal test is considered to be the most reliable measure of type-specific antibody, we wished to determine the correlation of TSA with bactericidal activity. Figure 1 contains data relating these antibody responses in 293 patients. Coefficients of correlation were calculated between TSA titer and corresponding bactericidal index and were found to be highly significant ($P < 0.001$). Most observations fell within two standard deviations of the regression line. In general, sera with TSA titers of ≥ 160 showed bactericidal activity against the homologous serotype.

TSA response in chimpanzees after induced infection with group A type 12 is shown in Fig. 2 and 3. TSA began to develop at approximately 2 weeks after onset, as does antistreptolysin O and antihyaluronidase. However, maximal titers were reached at varying times after onset, depending upon the individual. Detectable antibodies to types 1, 4, 5, and 6 did not develop during the course of these experiments.

Some typical responses in patients after known naturally occurring infections by various group A serotypes are given in Table 4. Homologous TSA titers developed, whereas heterologous response was absent.

DISCUSSION

Many investigators have appreciated the value of measurement of type-specific antibody after streptococcal infection. Several methods have been proposed, such as mouse protection, long chain, and bactericidal tests, which have proved

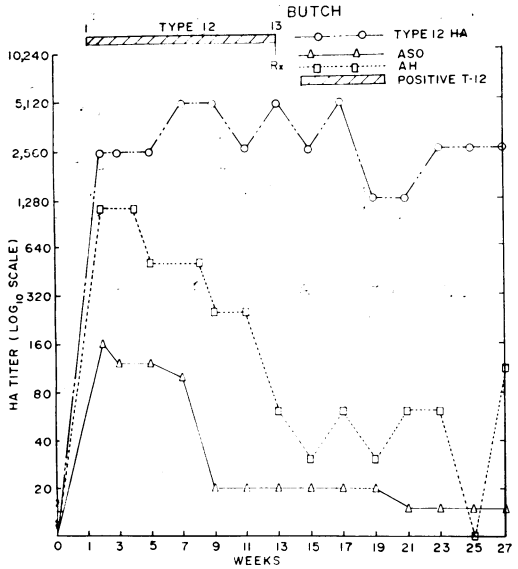

FIG. 2. *Development of streptococcal antibodies in a chimpanzee after induced group A, type 12 infection.*

to be impractical owing to their cumbersome and time-consuming nature. Most of the difficulties described by investigators attempting to utilize the hemagglutination technique for type-specific antibody titration have been due to lack of specificity. The most effective method in overcoming this obstacle has been by the application of adsorption techniques applied to the test sera. These

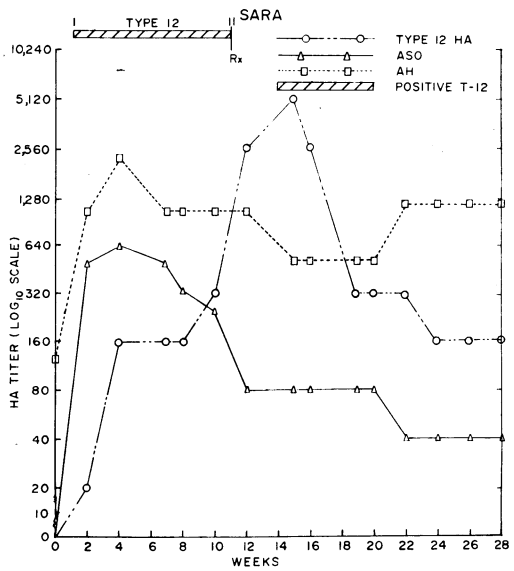

FIG. 3. *Development of streptococcal antibodies in a chimpanzee after induced group A, type 12 infection.*

TABLE 4. *Time relationship of infection by a known streptococcal serotype to specific hemagglutinin response*

Patient and nature of infection	Weeks after detection of infection		Type-specific antibody titer			
			Type 1 antigen	Type 5 antigen	Type 6 antigen	Type 12 antigen
Streptococcal pharyngitis, type 1 (patient remains chronic carrier of same serotype)	1		—[a]	—	—	40
	26		2,560	—	—	80
	78		1,280	—	20	320
	116		40,960	—	40	40
	127		10,240	—	—	—
	153		40,960	—	—	—
Streptococcal pharyngitis, type 5	Preinfection		20	40	—	—
	14		80	320	—	—
	66		80	20,480	20	80
Streptococcal pharyngitis, types 1 and 6 concurrent	Type 1	Type 6				
	23	2	1,280	40	80	40
	77	29	640	320	80	80
	113	83	80	80	1,280	40
	126	119	160	160	640	40
	138	132	160	80	640	80
Streptococcal pharyngitis, types 1 and 12 separately	Type 1	Type 12				
	30	13	320	40	20	40,960
	115	90	2,560	40	20	2,560
	129	103	5,120	160	20	5,120
	157	128	320	160	20	2,560
	185	156	40	160	40	5,120

[a] No detectable titer.

manipulations render the test impractical for use on large numbers of specimens.

Our observations show that, with relatively simple purification procedures and careful determination of optimal concentration, an antigen can be prepared which is sensitive and specific in the hemagglutination system. Although there are significant crosses of type 12, for example, with types 31 and 39 antisera (Table 2), this phenomenon does not interfere with the detection of type 12 antibody response following infection by this serotype. Comparable or better specificity is obtained with types 1, 4, 5, 6, and 9 antigen preparations.

The specificity of the TSA titer is also demonstrated by specific blocking of homologus antigen-antibody reaction after adsorption of test sera with appropriate antigen. The significant correlation of TSA titer with homologous bactericidal activity is another determinant of such specificity.

We performed thousands of TSA titrations for types 1, 4, 5, 6, 9, and 12 in our laboratory during the past year. These determinations were made on sera collected from individuals participating in our longitudinal studies as well as clinical specimens obtained during epidemic investigations. Clinical and bacteriological data are available on these persons for periods up to 5 years. Of these patients, 84% developed homologous TSA titers of $\geq$160 after acquisition of known group A serotypes. Induced infections in our studies with chimpanzees elicited similar specific-antibody response.

Therefore, we suggest that this method of TSA measurement can be used to illuminate an individual's previous streptococcal experience even in the absence of bacteriological data.

Since manipulation of the test sera beyond adsorption with sheep RBC is unnecessary, this procedure provides a practical means of titration of streptococcal TSA on the sera of large numbers of patients, by using minimal quantities of serum and reagents and providing sensitive, specific, reliable, and reproducible results.

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