Enzyme-Linked Immunosorbent Assay for Streptococcal M Protein Antibodies

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The enzyme-linked immunosorbent assay (ELISA) described by Engvall and Perlmann, which uses antigen-coated tubes and enzyme-labeled anti-immunoglobulin, has been used for the detection of antibodies against streptococcal M protein. The antigen used in the assay was obtained by guanidine extraction of type M-12 streptococcal cell walls followed by hydroxyapatite chromatography. This antigen has the capacity to elicit bactericidal antibodies in rabbits. The results show that the ELISA is specific and highly sensitive for the detection of antibodies in rabbit and human antisera. Preliminary results suggest that, when M-12 antigen is used, the antibodies detected by ELISA are the same antibodies detected in the bactericidal test. The assay has been performed with human and rabbit sera. There was a 96% agreement between bactericidal and ELISA results with rabbit sera and 97.5% agreement with human sera. All bactericidal antibody-positive sera tested thus far yielded positive ELISA results.

Among the many virulence factors possessed by group A streptococci, M protein is the most important. M proteins impede phagocytosis and elicit type-specific protective antibodies (opsonins) in humans (16). There have been several tests designed to measure the type-specific M protein antibodies; however, only the bactericidal test (14, 17) can be considered the true measure of opsonic antibodies in humans. In a recent review, Fox (10) summarizes the various tests and the pitfalls of each. Briefly, the methods are the capillary precipitin test, the *in vitro* bactericidal assay, the "long-chain" test, and the passive mouse protection test.

The standard method of measuring M protein antibody response in rabbits is a precipitin reaction, which has been found to be associated with the opsonic activity in rabbit antiserum (16). The precipitin response in humans is unsatisfactory; therefore, it cannot be used to measure opsonic antibody response. The standard bactericidal test (16) and the mouse leukocyte test, which is a modified bactericidal test (4), appear to be highly specific but are tedious and inconvenient to perform. The latter tests are therefore susceptible to laboratory error. The mechanism of the long-chain test of Stollerman and Ekstedt (22) is not well understood; however, correlation of results of this test and the bactericidal test is generally good with strong bactericidal sera but poor with weak bactericidal sera. The passive mouse protection

test, once thought to correlate with bactericidal tests, must be interpreted with care. Becker et al. (3) recently demonstrated mouse virulence factors other than M protein and hyaluronic acid capsules. The accessory mouse virulence factor possessed by group A streptococci makes interpretation of the mouse protection test for the detection of M protein antibody very difficult.

The specificity of hemagglutination (7, 20, 23, 25), complement fixation (24), agglutination of latex particles (13), and radioimmuno-electrophoresis (1, 11) techniques is dependent upon the purity of the M protein antigen used in each test. The latter tests have not received wide acceptance due to limited specificity and poor agreement with indirect bactericidal test results.

Beachey et al. (2) concluded from their studies that the complement fixation test could not distinguish between type-specific and non-type-specific antibodies. Anthony (1) cautioned that there was generally a linear relationship between the antigen-binding (radioimmunoassay) and the precipitating capacity of different rabbit sera, but a direct relationship between the antigen-binding and bactericidal activity of individual sera was not apparent. This lack of correlation was probably due to impurities in the antigen preparation used.

The newer methods of measuring M protein antibodies are less than satisfactory, especially

in terms of specificity. We wish to describe a simple, convenient, specific antibody test to measure M protein antibodies. This test is based on the enzyme-linked immunosorbent assay (ELISA) method of Engvall and Perlmann (8) with guanidine-extracted, purified M protein antigen, fraction a (21).

MATERIALS AND METHODS

Bacterial strain. Lancefield's strain 12/27/1 (CDC-SS-635) of group A streptococcus, type M-12, was used in these studies. It was obtained from the Streptococcus Section of the Center for Disease Control (CDC), Atlanta, Ga.

Isolation of antigen. The bacteria were cultured in Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) supplemented with glucose, NaHCO_3 , and Na_2HPO_4 , as described by Fox (9). The organisms were heat killed at 60 C for 2 h and harvested by centrifugation. The cells were washed twice with phosphate-buffered saline, pH 7.2, and once with distilled water. Bacterial cell walls were prepared according to the method of Bleiweis et al. (5). The M protein antigen, fraction a, was extracted from cell walls by the guanidine method and was further purified by ammonium sulfate and pH 5 fractionation and by hydroxyapatite column chromatography, as described earlier (21).

Rabbit sera. Antiserum against group A streptococcal whole cells, type M-12 specific, was the standard CDC antiserum used in the Streptococcus Reference Laboratory at CDC. It was obtained from the Biological Reagents Branch of CDC. Unabsorbed antisera against isolated fractions of type M-12 cells and cell walls were obtained by immunizing rabbits with antigen mixed with Freund complete adjuvant (21) and through the courtesy of Kenneth Vosti (Stanford University, Stanford, Calif.). Normal rabbit sera were obtained from noninfected rabbits housed in the animal facilities at CDC.

Human sera. Human sera were obtained from the Streptococcal Serology Unit of the Bacteriology Immunology Branch and from laboratory personnel at CDC.

Indirect bactericidal tests. All sera were tested for opsonic antibodies by the technique of Lancefield (14).

Preparation of enzyme-conjugated anti-immunoglobulin. Conjugates were prepared by coupling alkaline phosphatase to goat anti-rabbit and anti-human immunoglobulins essentially as described by Engvall and Perlmann (8). The enzyme preparation used was alkaline phosphatase, Sigma type VII (Sigma Chemical Co., St. Louis, Mo.; specific activity, 990 U/mg of protein). A 0.3-ml portion of the enzyme-ammonium sulfate suspension was centrifuged in the cold for 10 min at 1,000 rpm. The supernatant was discarded, and 0.1 ml (5 mg/ml) of the specific goat anti-rabbit gamma globulin or goat anti-human immunoglobulin G was added to the pellet. After dialysis overnight against phosphate-buffered saline, 10 μl of glutaraldehyde was added to give a final concentration of 0.2%. The mixture was kept at room temperature (approximately 25 C)

for 2 h and diluted to 1 ml with phosphate-buffered saline. The resulting solution was dialyzed overnight against phosphate-buffered saline. The dialysate (conjugate) was diluted to 10 ml with 5% human serum albumin in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) containing 0.001 M MgCl_2 and 0.02% NaN_3 . The conjugate was stored at 4 C.

ELISA. ELISA was performed essentially as described by Engvall and Perlmann (8). Disposable polystyrene tubes (12 by 75 mm; Falcon Plastics, Division of Becton, Dickinson & Co., Oxnard, Calif.) were incubated with 1 ml of antigen solution (0.125 to 5 $\mu\text{g}/\text{ml}$) in 0.05 M carbonate buffer, pH 9.6, containing 0.02% NaN_3 for 3 h at 37 C. Prior to testing, the tubes were washed three times with 0.9% NaCl containing 0.05% Tween 20. The washed, antigen-coated tubes were then incubated with antisera diluted in phosphate-buffered saline with 0.05% Tween 20 and 0.02% NaN_3 for 6 h at room temperature. The tubes were washed three times as before, and 1 ml of anti-immunoglobulin-alkaline phosphatase conjugate diluted in phosphate-buffered saline with 0.05% Tween 20 and 0.02% NaN_3 was added. After incubation overnight at 37 C, the tubes were washed three times with 0.9% NaCl containing 0.05% Tween 20, and 1 ml of alkaline phosphatase substrate solution, *p*-nitrophenyl phosphate (1 mg/ml; Sigma Chemical Co.), in 0.05 M carbonate buffer, pH 9.8, containing 0.001 M MgCl_2 was added. The reaction was developed at 37 C for 1 h and was stopped by the addition of 0.1 ml of 1 M NaOH. Absorbance was read at 400 nm in a Beckman DU spectrophotometer (Beckman Instruments, Palo Alto, Calif.).

Determination of optimal antigen concentration for coating tubes. Polystyrene tubes were incubated with 1 ml of antigen (0.125 to 10 μg of protein per ml) in 0.1 M carbonate buffer, pH 9.6, with 0.02% NaN_3 and were tested against a standard dilution of homologous antiserum and a 1:500 dilution of conjugate as previously described.

Conjugate dilution. A working dilution of conjugate was determined for the ELISA by using a constant amount of antigen and by varying the concentration of conjugates in the test. Five dilutions were run (1:100 to 1:500), and the highest dilution yielding an absorbance of 0.500 or greater at 400 nm after a 1-h reaction time was chosen as an acceptable working dilution. A reference antiserum, i.e., one known to contain antibodies against the test antigen, was always included to check the stability of the conjugate.

RESULTS

Effect of antigen concentration on the coating process. Sensitization was obtained with antigen concentrations of 0.125 to 4 μg of protein per ml. Maximum absorbance was obtained with an antigen concentration of 0.5 $\mu\text{g}/\text{ml}$. Higher or lower concentrations of antigen yielded lower absorbance values at 400 nm after a 1-h reaction time (Fig. 1).

FIG. 1. Effect of antigen concentration on the tube-coating process (antigen titration). Tubes were coated with solutions of antigen of different concentrations and incubated with a 1:1,000 dilution of homologous antiserum, followed by incubation with enzyme-conjugated anti-immunoglobulin. Absorbances at 400 nm were obtained after 1 h at 37 C.

Conjugate dilution. When freshly prepared conjugate was used in the ELISA it was diluted 1:500. Absorbances at 400 nm ranged from 0.500 to 1.000. Conjugates were kept at 4 C and had been used for at least 1 year. After approximately 6 months, some of the enzymatic activity was lost; however, this was compensated for by using a more concentrated solution of conjugate.

Bactericidal test. Sera that inhibited 50% of streptococcal growth (1/2 total generations) in the presence of fresh human blood were considered bactericidal. However, all sera cited as bacterial in this study yielded greater than 50% inhibition. It has been our experience that, if there is less than 50% inhibition, the results are not reproducible. To test the specificity of the bactericidal test, human and rabbit sera known to contain bactericidal antibodies against a given M-type streptococcus were run against several other streptococcal M types. There was no inhibition of growth of the heterologous M types, indicating the specificity of the bactericidal test.

Antibody detection in rabbit sera. Protein fraction a from type M-12, group A streptococcal cells elicits bactericidal antibodies in rabbits and has opsonic inhibitory activity (21). Therefore, fraction a was used in the ELISA to test for opsonic antibodies in rabbit antisera.

Fourteen rabbit sera diluted to 1:1,000 and known to contain opsonic antibodies yielded absorbance values greater than 0.200 optical density unit in the ELISA (range, 0.242 to 0.400). Indirect bactericidal tests (14) were run

simultaneously to confirm the presence of opsonic antibodies in the sera. The ELISA was declared positive for opsonic antibodies if a 1:1,000 dilution of serum yielded an absorbance value greater than 0.200 optical density unit at 400 nm after a 1-h reaction time. However, the latter was valid only if positive and negative control sera were run. The controls consisted of sera known to contain or not contain opsonic antibodies on the basis of indirect bactericidal test results. ELISA-negative sera were arbitrarily chosen as those yielding absorbance values of 0.100 optical density unit or less.

Twenty-six rabbit sera, from normal and immunized rabbits but known to be free of detectable opsonic antibodies by the bactericidal test, were tested by the ELISA. On the basis of the criteria established above, 25 of the sera yielded negative results and 1 was positive. The ELISA-positive serum was from an immunized rabbit. The discrepancy could be due to the greater sensitivity of the ELISA test.

To determine the specificity of the fraction a antigen, the ELISA was performed on 46 different group A streptococcal M-type-specific rabbit antisera (CDC M antisera). Thirty-nine of the heterologous antisera yielded negative ELISA results, whereas seven yielded questionable results. The latter antisera were cited as questionable because all yielded absorbance values greater than 0.100 (range, 0.125 to 0.290). However, four of the seven antisera yielded negative ELISA results at a dilution of 1:4,000, and the remaining three were negative at 1:8,000, 1:16,000, and 1:64,000 dilutions, respectively. Although not all sera were titrated, the CDC M-12 antisera had an ELISA titer of 128,000.

The latter three heterologous antisera, types M-21, M-37, and M-42, were run in an indirect bactericidal test with type M-12 streptococci as the test organism. Each of the three heterologous antisera had some bactericidal activity against M-12 streptococci.

Guanidine-extracted antigens from streptococcal M-types 1, 4, 12, and 14 were cross-tested by the ELISA with homologous and heterologous rabbit antisera. The tests were positive with homologous sera only. The latter results further illustrate the specificity of the tests.

Detection of M protein antibodies in human sera. The ELISA was performed with a total of 51 human sera. Indirect bactericidal tests were performed on all sera at the same time. Fifteen of the sera had opsonic antibodies, and all of the same sera yielded positive ELISA results. Thirty-six of the sera had no detectable opsonic antibodies, and 34 of the same sera yielded negative ELISA results (Table 1). There were two discrepancies between results of the op-

TABLE 1. Agreement of bactericidal test results with ELISA results

No. and source of sera	Antibody tests		Agreement (%)
	Bactericidal	ELISA	
Rabbit			
1	—	+	97.5
14	+	+	
25	—	—	
Human			
2	—	+	96.1
15	+	+	
34	—	—	

sonic antibody test (indirect bactericidal test) and the ELISA. Two of the antisera that had no detectable opsonic antibodies were positive in the ELISA. There is a possibility that the latter sera had opsonic antibodies in low concentrations, which could not be detected with the M-rich strain that was used in the bactericidal test. The agreement between the bactericidal test and ELISA was 96%.

DISCUSSION

The immunoenzyme method has been used by several investigators for the detection of antibodies against various organisms. Patramanis et al. (18) used the method to detect antibodies against *Treponema pallidum* and *Toxoplasma gondii*. Carlsson et al. (6) quantitated antibodies against *Salmonella* O antigens. Other studies include detection of antibodies against *Vibrio cholerae* (12), quantitation of immunoglobulins (8), and demonstration of tissue antibodies (19). As far as we know, there is no previous report concerning the application of this method to the detection of streptococcal M protein antibodies.

Group A streptococcal infections are still of major concern in the United States and other countries. There is a need for a sensitive and specific test to measure streptococcal M protein antibodies. The latter antibodies confer immunity in individuals once they are infected with a given M type (16). However, certain questions concerning the persistence of protective antibodies in individuals and the ability of these antibodies to confer cross-protection are yet to be answered. The bactericidal and chain elongation tests (14, 17) are highly specific, but they are cumbersome and time consuming. The most widely accepted test, the bactericidal test, requires fresh human blood, which is sometimes difficult to acquire. The blood donor must be free of existing M protein antibodies against the particular M-type streptococci under study. The strain of bacteria used in the test has to be carefully selected for its suitability, since the

test depends on the amount of M protein it produces and the potency of the antisera being tested (15).

We have evaluated the efficacy of applying the ELISA to measure streptococcal opsonic antibodies in humans and rabbits. Results are summarized in Table 1. The bactericidal test was performed on all sera to determine if there was any correlation between the type of antibodies measured in the two tests. Ninety-eight percent agreement between bactericidal antibody results and ELISA results was obtained with rabbit sera. There was a 96% agreement between bactericidal antibody results and ELISA results with human sera. There were no bactericidal antibody-positive sera with corresponding ELISA-negative results. Two sera yielded ELISA-positive results and bactericidal antibody-negative results. The latter results could possibly reflect the greater sensitivity of the ELISA test. If our results are confirmed by further testing, it is possible that the ELISA technique can be performed routinely to measure specific M protein antibodies in humans and in experimental animals. The ELISA has been performed with type M-6 and M-14 antigen and homologous antisera. Results agree closely with bactericidal antibody results. Although this is not intended to be a definitive study, preliminary results indicate that the test can be used as a potential new tool in studying the M protein immune response in humans and rabbits. The test should be expanded and performed with purified antigen from more types.

The ELISA is simple to perform. The reagents are very stable. Conjugate can be used for at least 1 year. The assay is extremely sensitive and highly specific, with the antigen being the only limiting factor. Only very small amounts of antigen are used, which makes the assay relatively inexpensive to perform. Of practical importance is that the assay requires no special equipment, and the individual performing the assay does not have to be highly skilled in laboratory techniques.

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