Assay of Antibody to Group A Streptococcal Carbohydrate by Enzyme-Linked Immunosorbent Assay

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An indirect enzyme-linked immunosorbent assay system for determination of antibody levels to the group A streptococcal cell wall carbohydrate antigen is described. Optimal conditions for antigen preparation, purification, and conjugation to poly-L-lysine for adequate adsorption to the solid phase are presented. Antibody titers of unknown sera were determined by comparison to known reference standard pool sera. A highly significant correlation ($p < 0.0001$) was found between enzyme-linked immunosorbent assay antibody titers and antigen-binding capacity in a previously described radioimmunoassay. Utilizing an isotype-specific anti-immunoglobulin reagent and immunoabsorbent-purified antibody to group A streptococcal cell wall carbohydrate antigen, we were able to detect nanogram quantities of antibody by the enzyme-linked immunosorbent assay technique. This system will provide for more generalized use of group A streptococcal cell wall carbohydrate antigen antibody determinations for the study of immune responses after streptococcal infections and their complications.

After the description of the antistreptolysin O test, several methods for measuring antibody responses to other group A streptococcal antigens were introduced. These included the anti-DNase and the anti-NADase tests (3, 24), designed to measure antibody to streptococcal extracellular products. Subsequently, Halpern and Goldstein (10) described a radioimmunoassay for antibody to a cell wall antigen, the streptococcal group-specific carbohydrate. This test has been used to measure antibody to the group A carbohydrate (A_{CHO}) in the serum of patients with group A streptococcal infections and their complications, e.g., nephritis or rheumatic fever (7).

The anti-A_{CHO} assay is a radioimmune precipitation technique requiring the use of radiolabeled, purified streptococcal cell wall polysaccharide. The need to use substantial amounts of radioactive precursors, the requirement for extensive processing to prepare small amounts of labeled antigen, and the potential for aberrant results resulting from the presence of contaminants in the antigen preparation limit the availability and the generalized usefulness of the technique. The present study was therefore undertaken to determine whether the enzyme-linked immunosorbent assay (ELISA) could offer a more practical alternative to the radioimmunoassay technique.

MATERIALS AND METHODS

Group A streptococcal carbohydrate antigen. For most experiments the antigen consisted of formamide-extracted, group-specific A_{CHO} prepared as follows. Group A streptococci were grown in Todd-Hewitt broth overnight, and the cells were collected and washed. Cell walls were prepared by Mickle disintegration and differential centrifugation by the method of Salton and Horne (20). Carbohydrate was extracted from lyophilized cell walls by the hot formamide method of Fuller as modified by Heymann et al. (11) and assayed for the rhamnose and glucosamine content as previously described (6, 19). For selected experiments, the A_{CHO} antigen was alternatively prepared by acid extraction or phage lysis of group A streptococci as previously described (13, 14) or by extraction with the *Streptomyces albus* enzyme (16), which was kindly furnished by Maclyn McCarty, Rockefeller University, New York, N.Y.

Antigen conjugation to PLL. A_{CHO}, like other bacterial polysaccharides, adsorbs poorly to plastic surfaces such as the tubes or microtiter plates used in ELISA assays, due to the net negative charge conferred by acidic groups on the antigen. To overcome this problem we conjugated A_{CHO} to poly-L-lysine (PLL), which then adsorbs to plastic supporting materials, by the method of Gray (9). Briefly, 2 mg of formamide-extracted A_{CHO} (in 0.2 ml of distilled water) was added to 1.0 ml of 0.01 N NaOH (pH 12.0) and mixed gently for 10 s. This solution was then transferred to a tube containing approximately 5.0 mg of cyanuric chloride

(Aldrich Chemical Co., Milwaukee, Wis.) and mixed gently with continuous monitoring of the pH of the solution. When the pH had fallen to 8.3 to 8.4, the supernatant was quickly transferred to a tube containing 0.2 ml of PLL (molecular weight 40,000; Sigma Chemical Co., St. Louis, Mo.), 1.0 mg/ml in 0.05 M Tris buffer (pH 8.1), avoiding transfer of any of the cyanuric chloride crystals. The final pH after this step was between 8.0 to 8.2. The solution was dialyzed at 4°C for 18 h against phosphate-buffered saline (PBS; pH 7.4). The A_{CHO}-PLL conjugate may be stored at 4°C for up to 6 months without significant loss of activity.

Buffers. PBS (pH 7.4) was found superior to bicarbonate buffer (pH 9.6) as a coating buffer for preparation of antigen-coated plates. Washing buffer consisted of PBS (pH 7.4) containing 0.05% (vol/vol) polysorbate (Tween 20; Sigma), and 0.5 M NaCl (PBS-T-NaCl). Preliminary experiments demonstrated that the PBS-T-NaCl buffer was superior to PBS alone, PBS with 0.05% Tween 20, or PBS with 0.5% Tween 20 and 0.5 M NaCl in inhibiting the nonspecific adherence of nonimmune human sera to the antigen-coated plate.

Preparation of antigen-coated plates. Flat-bottom 96-well polystyrene tissue culture plates (Linbro; Flow Laboratories, McLean, Va.) were used for the solid phase for the ELISA technique. The A_{CHO}-PLL antigen was diluted in PBS (pH 7.4) and dispensed in 0.05-ml samples into the wells of the microtiter plate. The optimal dilution of A_{CHO}-PLL for coating was determined for each conjugate preparation as described below. After incubation at room temperature (24°C) for 18 h, the plate was washed three separate times by immersion in distilled water, followed by a single 10-min soak in washing buffer. Experiments have shown that plates coated with the antigen may be stored at 4°C for a least 10 days without loss of potency.

Alkaline phosphatase labeling of anti-human immunoglobulins. The immunoglobulin (IgG) fraction of sheep antiserum to human immunoglobulins or rabbit antiserum to human IgG, gamma-chain specific (Cappel Laboratories, Cochranville, Pa.), was conjugated covalently to alkaline phosphatase (type VII, bovine intestine, 1,260 U/mg of protein; Sigma) by the one-step method of Avrameas (1). The conjugate was dialyzed extensively against PBS (pH 7.4) and then Tris buffer (pH 8.8) and stored at 4°C in the dark until used. The working dilution of the conjugate was determined by checkerboard titration against various concentrations of diethylaminoethyl-purified human IgG. Enzyme substrate consisted of *p*-nitrophenylphosphate, 1.0 mg/ml, in 0.05 M carbonate buffer (pH 9.8) containing 1.0 mM MgCl₂.

Human sera. Serum samples from normal volunteers were stored at -20°C without preservative until used. In some experiments, a reference pool of 10 sera was prepared from samples previously shown to be positive for antibody to A_{CHO} by the radioimmune precipitin assay (S. T. Shulman and E. M. Ayoub, Clin. Immunol. Immunopathol., in press).

For some experiments, purified human antibody to A_{CHO} was prepared by affinity chromatography with

an immunoabsorbent column consisting of A_{CHO} covalently coupled to Affi-Gel 10 (Bio-Rad Laboratories, Rockville Center, N.Y.) in 0.1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH at 7.5) for 4 h at 4°C. The immunoglobulin fraction of serum containing a high level of antibody to A_{CHO} (previously determined by the radioimmunoassay) was obtained by precipitation with 40% ammonium sulfate. After extensive dialysis against PBS, the immunoglobulin fraction was applied to the A_{CHO} immunoabsorbent column, and nonspecific immunoglobulin was removed by washing with 0.1 M HEPES buffer (pH 7.5). Affinity-purified antibody was then eluted from the column with 0.1 M glycine-hydrochloride buffer (pH 2.0). After dialysis against PBS (pH 7.4), the eluted fractions were assayed for antibody to A_{CHO} by radioimmunoassay. Those fractions containing antibody were then assayed for total IgG content by a competitive binding radioimmunoassay with ¹²⁵I-protein A (15).

ELISA procedure. The ELISA was performed by adding 0.05 ml of test serum or 0.05 ml of the reference

FIG. 1. Determination of serum A_{CHO} antibody level by comparison to reference serum standard. The antibody content of the test sera (log antibody titer) is expressed as the reciprocal of the dilution of the test serum that gave the same absorbance reading as 1:1,600 dilution of the reference serum standard. The log antibody titer of test serum A is 2.85, and that of test serum B is 3.85.

FIG. 2. Specificity of antibody binding to ACHO-PLL antigen. Serum A, shown to contain ACHO antibody by radioimmunoassay, was tested for binding to microtiter wells coated with the ACHO-PLL antigen (●) and to wells coated with PLL alone (○). Antibody was readily detected in the ACHO-PLL-coated wells, whereas only background binding occurred in the PLL-coated wells. Serum B (▲), which did not contain ACHO antibody, did not bind to the ACHO-PLL wells above background levels.

pool serum to the wells of an ACHO antigen-coated plate. Serial twofold dilution of the sera was achieved by adding the initial serum dilutions to the top wells in the plate, dispensing equal amounts (0.05 ml) of the PBS-T-NaCl buffer to the wells in the lower rows, and making serial dilutions with a multichanneled pipette (Titertek; Flow Laboratories, Inc., Rockville, Md.). Sera were incubated in the plates for 2 h at 37°C. The plate was then washed three times in distilled water and once in PBS-T-NaCl as described above. Next, 0.05 ml of an appropriate dilution of alkaline phosphatase-conjugated sheep anti-human immunoglobulin in PBS-T-NaCl was added to the wells and incubated for 2 h at 37°C. The plate was then washed as above, 0.1 ml of *p*-nitrophenylphosphate substrate (1.0 mg/ml) was added to each well, and plates were incubated at 37°C for 30 min. Absorbance of the substrate was measured at 405 nm in a multichanneled photometer (Titertek Multiskan; Flow Laboratories).

The absorbance of the test sera was compared with that of the serial dilutions of the reference pool serum which served as a constant internal control. A plot of optical density (OD) versus serum dilution was made for the reference pool and test sera on a semilog scale (Fig. 1). The reference pool generally gave an OD of 0.400 at a 1:1,600 dilution. The antibody content of the test serum was arbitrarily expressed as the reciprocal dilution of the test serum that gave the same OD reading as a 1:1,600 dilution of the reference pool.

RESULTS

Specificity for ACHO antigen. Preliminary studies were performed to determine the capacity of

human serum antibody to bind selectively to ACHO-PLL by the ELISA technique. Formamide-extracted carbohydrate (1 mg/ml) was conjugated to PLL (1 mg/ml). An antigen control consisting of PLL without ACHO was processed in the same manner. Microtiter plates were coated with both preparations followed by the addition of various dilutions of a reference serum previously assayed for antibody to ACHO by the radioimmunoassay and determined to have an antigen-binding capacity of 2.5. The results revealed that serum antibody bound effectively to the ACHO antigen-coated wells. The amount of antibody bound decreased with successive dilutions of the serum (Fig. 2). Only minimal background binding occurred in the wells coated with PLL without ACHO antigen. The binding of the reference serum was compared to serum shown to contain no antibody by the radioimmunoassay. OD readings for the latter serum at dilutions of 1:100 and higher did not exceed background (Fig. 2).

Antigen preparation: effect of ACHO concentrations. The concentration of the ACHO antigen needed to yield an optimal conjugate with the PLL was investigated. The rhamnose content of a formamide-extracted ACHO preparation was determined and found to be 65%. Samples (0.2 ml) of this preparation containing 1.0, 2.0, 3.0, and 6.0 mg of rhamnose per ml were conjugated with 0.2 ml of PLL (1 mg/ml) by the procedure described above. Microtiter plates coated with various dilutions (1:50 to 1:400) of the four ACHO-PLL preparations were used to assay the relative binding of the reference serum and two other sera. Binding of serum antibody increased with increasing rhamnose content of the ACHO-PLL preparations, reaching a plateau at 3.0 to 6.0 mg of rhamnose per ml (Table 1). For subsequent conjugations of the ACHO to PLL, 0.2 ml of the formamide-extracted ACHO (at 10 mg/ml), equivalent to 6.0 mg of rhamnose per ml, was used.

Source of ACHO for antigen preparations. ACHO preparations extracted by different methods were tested for their efficacy in measuring

TABLE 1. Antigen preparation and effect of ACHO concentration

ACHO concn (mg of rhamnose per ml)	OD ₄₅₀ for serum ^a (dilution):		
	A (1:640)	B (1:640)	C (1:320)
1.0	0.548	0.828	0.799
2.0	0.843	1.024	0.946
3.0	0.974	1.002	1.328
6.0	1.027	1.124	1.499

^a A, Reference serum pool; B and C, obtained from patients, were shown to contain anti-ACHO antibody by radioimmunoassay.

FIG. 3. Correlation between ELISA titer and radioimmunoassay for antibody to A_{CHO} . Sixty sera containing a wide range of antibody levels to A_{CHO} as determined by antigen-binding capacity in the radioimmunoassay were tested in the ELISA system. A comparison of antigen-binding capacity with log antibody titer in ELISA gave a correlation coefficient of 0.664 ($P < 0.0001$).

antibody by the ELISA technique. Solutions containing 1.0, 3.0, and 5.0 mg of rhamnose per ml of formamide-extracted, acid-extracted, *Streptomyces albus* enzyme-extracted, and phage lysin-extracted carbohydrates were conjugated to PLL. The standard reference pool serum was then assayed on microtiter plates coated with these A_{CHO} -PLL preparations. The different preparations yielded similar antibody titers at equal antigen concentrations, with the A_{CHO} -PLL prepared by using 5.0 mg/ml of the rhamnose yielding the higher antibody titers in all preparations.

The effect of antigen purity on antibody titers was then studied. Samples of the above A_{CHO} extracts were fractionated on a Sephacryl S-200 column, and the peak containing the antigen was concentrated and dialyzed against distilled water. Preparations containing 1.0 mg of rhamnose per ml were then conjugated to PLL. Assays of the reference serum pool with the different antigens gave titers that varied by no more than 1 doubling dilution. In general, the Sephacryl-fractionated antigen gave slightly lower antibody titers than did the nonfractionated antigen prep-

arations. This difference is probably due to the presence of small quantities of other streptococcal cellular antigenic components in the unfractionated extracts.

Correlation between radioimmunoassay and ELISA. Sixty sera that had been assayed for antibody titers to carbohydrate by the radioimmune precipitin technique were assayed in parallel for antibody by the ELISA technique. The sera were selected to represent a wide range of titers. The reference serum pool was used with each run as an internal standard. The antibody titers for the test sera (as calculated above) were compared with the antigen-binding capacity for the serum determined by the radioimmunoassay (Fig. 3). The correlation coefficient for corresponding titers in the 60 sera was $r = 0.664$, giving a T value of 6.77 and a P value of < 0.0001 .

Quantitation of A_{CHO} -specific IgG antibody. To examine the sensitivity of the ELISA and to express the results in absolute or gravimetric terms rather than relative titer values, we assayed affinity-purified A_{CHO} antibody in parallel with the reference standard pool serum by the ELISA technique. Bound IgG antibody was detected by using an isotype-specific, alkaline phosphatase-conjugated anti-human IgG reagent in these experiments. The purified antibody to A_{CHO} gave linear absorbance readings between 20 and 300 ng of IgG per ml (1.05 to 15 ng of IgG added per well) (Fig. 4). Dilutions of the refer-

FIG. 4. Quantitation of A_{CHO} -specific IgG antibody. Immunoabsorbent purified A_{CHO} antibody (●) was tested in the ELISA assay by using an alkaline phosphatase-labeled anti-IgG (gamma-chain specific) reagent. Purified A_{CHO} antibody gave linear absorbance readings between 20 and 300 ng of IgG per ml. When the pooled reference serum standard was assayed for IgG antibody (■), linear absorbance readings were obtained between dilutions of 1:200 and 1:6,400, corresponding to 20 to 200 ng of IgG anti- A_{CHO} per ml as read from the purified anti- A_{CHO} curve.

ence standard pool serum between 1:200 and 1:6,400 gave linear absorbance readings between approximately OD 0.2 and 1.0, corresponding to 20 to 200 ng of IgG anti-A_{CHO} per ml (1 to 10 ng of IgG anti-A_{CHO} per well), as read from the purified anti-A_{CHO} standard curve.

DISCUSSION

Antibodies to the A_{CHO} antigen have been detected in patients with streptococcal infections and their complications such as rheumatic fever or nephritis. Persistence of high levels of A_{CHO} antibody has been demonstrated in patients with rheumatic heart disease and has been utilized as a marker for persistent rheumatic mitral valvular disease (2, 7, 23). Previous investigators have used radioimmune precipitation (7, 10), double diffusion in gel (4, 25), or the tanned erythrocyte hemagglutination techniques (8, 12, 17, 21). The ELISA technique offers advantages over these other methods of measuring antibody to A_{CHO} in that the assay is simple to perform and utilizes reagents that are easy to prepare and are stable for prolonged periods of time. In addition, the ELISA technique avoids the biohazards and expensive detection equipment required for the radioimmunoassay.

Previous observations on A_{CHO} antibody levels in a variety of pathological conditions have given conflicting results. For example, Zimmerman et al. (25), using a radial immunodiffusion technique, were not able to confirm the persistently high A_{CHO} antibody levels in rheumatic fever patients reported by Dudding and Ayoub (7). This was probably due to the fact that results with the different assay techniques are influenced by differences in antibody isotype, affinity, and possibly subclass (5, 22). We have recently shown that there is poor correlation between A_{CHO} antibody levels determined by radioimmunoassay and those determined by radial immunodiffusion (Ayoub, unpublished data). In contrast, results in this report show that antibody titers in the ELISA system, detected by polyvalent antibody to human immunoglobulins, correlate highly with the antigen-binding capacity by radioimmunoassay. Thus, the ELISA would be an excellent alternative assay to confirm these earlier findings.

The ELISA assay described here was designed to assess the total immunoglobulin or polyvalent antibody response to A_{CHO}. Riesen et al. have suggested that human antibody to A_{CHO} is restricted to the IgG isotype and the IgG2 subclass (18). This finding can be confirmed by using a modification of our ELISA method. By altering the specificity of the alkaline-phosphatase conjugated anti-human immunoglobulin reagent, an isotype-specific or subclass-specific assay can be developed.

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