Enzyme-Linked Immunospecific Antibody Test for Detecting Antibody to *Klebsiella*

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The enzyme-linked immunospecific antibody test was performed in standard test tubes and microtiter plates to measure high-titer antibody against *Klebsiella* capsular polysaccharide. Initial studies were conducted with rabbit sera; other studies were conducted with the serum of a patient infected with type 9 *Klebsiella*. Both immunized rabbits and an infected patient disclosed high titers of anticapsular antibody. Control sera from other immunized rabbits and other infected humans failed to show this substantial antibody titer against type 9 *Klebsiella*. Comparisons between counterimmunoelectrophoresis and indirect immunofluorescence disclosed that the sensitivity of the enzyme-linked immunospecific antibody test for anti-*Klebsiella* antibody ranged between 400 and 10,000 times that of these tests.

The enzyme-linked immunospecific antibody (ELISA) test was first described by Engvall and Pearlman, in 1971, to measure immunoglobulin G (IgG) (7). The test has been used to detect antibody in parasitic diseases: malaria, toxoplasmosis, and Chagas' disease (12-14); and also in viral diseases: measles, cytomegalovirus, and rubella (11). Work directed at the detection of antibodies against bacteria with the ELISA technique has dealt primarily with *Salmonella* (2, 3). However, detection of antibody in sera of patients with bacterial disease by precipitin techniques has been successful in detecting staphylococcal endocarditis (6). Antibodies against *Pseudomonas* and *Klebsiella* have been found in sera of patients infected with these species (4, 5).

Because of these results, we elected to explore the feasibility of the ELISA test to detect antibodies to *Klebsiella*. We were particularly concerned with developing methods that allowed testing in microtiter plates with simplified methodology. Initial studies were conducted with rabbit sera. Additional studies were done on sera from infected humans.

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MATERIALS AND METHODS

Bacterial typing. *Klebsiella* organisms were typed

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by using conventional quellung reactions and Difco antisera.

Antigen. *Klebsiella* capsular polysaccharide (KCP) (type 9) was extracted from cells cultured from a patient with *Klebsiella pneumoniae* pneumonitis by a modification of the method of Campbell and Pappenheimer (1). *Klebsiella* organisms were mass cultured in Trypticase soy broth (TSB), harvested by centrifugation, washed twice in phosphate-buffered saline (PBS), lysed in 1% phenol-water, autoclaved, and centrifuged, and the supernatant was extracted with 95% ethanol, containing 50 g of sodium acetate per liter. Precipitated polysaccharide was redissolved in 5% sodium acetate, shaken with a 1/5-volume of chloroform-butanol (5:1), and dried in vacuo.

Antisera. Rabbit antisera to *Klebsiella* (Difco Laboratories, Detroit, Mich., pool three and type 9) were used. Goat anti-rabbit IgG (heavy-chain specific, lot 9195; 2.4 mg of antibody protein per ml, Cappel) and anti-human IgG (gamma-chain specific, lot 9666; 1.5 mg of antibody protein per ml, Cappel) were conjugated with alkaline phosphatase (AP).

Conjugations. The method of Engvall and Perlman was used (8). A 0.3-ml portion (1.5 mg of protein) of AP (Sigma type 7) was centrifuged for 10 min at 4,600 × g. The supernatant was discarded, and 0.2 ml of antiglobulin (0.5 mg of protein) was added and dialyzed against PBS overnight. Reagent-grade glutaraldehyde was diluted to 0.2% with water, and 80 µl was added and incubated at room temperature for 2 h. The reactant mixture was diluted to 2 ml with PBS (pH 7.2) and dialyzed against two 5-liter exchanges of PBS overnight. Conjugate was diluted to 10 ml in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0) with 0.001 M MgCl₂ and stored until needed at 4°C. Working dilutions of antiglobulin conjugate were from this 10 ml at 1:125 to 1:500 into PBS-Tween 20 (Tween 0.05%).

Substrate. *p*-Nitrophenol phosphate (NPP; 40 mg/tablet) (Sigma Chemical Co., St. Louis, Mo.) was thawed and diluted to a concentration of 1 mg/ml in 0.05 M Na₂CO₃ (pH 9.8) with 0.001 M MgCl₂.

Other serological testing. Indirect immunofluorescence (IFA) was modified slightly from the method of Goldman (9). Whole, washed cells (50 to 100 per oil immersion field) were air dried and fixed with Kirkpatrick fixative. Dilutions of sera were incubated for 45 min at 37°C and washed three times for 5 min with PBS (pH 7.2). Anti-human or anti-rabbit fluorescein-conjugated IgG was incubated for 45 min, and slides were washed three times for 5 min with PBS. Slides were prepared with Difco mounting fluid; a cover slip was added; and reading was at $\times 1,000$ on an AO Fluorestar by an operator unaware of the source of the material. Counterimmunoelectrophoresis (CIE) was performed as described by Rytel (10). Plates (1.25 by 3.25 inch; ca. 31.75 by 82.55 mm) contained 1.0% agarose and barbital buffer (pH 8.6) ($I = 0.05$). Wells were 4 mm in diameter and 3 mm apart, edge to edge. Plates were subjected to electrophoresis at 45 V and 8 ml for 45 min.

Sera. Normal sera were obtained from 9 laboratory personnel; control patient sera were obtained from 10 patients with bacteremia as follows: *Bacteroides fragilis*, 2; *Escherichia coli*, 3; *Pseudomonas aeruginosa*, 2; *Haemophilus influenzae*, 1; *Streptococcus pneumoniae*, 2. Human sera were collected with informed consent from a patient with clinical and microbiological evidence of bacteremic type 9 *K. pneumoniae* pneumonitis, as well as from three patients with other *K. pneumoniae* infections. Two patients had pulmonary infections, and one had spontaneous peritonitis.

Testing. Testing was in polystyrene tubes (10 by 75 mm; Kimble) and in polystyrene microtiter plates (Cooke Engineering Co., Alexandria, Va.). KCP was diluted to varying concentrations (25 to 100 μ g/ml) and passively adsorbed for 3 h in 0.05 M carbonate buffer, pH 9.6. Tubes received 1 ml and wells 0.25 ml of KCP. Both were emptied by aspiration, and 1 ml or 0.25 ml of bovine serum albumin (BSA) was added to tubes or wells, respectively, at concentrations of 50 to 10,000 μ g/ml in 0.05 M carbonate buffer, pH 9.6, and 0.02% sodium azide. After a 2-h incubation, the tubes or wells were aspirated and washed three times with PBS. Initially, type-specific or pooled rabbit antiserum was diluted, added (tubes, 1 ml; wells, 0.25 ml), incubated for 2 h, and bound to the KCP. In other studies, human serum was handled similarly. Tubes or wells were aspirated and washed three times with a PBS-Tween 20 mixture containing 0.05% Tween. Goat anti-rabbit IgG AP conjugate or rabbit anti-human IgG AP conjugate was diluted to a suitable working concentration, and 1 ml was added to tubes or 0.25 ml to microtiter wells. Working concentrations were determined by visual assessment of modest yellow color in tubes or wells sensitized directly with rabbit or human IgG. Reactants were aspirated, wells and tubes were washed three times with PBS-Tween (pH 7.2), and AP substrate (*p*-nitrophenol phosphate) was added, 1 ml to tubes and 0.25 ml to microtiter wells. Incubation was at 37°C for 100 min, and the resulting colorimetric reaction was monitored at 400 nm and visually. Reaction was stopped by addition of 1 N

NaOH; 100 μ l was added to tubes, 25 μ l to wells. Optical density was assessed in a Coleman II Junior spectrophotometer in small cylindrical cuvettes (light path, 2 mm).

RESULTS

Initial studies showed that 50 μ g of KCP per ml gave satisfactory antigen binding. Increasing the concentrations to 100 μ g/ml did not significantly change subsequent optical absorption, whereas decreasing the concentrations to 25 μ g/ml resulted in substantially less absorption. A 50- μ g/ml concentration was used in all further tests. Other studies determined that working antibody conjugate dilutions of 1:500 were less active. Working dilutions of 1:250 were used throughout. The need for vigorous washing procedures, as well as the necessity to aspirate reactant materials, was repeatedly confirmed. BSA concentrations were studied holding other reactants constant. When large concentrations of BSA (used to stop nonspecific binding) were studied, enzymatic activity decreased. This suggests that high concentrations of BSA may compete with previously adsorbed KCP and displace it. Whereas these changes were not large, 50 μ g of BSA per ml was used in subsequent experiments. When no BSA was used at this step, absorption was subsequently considerably higher, suggesting that either or both initial antiserum or subsequent antiglobulin conjugate was nonspecifically adsorbed to the polystyrene surface. The net result of either was localization of non-immunologically mediated enzyme and false positive reactions.

The ELISA test was used to determine the titer of specific antibody in rabbit antiserum. Results are displayed in Fig. 1 that show optical density with antiserum against type 9 *K. pneumoniae*. Pool three antiserum (against types 8, 9, and 10) was one order of magnitude less active (not shown). Optical density correlated well with

FIG. 1. Optical density measuring bound antibody against *Klebsiella* type 9 antigen with type 9-specific and heterologous antisera.

simple visual assessment. Control heterologous rabbit antisera (anti-pneumococcal omniser) appear on the same graph. Other tested, pooled anti-pneumococcal antisera were similar. Results were achieved consistently with both tubes and microtiter plates. When the reaction was repeated several months later with the same reagents, essentially identical figures were reproduced.

Human study results are presented in Fig. 2. Solid squares represent dilutions of serum from a patient with bacteremic pneumonia caused by *K. pneumoniae* type 9. Results presented are mean optical density of four such tests; vertical bars disclose the standard error of the mean. Control sera from 9 normal persons and 10 bacteremic patients are presented in closed circles and open squares, respectively; results presented are mean optical density for each group; vertical bars indicate the standard error of the mean. All tests were run in duplicate. Sera from three patients with *Klebsiella* infections, not type 9, were also analyzed with type 9 KCP but are not presented graphically. One serum (patient infected with type 8) had a titer of 1:512; the other two (patients infected with unknown types from pools 7 and 10) had titers of 1:64 and 1:128, respectively. The above *Klebsiella* type 9 rabbit antiserum and human serum were also studied by IFA staining and by conventional CIE methods. Rabbit anti-*Klebsiella* type 9 antiserum had

a maximum titer of 1:64 by IFA and 1:32 by CIE; the tested human serum had titers of 1:64 by both techniques. The ELISA test detected antibody from 400 to 10,000 times these dilutions. As described and implemented in our laboratory, the ELISA, CIE, and IFA tests all require from 2 to 3 h; thus, the increased sensitivity of the ELISA method is not related to longer incubation.

DISCUSSION

Though our initial studies were performed with polystyrene tubes, results were equally satisfactory with microtiter plates, and subsequent experiments were conducted with these. The small volume of the microtiter plates necessitated a very short light path (2 mm maximum) on our modified spectrophotometer; a conventional 10-mm light path would be more desirable.

The ELISA test was proven able to discern high-titer antibodies against *Klebsiella* type 9. Minimal dilutions of normal human sera were remarkably free of this antibody. Similarly diluted sera from patients with other active bacterial infections were also free of this type-specific antibody.

Two of the sera from three patients with *Klebsiella* infections, not type 9, had increased antibody for the type 9 antigen. This antibody activity probably represents cross-reactivity to the

FIG. 2. Binding of human serum from *Klebsiella*-infected patients compared to sera of normal controls and of other bacteremic patients.

type 9-specific antigen. Increasing the number of antigens to include strains commonly encountered in clinical practice may allow detection of *Klebsiella* infection in patients. This might be immensely helpful in sorting out those patients who are colonized from those who are infected with this frequent nosocomial pathogen.

The advantages for the method presented are obvious and include: (i) reagents are long-lived, maintaining enzymatic activity; (ii) expensive monitoring equipment used in other antibody tests is not required; (iii) materials are inexpensive and easily disposable; (iv) substantial dilutions of serum are allowed by the sensitivity of the test; and (v) the probability of automation is high.

We believe that the ELISA test lends itself to the relatively rapid screening of sera for the presence of specific antibodies and that the test is semiquantitative. We feel that additional studies are warranted and that antibody-mediated detection of bacterial diseases in humans using the ELISA technique is probable.

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