Detection of *Pseudomonas aeruginosa* Antigenemia in Granulocytopenic Rabbits by Radioimmunoassay

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We assessed the ability of a solid-phase radioimmunoassay, modified to allow antigen detection in serum, to detect circulating antigens in granulocytopenic rabbits with *Pseudomonas aeruginosa* bacteremia. In vitro experiments with *Pseudomonas* lipopolysaccharide indicated that treatment of serum-lipopolysaccharide mixtures with heat, chloroform, or heparin improved the sensitivity for detecting lipopolysaccharide 8- to 16-fold. Chloroform treatment permitted antigen detection in serum or plasma of bacteremic rabbits in which antigen could be detected poorly or not at all in untreated specimens. However, chloroform-treated specimens occasionally caused visible dissolution of plastic tubes, resulting in nonspecific binding of 125I-labeled anti-*Pseudomonas* immunoglobulin G. Heating sera at 56°C for 30 min improved antigen detection both in lipopolysaccharide-serum mixtures and in bacteremic rabbits. Antigen was detected in the heated serum or plasma of 20% of 20 culture-positive granulocytopenic rabbits, none of 15 culture-negative granulocytopenic rabbits, and none of 35 normal rabbits. Antigen was detected in none of 15 rabbits with 2 to 300 colony-forming units of *P. aeruginosa* per ml of blood and in 4 of 5 rabbits with >10⁵ colony-forming units per ml. We conclude that circulating antigens are present in the blood of rabbits with high-level *P. aeruginosa* bacteremia and that these antigens can be detected by solid-phase radioimmunoassay. Further improvements in assay sensitivity will be required to detect antigens, if present, in animals with lower levels of *P. aeruginosa* bacteremia.

*Pseudomonas aeruginosa* remains an important human pathogen, especially in hospital-acquired infections and in infections acquired by granulocytopenic patients (2, 4). A noncultural rapid method for diagnosing these infections should have important applications. First, early institution of specific antibiotic combinations, such as tobramycin and ticarcillin, appears to improve survival in *P. aeruginosa* infections (1, 17). Such combinations are probably not optimal for certain other pathogens such as *Klebsiella pneumoniae* (8). Thus, a rapid diagnostic test would allow proper selection of initial antibiotic therapy. Secondly, in nosocomial pneumonias, a large variety of organisms must be suspected as etiologies (2, 12). Sputum culture results are usually difficult to interpret since upper airway contaminants may grow in addition to the true pathogen(s). Detection of circulating *P. aeruginosa* antigens in such a patient would suggest the presence of large numbers of *P. aeruginosa* in the patient, implicating it as a true pathogen. We, therefore, developed a solid-phase radioimmunoassay for detecting *P. aeruginosa* antigens (7). The assay was useful for detecting antigens in crystalloid solvents, including urine (7), but not in serum. We have subsequently found methods to improve the sensitivity of the assay for detecting antigens in serum. We report here an evaluation of this assay, modified to improve antigen detection in serum, for detecting antigens in granulocytopenic rabbits with *P. aeruginosa* sepsis.

MATERIALS AND METHODS

Solid-phase radioimmunoassay. The basic radioimmunoassay technique has been described in detail elsewhere (7). In brief, 0.2-ml portions of immunoglobulin G (IgG) antibody, 20 μg/ml in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.0, were incubated in polystyrene tubes for 1 h at 37°C and then aspirated. The IgG was obtained from a rabbit immunized over a 12-week period with serotype 6 *P. aeruginosa* organisms. Next, 5% bovine serum albumin in 0.01 M Tris-hydrochloride, pH 7.0, was similarly incubated and aspirated. Next, 0.2 ml of test sample, either antigen-solvent mixtures prepared in vitro or serum from infected or control rabbits, was incubated for 1 h and aspirated. Finally, rabbit anti-*P. aeruginosa* IgG, labeled with 125I, was incubated for 1 h and aspirated. Specimens were tested in triplicate. Results (mean ± standard deviation) were expressed as nanograms of 125I-labeled IgG antibody bound to the tubes after the final step of the assay.
Chloroform treatment of serum. Some specimens were tested in the radioimmunoassay after chloroform extraction as modified from Levin et al. (9). One part chloroform was mixed with four parts serum, blended vigorously in a Vortex mixer for 1 min, and then centrifuged for 10 min on a table top centrifuge. The cloudy, upper, aqueous layer was then tested in the assay.

Experimental P. aeruginosa sepsis. A previously described rabbit model was used (21). To induce granulocytopenia, rabbits were injected intravenously with nitrogen mustard, 3 mg/kg, on day 1. Beginning on that same day, the rabbits were given drinking water and daily eye drops containing serotype 6 P. aeruginosa organisms. Procaine penicillin G, 625 mg intramuscularly daily, was used to prevent Pseudomonas multocida infections. Beginning on day 3 the rabbits were bled twice daily by ear venipuncture. Blood was cultured qualitatively and, usually, quantitatively; remaining blood was frozen as serum or plasma for later testing in the radioimmunoassay. Rabbits appearing moribund were exsanguinated by cardiac puncture, and the blood was handled similarly. Control samples consisted of serum from normal rabbits; culture-negative specimens from granulocytopenic rabbits were used as additional controls.

P. aeruginosa lipopolysaccharide. The lipopolysaccharide antigen used in these experiments was prepared by hot phenol-water extraction and ultracentrifugation as described by Westphal and Jann (20).

RESULTS

Lipopolysaccharide detection in serum and in saline. Serotype 6 P. aeruginosa lipopolysaccharide was dissolved in either 0.1 M Tris–0.15 M NaCl or normal human serum. Whereas the lipopolysaccharide detection limit in buffered saline was 0.05 μg/ml, the detection limit in serum was only 10 μg/ml (Fig. 1). Thus, serum dramatically inhibited detection of lipopolysaccharide in the assay.

Attempts to diminish the inhibitory effects of serum on antigen detection. Initial attempts to eliminate the inhibitory activity of serum were based on Levin’s observations that detection of lipopolysaccharide with the Limulus lysate assay is enhanced by using plasma, rather than serum, as the test medium and that chloroform extraction of plasma further enhances the sensitivity of the assay (10). Chloroform treatment of serum-lipopolysaccharide mixtures enhanced lipopolysaccharide detection fourfold (Fig. 2). Also, the use of plasma as the lipopolysaccharide solvent, rather than serum, improved the sensitivity. It was found, however, that addition of heparin to serum resulted in an eightfold enhancement of sensitivity (Fig. 2). Since this was similar to the sensitivity with lipopolysaccharide dissolved in plasma (data not shown), it appeared that the presence of the anticoagulant, heparin, rather than the absence of consumed clotting factors, accounted for the improved sensitivity in plasma. Heating the serum-lipopolysaccharide mixtures at 56°C for 30 min also improved the sensitivity for antigen detection approximately 16-fold over untreated serum (Fig. 2). Thus, chloroform extraction, heparin, or heating partially eliminated the inhibitory effect of serum on antigen detection.

Detection of P. aeruginosa antigens in granulocytopenic rabbits. Plasma specimens of P. aeruginosa lipopolysaccharide was dissolved in normal human serum or 0.1 M Tris–0.15 M NaCl, pH 8.0, and tested in the solid-phase radioimmunoassay. The detection limit in serum was 10 μg/ml, and that in buffered saline was 0.625 μg/ml. Bar = ± 1 standard deviation of triplicate tubes. Units of the y axis represent nanograms of 125I-labeled anti-P. aeruginosa IgG bound to tubes after the final step of the assay.

Fig. 1. Comparison of lipopolysaccharide detection in serum versus saline. P. aeruginosa lipopolysaccharide was dissolved in normal human serum or 0.1 M Tris–0.15 M NaCl, pH 8.0, and tested in the solid-phase radioimmunoassay. The detection limit in serum was 10 μg/ml, and that in buffered saline was 0.625 μg/ml. Bar = ± 1 standard deviation of triplicate tubes. Units of the y axis represent nanograms of 125I-labeled anti-P. aeruginosa IgG bound to tubes after the final step of the assay.

Fig. 2. Attempts to improve antigen detection sensitivity in serum by radioimmunoassay. Serum-lipopolysaccharide mixtures were tested by heating at 56°C for 30 min, adding heparin (1,000 U/ml), or treating with chloroform, and then tested in the radioimmunoassays. The sensitivities after these treatments were as follows: no treatment, 10 μg/ml; heat, 0.625 μg/ml; heparin, 1.25 μg/ml; chloroform, 2.5 μg/ml. Symbols and units are as in Fig. 1.
from eight culture-positive granulocytopenic rabbits, eight culture-negative granulocytopenic rabbits, and 10 normal controls were tested in the assay after treatment with chloroform. As shown in Fig. 3, three of eight specimens from culture-positive rabbits produced higher results in the assay than specimens from culture-negative or normal control rabbits. However, it was noted that occasional assay tubes became visibly distorted during incubation of chloroform-treated plasma, resulting subsequently in increased binding of $[^{125}I]IgG$ to the tubes. This effect was probably due to residual chloroform in the plasma causing partial dissolution of the polystyrene tubes. Neither prolonging the centrifugation to 1 h to improve separation, placing the treated specimens in a vacuum concentrator to selectively evaporate chloroform, nor attempting to absorb out the chloroform with paraffin eliminated the residual chloroform. Chloroform treatment, therefore, was not further evaluated.

Subsequent rabbit specimens were tested after heating for 30 min at 56°C. Of 20 culture-positive granulocytopenic rabbits so tested, 20% were positive in the radioimmunoassay (Fig. 4). None of the 53 rabbits in the two control groups was positive. Quantitative cultures were performed on blood from 17 of the 20 culture-positive rab-

bits. Of five rabbits with $10^3$ or more colony-forming units per ml of blood, four were positive in the assay (Fig. 5). Colony counts ranged from 2 to 300/ml in the remaining 12 rabbits, none of which was positive. Thus, antigens were detected in animals with overwhelming bactere-

mia.

We have previously shown that the radioimmunoassay distinguishes $P. aeruginosa$ antigens from other bacterial antigens both in broth culture supernatants and in urine from bacteriuric patients (7). As an additional test of specificity, serum from an antigen-positive bacteremic rabbit was absorbed with gamma globulins, prepared by ammonium sulfate precipitation, from rabbits hyperimmunized with sonic extracts of an Escherichia coli isolate and of a Salmonella minnesota Re mutant strain. As shown in Table 1, absorption with the anti-$S. minnesota$ gamma globulin did not inhibit detection. Absorption with the $E. coli$ gamma globulins slightly diminished the amount of antigen detected, but not to the degree seen with the anti-$P. aeruginosa$ gamma globulin. Thus, the antigen detected was of bacterial origin and not rheumatoid factor or similar activity.

**DISCUSSION**

Our initial attempts to detect antigens by solid-phase radioimmunoassay indicated that, although $P. aeruginosa$ urinary tract infections could be detected with ease (7), components of human serum markedly inhibit antigen detection. Lipopolysaccharide was used as the test antigen because it is relatively simple to purify...
and seemed a reasonable candidate for detection in septic humans; patients with *P. aeruginosa* sepsis may have a positive *Limulus* lysate assay result (9). Serum components inhibit detection of lipopolysaccharide in the *Limulus* lysate assay (10). Chloroform, which diminishes inhibition in the *Limulus* lysate assay, also partially diminished inhibition in the radioimmunoassay. The mechanism for the effect of chloroform is not clear, although elimination of protein inhibitors by denaturation seems likely. Unfortunately, after blending the serum/chloroform mixture in a Vortex mixer, enough chloroform remained in the aqueous phase to cause distortion of occasional polystyrene assay tubes. Alternative means of removing inhibitory effect of the serum were sought.

Using plasma, rather than serum, as the lipopolysaccharide solvent, improved the sensitivity of the assay. A similar effect is known for the *Limulus* lysate assay (10). However, we subsequently noted that addition of heparin to serum was as effective as using plasma as the lipopolysaccharide solvent. Since heparin inhibits complement activation (11, 19), heating serum at 56°C for 30 min was tried and found to be equally effective. We have subsequently found that detection of staphylococcal and hepatitis B surface antigens by solid-phase radioimmunoassay is also improved by heating at 56°C (18).

The inhibitory effect of complement is at least partially due to activation of the complement system by the IgG-coated tubes (18), probably resulting in coating of the IgG by complement components, thus rendering them unavailable for attachment to antigens. This inhibitory effect would probably apply to any antigen detection system which uses an antibody-coated solid phase. Additionally, some antigens, including lipopolysaccharides (5, 14), may themselves activate complement, resulting in a complement coat which interferes with binding to antibodies.

Using 56°C heated test specimens the radioimmunoassay detected circulating antigens in four of five granulocytopenic rabbits with 2,400 to 30,000 bacteria per ml of blood. Since the assay does not detect antigens in the broth of growth-phase cultures until >10³ organisms are present (6), it seems unlikely that the antigens detected were strictly cell associated. Growing bacteria are known to release cell wall materials into the growth medium (13). It seems likely that numerous metabolically active bacteria are required to produce these materials in sufficient quantity to be detected in the assay, since the body rapidly clears at least some bacterial components (15). It is possible that a different infection model consisting of local collections of large numbers of bacteria, such as pneumonia or abscesses, would have resulted in more antigen production at lower levels of bacteremia.

Whether failure to detect antigens in rabbits with fewer than 10³ bacteria per ml of blood represents effective host clearance of antigen or inadequate assay sensitivity is unclear. The test antigen, *P. aeruginosa* lipopolysaccharide, was detectable in heated serum at 0.625 µg/ml. Lower levels of circulating bacterial antigens have been detected in certain other infections (3, 5, 16). Improvement of the sensitivity of the assay would probably allow detection of antigens in more animals. However, residual inhibitory activity of serum persists, even after heating at 56°C, addition of heparin, or treatment with
chloroform. Since the assay in its present form has demonstrated the presence of detectable circulating antigens in *P. aeruginosa* sepsis, further efforts are being expended in our and other laboratories to improve the sensitivity of the assay.

Finally, although the test antigen used to develop this assay is lipopolysaccharide, we do not know the identity of the antigen(s) present in the bacteremic rabbits. The antisera used in the assay was elicited by immunization with whole *P. aeruginosa* organisms. Analysis of this serum by crossed gel electrophoresis with a sonic extract of the serotype 6 *P. aeruginosa* organisms as antigen revealed at least 14 precipitin arcs. Thus, the assay system is theoretically capable of detecting many different components of the *P. aeruginosa* organisms in addition to the lipopolysaccharide. Knowledge of which component(s) were detected should allow improvement of the sensitivity of the assay by using monospecific antibodies as the assay second antibody. Experiments to identify the antigen(s) are in progress.

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