Solid-Phase Radioimmunoassay with Protein-A-Bearing Staphylococcus aureus Cells Used To Assay a Protein (Ferritin) and a Hapten (Digoxin)

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We compared use of protein-A-containing Staphylococcus aureus bacteria with conventional ammonium sulfate precipitation and second-antibody methods of separating bound and free antigen in the radioimmunoassay of a hapten (digoxin) and protein (ferritin) in human sera. In each case, values obtained with the heat-killed, formalin-fixed bacteria correlated well with those found by established methods. No matrix effects were detected in either hapten or protein measurements. Because of the affinity of S. aureus for rabbit IgG, rabbit antisera could be used with a small number of bacteria to detect antigen in the presence of 50-fold excess human IgG. The availability of S. aureus and ease of handling make this reagent a rapid, economical alternative of general applicability in radioimmunoassay.

Additional Keyphrases: intermethod comparison • separation of bound and free antigen

With the development by Yalow and Berson (1, 2) of the procedures of radioimmunoassay, it is possible to detect many of the proteins and drugs, present in low concentrations in plasma, that are of biological and clinical interest. One of the difficult steps in any radioimmunoassay is the separation of free ligand from bound. Approaches previously described in the literature include the use of salting-out techniques (e.g., with ammonium sulfate), activated charcoal adsorption, and, more recently, the use of a second antibody (3). All of these methods have inherent difficulties in the separation phase and in the disturbance of the antigen/antibody equilibrium such as occurs with activated charcoal and ammonium sulfate.

Jensen (4, 5) noted that most Staphylococcus aureus bacteria contain an antigen that interacts specifically with human serum immunoglobulin G (IgG). This interaction has been attributed to “protein A,” a protein on the cell wall of the bacteria (6, 7). Forsgren and Sjoquist (8) and Kronvall and Frommell (9) showed that protein A reacts specifically with the Fc portion of the IgG molecule; phylogenetic studies (10, 11) indicated that a variable portion of the serum IgG of most mammalian species exhibited reactivity with the protein A. However, the ability of IgG to combine with protein A is restricted to particular subclasses, such as IgG1, IgG2, and IgG4 in humans (12); IgG2a, IgG2b, and IgG3 in mice (13). The percentage of protein A-reactive IgG in sera ranges from well over 90% in rabbits (11, 14) and humans (14) to a much smaller proportion in sheep (11).

These and related properties make chemically modified protein-A-bearing staphylococci the ideal type of solid-phase adsorbent for antigen/antibody complexes. It has been utilized in antigen isolation studies (15-18) and has been shown to be useful in the separation of bound and free portions in a radioimmunoassay for alpha-fetoprotein (19).

We attempted to use S. aureus—Cowan strain 1 as a general solid-phase reactant in radioimmunoassay. The bacteria can be grown in large quantities and could be a less expensive alternative to the second-antibody approach. We tested the general applicability of this method by assessing the performance of S. aureus in the radioimmunoassay of a protein (ferritin) and a hapten (digoxin) of low relative molecular mass.

During the preparation of this manuscript, a publication by Gupta and Morton (20) confirmed the applicability of this approach, demonstrating excellent correlation with the second-antibody method for the measurement of human serum albumin.

Materials and Methods

Growth of S. aureus

The method of growth was adapted from Kessler (18). The protein-A-bearing strain of S. aureus—Cowan I (ATCC 12598), was obtained from Dr. H. Richardson, Department of Pathology, McMaster University.

After killing the bacteria by heating, we stored the suspension (100 g/L) at 4 °C or −20 °C in phosphate-buffered saline (150 mmol/L NaCl, 40 mmol/L phosphate, pH 7.2) containing 0.5 g of sodium azide per liter.

Immediately before use, the bacteria were pelleted by centrifugation at 2000 × g for 30 min at 4 °C. It was resuspended and incubated in “NET” buffer [pH 7.4, containing, per liter, 150 mmol of NaCl, 5 mmol of ethylenediaminetetraacetate, 50 mmol of tris(hydroxymethyl)aminomethane, and 0.2 g of sodium azide] 5 g of Triton X-100 per liter for 15 min at room temperature (25 °C). The bacteria were washed once in NET buffer containing 0.5 g of Triton X-100 per liter and finally resuspended again to give a 100 g/L concentration in NET buffer containing 0.5 g of Triton X-100 per liter.

Characterization of S. aureus—IgG Binding

IgG from normal human, rabbit, and sheep sera was isolated by a combination of ammonium sulfate fractionation, ion-exchange chromatography on DEAE-cellulose, and molecular-sieve chromatography on Sephadex G-200 (25). The purified proteins were labeled with 125I by the Chloramine-T method (21), and the protein-associated 125I was determined by precipitation with trichloroacetic acid.

The labeled protein (10 μL) was added to 500 μL of rabbit serum diluted fivefold with phosphate-buffered saline, 500 μL of a 200 g/L trichloroacetic acid solution was added, and the mixture was vortex-mixed and centrifuged. The counts associated with the precipitate represented protein-bound 125I.

In all of the binding experiments, 125I-labeled IgG in various amounts was mixed with 50 μL of S. aureus suspension in NET buffer containing 0.5 g of Triton X-100 per liter and left at room temperature for 30 min in 1.5-mL polypropylene Eppendorf microtubes. The tubes were centrifuged, the supernatants removed by aspiration, and the packed bacteria washed twice with NET buffer containing 0.5 g of Triton X-100 per liter and counted in a gamma counter (Beckman Instruments, Inc., Fullerton, CA 92634).

[125I]Digoxin (NEA-020T) and [125I]digoxin (NEA-056T) were purchased from New England Nuclear, Boston, MA.
02118. Human ferritin was purified as previously described (22). All antisera used were prepared locally.

Results

Binding Characteristics of S. aureus

Purified 125I-labeled IgG from rabbit human and sheep sera was used to characterize the appropriate time of reaction, the maximum binding capacity, and potential interference/cross reactivity characteristics of the S. aureus.

Various amounts of 125I-labeled rabbit IgG in a final volume of 0.5 mL of NET buffer containing 0.5 g of Triton X-100 per liter were mixed with 50 μL of S. aureus suspension.

Figure 1 shows that 50 μL of a 100 g/L S. aureus suspension will optimally bind up to 60 μg of rabbit IgG.

We compared the reactivity of S. aureus for IgG of various species, using labeled human (117 mg/L), sheep (90 mg/L), and rabbit (123 mg/L) IgG. These were reacted with 50 μL of S. aureus suspension for 30 min at room temperature. After the bacteria were removed by centrifugation, a second 50 μL of S. aureus suspension was added to the supernate. After 30 min the mixture was centrifuged and the radioactivity in the bacteria pellet was counted.

Table 1 demonstrates that S. aureus will bind only 90% of rabbit, 64% of human, and 56% of sheep IgG under the assay conditions described, even in the presence of an excess of S. aureus cells.

To assess the binding competition between rabbit and human IgG, we mixed 500 μg of human IgG and 20 μg of 125I-labeled rabbit IgG with 50 μL of S. aureus suspension. After 30 min at room temperature, the mixture was centri-fuged. This resulted in the binding of 90% of the rabbit IgG to the S. aureus. If we assume an average IgG concentration in rabbit and human serum of 10 g/L, 50 μL of the 100 g/L S. aureus suspension could be used to bind up to 100 μL of rabbit antibody (diluted 50-fold) in the presence of up to 50 μL of undiluted human serum. To achieve a margin of safety, we used 200 μL of S. aureus suspension and 25 μL of undiluted human plasma with rabbit antiserum at dilutions from 2000- to 50 000-fold.

Radioimmunoassay Comparisons

Ferritin. Second-antibody separation was done as previously described (22).

For S. aureus separation, 100 μL of rabbit anti-ferritin antibody diluted 50 000-fold in phosphate-buffered saline-EDTA (pH 7.8, containing, per liter, 10 mmol of PO4, 8.77 g of sodium chloride, and 20 g of disodium EDTA) was incubated with 100 μL of labeled ferritin (125I, 12 000 cpm, diluted in phosphate-buffered saline containing 20 g/L of bovine serum albumin), and 50 μL of phosphate-buffered saline containing 10 g of bovine serum albumin per liter, 50 μL of standard, or 25 μL of serum plus 25 μL of phosphate-buffered saline with 10 g of bovine serum albumin per liter, in Brinkmann Eppendorf centrifuge tubes for 18 h (overnight) at 4°C; 200 μL of S. aureus suspension in NET buffer containing 0.5 g of Triton X-100 per liter was added. Thirty minutes later, the tubes were centrifuged, the supernatant fluid was aspirated, the bacteria were washed twice, and the radioactivity was counted.

Seventy samples submitted for routine determination of ferritin were analyzed by both methods. Figure 2 shows excellent correlation (r = 0.968) over the entire range of values.

We have previously demonstrated a lack of plasma matrix effects in the second-antibody method for the determination of ferritin (22). The correlation coefficient, and slope and intercept data shown in Figure 2 indicate that the use of S. aureus does not introduce a matrix effect. We confirmed this by analyzing sera at two volumes, 15 and 25 μL. There was an equal distribution of positive and negative differences between the calculated values, with a correlation coefficient of 0.991.

Digoxin. We measured digoxin, using [H3]digoxin and (NH₄)₂SO₄ separation as described by Farr (3) and using

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Table 1. Binding of IgG by 100 g/L Suspension of S. aureus

<table>
<thead>
<tr>
<th>IgG, μg</th>
<th>50 μL added</th>
<th>2nd 50 μL added</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>89.1</td>
<td>1.4</td>
<td>90.5</td>
</tr>
<tr>
<td>123</td>
<td>63.4</td>
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<td>88.5</td>
</tr>
<tr>
<td>Human IgG</td>
<td></td>
<td></td>
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<tr>
<td>23</td>
<td>60.9</td>
<td>2.0</td>
<td>62.9</td>
</tr>
<tr>
<td>117</td>
<td>63.9</td>
<td>2.0</td>
<td>65.9</td>
</tr>
<tr>
<td>Sheep IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>45.0</td>
<td>10.9</td>
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<tr>
<td>90</td>
<td>42.9</td>
<td>12.3</td>
<td>55.2</td>
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Fig. 1. Binding capacity for rabbit IgG of a 100 g/L suspension of S. aureus

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Fig. 2. Correlation between second-antibody and S. aureus methods for assay of ferritin


**Discussion**

We have attempted to demonstrate the suitability of *S. aureus* cells for separating bound and free antigen in a radioimmunoassay of both a protein (ferritin) and a hapten (digoxin) in human sera. In each case, good correlation was found with existing salt fractionation and second-antibody separation methods.

We made no attempt to determine within-run and between-run precision, though the results of Gupta and Morton (20) would indicate that coefficients of variation similar to that encountered in the double-antibody method would be expected (10–15% within-run and between-run).

*S. aureus* has been used to separate free and bound antigen in the radioimmunoassay of α-fetoprotein (19), viral antigens

\[ y = b_0 + b_1 x \]

**Fig. 3. Digoxin measurement by *S. aureus* method at two plasma volumes**

Units on the axes are μg/L.

\[ y = b_0 + b_1 x \]

\[ r = 0.892 \]

\[ b_0 = -0.26 \]

\[ b_1 = 1.12 \]

\[ y = b_0 + b_1 x \]

\[ r = 0.892 \]

\[ b_0 = -0.26 \]

\[ b_1 = 1.12 \]

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\[ y = b_0 + b_1 x \]

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(23), and human serum albumin (20). Gupta and Morton raised the problem of cross reacting IgG in determinations involving human sera.

We have shown that S. aureus will maximally bind 90%, 64%, and 55% of available rabbit, human, and sheep IgG, respectively, and that the bacteria will bind rabbit IgG preferentially in the presence of a 50-fold excess of purified human IgG.

These values are in agreement with that reported by Lind and Mansa for rabbit IgG (14) with use of S. aureus cells. The rabbit/human competition data are at variance with the findings of Langone (24), who reported that soluble 125I-labeled protein A bound to rabbit and human IgG with equal affinity; he, too, found that sheep IgG was bound much less actively.

However, it is possible that in the purification of protein A or the solubilization process, or both, some alteration occurs in the specificity of reaction with IgG. In our experiments on S. aureus cells, rabbit IgG was more effectively bound than human IgG. S. aureus is best used with rabbit antisera, but one can increase the binding efficiency to other antisera by using a rabbit antibody to bridge (e.g., rabbit anti-sheep IgG should increase the S. aureus binding of sheep IgG to 100%).

We did routine assays, using 25 μL of human serum and 200 μL of 100 g/L S. aureus suspension. Use of larger quantities of human serum would eventually cause quantitation difficulties (500 μL of human serum causes the rabbit IgG binding to 100 μL of S. aureus to decrease by 80%). This would necessitate the use of a larger volume of S. aureus, which may increase the nonspecific binding of label.

The bacteria sediment easily and pack well on centrifugation, allowing easy removal of supernate. The production of S. aureus and its appropriate utilization in radioimmunoassay represent a rapid and economical alternative to other methods of separation.

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References