Binding of Immunoglobulin G Aggregates and Immune Complexes in Human Sera to *Staphylococci* Containing Protein A

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ABSTRACT Using the Cowan I strain of *Staphylococcus aureus*, we compared the binding properties of human monomeric immunoglobulin (Ig)G and oligomeric or complexed IgG. Heat-aggregated IgG served as a model for complexed IgG and heat-killed, formalin-fixed *S. aureus* (StaphA) as a cellular receptor for IgG, in determining the parameters for oligomeric and monomeric binding. Because of its capacity for multipoint attachment, complexed IgG binding was favored over monomeric IgG binding, and this preferential binding was demonstrated kinetically in equivalent forward rates of binding but in a much slower rate of release from StaphA receptors.

From binding studies, we determined which conditions maximize complexed IgG binding and minimized monomeric IgG binding and applied them to the development of an assay for aggregated IgG and immune complexes in human sera. The StaphA binding assay that was devised is quantitative, sensitive, and not complement dependent. It is relatively unaffected by factors such as heparin, complement fixation, native antibodies, and immunoglobulin concentrations, but is affected by the presence of rheumatoid factors. It compares favorably with two other complement-dependent assays of immune complexes, the ^{125}I-Clq binding assay and the Raji cell assay, in terms of sensitivity and the size of immune complexes detected. Studies on the potential of the assay for detecting, isolating, and characterizing immune complexes in biological fluids are presented.

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INTRODUCTION

Indirect methods for detecting circulating complexes of antigen and antibody, or immune complexes (IC),[1] generally rely on properties of complexed immunoglobulin that can be distinguished from those of free immunoglobulin. These include physical properties such as size or solubility, reactivity with complement or complement components, and binding properties with soluble or cellular receptors.

Cellular receptors appear to bind complexed immunoglobulin (Ig)G more avidly than they do free immunoglobulin because of the potential for multipoint attachment. In several systems, it has been confirmed in thermodynamic terms that complexes bind with substantially greater affinity than does monomeric immunoglobulin (1, 2). The feasibility of using any cellular immunoglobulin receptor in an IC assay depends on whether the affinity properties of the receptor can be manipulated by creating test conditions that cause preferential binding or activation by IC. Such conditions have been found for a number of cellular receptors and form the basis of several assays for IC. These include interaction with complement receptors (3), interaction with platelet Fc receptors (4), inhibition of antibody-dependent, cell-mediated cytotoxicity (5, 6), and inhibi-

[1] *Abbreviations used in this paper:* Agg-IgG, aggregated IgG; anti-HB_sAg, antibody to HB_sAg; Az, sodium azide; BSA, bovine serum albumin; ClqBA, ^{125}I-Clq binding assay; HB_sAg, hepatitis B surface antigen; IC, immune complex(es); KLH, keyhole limpet hemocyanin; Mon-IgG, monomeric IgG; NHS, normal human serum; PEG, polyethylene glycol; PBS, phosphate-buffered saline; RA, rheumatoid arthritis; RF, rheumatoid factor, SBA, StaphA binding assay; SLE, systemic lupus erythematosus; StaphA, heat-killed, formalin-fixed Cowan I strain of *S. aureus*.

tion of the phagocytosis of immunoglobulin aggregates by macrophages (7).

Most strains of heat-killed, formalin-fixed Cowan I strain of *Staphylococcus aureus* (StaphA) contain a cell surface component, protein A, that binds to the Fc portion of subclasses IgG_1, IgG_2, and IgG_4 (8, 9) as well as to some but not all IgA and IgM preparations (10, 11). The *Staphylococcus* protein A has been used in two types of IC assays, but they depend on its general immunoglobulin binding reactivity rather than on any preferential binding properties it might have (12, 13). Using an insoluble, relatively inert, cellular receptor for IC seemed to us to offer an advantage, particularly with regard to the isolation and characterization of IC material. With this in mind, the IgG binding properties of StaphA were examined to devise conditions in which complexed immunoglobulin is preferentially bound. These studies led to the development of a StaphA binding assay (SBA) for IC in human serum. In this report we describe the development of this assay in terms of specificity, sensitivity, factors influencing the test, and its application to the detection and isolation of IC.

METHODS

Reagents and buffers. Human IgG (Cohn fraction II, Pentex, Kankakee, Ill.) was further purified by DEAE cellulose chromatography under the same conditions used for rabbit antisera (see below). Rabbit IgG (Pentex Biochemical, Kankakee, Ill.) was used without further purification. Polyethylene glycol (PEG, 6,000 mol wt; J. T. Baker Co., Phillipsburg, N. J.) was dissolved in borate saline buffer (0.1 boric acid, 0.25 M sodium tetraborate, 0.75 M NaCl, pH 8.3) at a 15% wt/vol stock solution and further diluted in the same buffer for use in the SBA. EDTA buffer consisted of 0.2 M disodium EDTA, adjusted to pH 7.4 with 1 N NaOH. Phosphate-buffered saline with sodium azide (PBS-Az) was 0.01 M phosphate, 0.15 M NaCl, 0.1% Az, pH 7.2–7.4. Bovine serum albumin (BSA, Pentex Biochemical) was added to PBS-Az at 0.5% wt/vol and the pH adjusted to 7.2–7.4 with 1 N NaOH (PBS-BSA-Az).

Sera and synovial fluids. Blood was allowed to clot at room temperature for 1–2 h. Serum was removed by centrifugation and stored at −70°C in small portions. Portions were then thawed just before use and used only once. Synovial fluids were treated with hyaluronidase (Worthington Biochemical Corp., Freehold, N. J.), centrifuged at 1,500 *g* for 10 min, and processed like sera.

Staphylococcus preparations. The Cowan I and Wood 46 strains of *S. aureus* were obtained from Dr. J. Cohen, Center for Disease Control, Atlanta, Ga. Heat-killed, formalin-fixed preparations of the *Staphylococci* were prepared as described by Kessler (14) with two modifications. Trypticase soy broth (BBL Microbiology Systems, Becton, Dickinson & Co., Cockeysville, Md.) supplemented with IsoVitaleX (BBL) and 0.3% wt/vol yeast extract (Difco Laboratories, Detroit, Mich.) was used for growing the organisms, and heat killing was done at 80°C for 2.5 min with rapid equilibration. StaphA was stored at −70°C at 10% vol/vol in PBS-Az. Once thawed it was kept at 4°C for up to 4 wk. Before use in the SBA, the StaphA was washed three times and suspended in PBS-BSA-Az at 0.8% vol/vol.

IgG preparations and binding studies. For use as a standard in the SBA, IgG was heat aggregated at 20 mg/ml for 20 min at 63°C. The preparation was chromatographed on a Sephadex G-200 column (Pharmacia Fine Chemicals, Piscataway, N. J.). Fractions corresponding to void volume material were pooled, centrifuged at 18,000 *g* for 40 min, and the supernatant material stored at 2.0 mg/ml in small portions at −70°C. Sucrose density gradient ultracentrifugation indicated that the aggregated IgG preparation (Agg-IgG) contained <10% monomeric (7s) IgG.

IgG was radiolabeled with ^{125}I by the iodine monochloride technique (15). A portion was heat aggregated as above. Radiolabeled and unlabeled preparations of IgG and Agg-IgG were chromatographed with Sephacryl S-200 (Pharmacia Fine Chemicals). Binding studies were performed within 24 h of chromatography. Monomeric IgG (Mon-IgG) and Agg-IgG preparations were adjusted to the desired concentration and sufficient radiolabeled preparations were added such that 100 μl contained 5,000–10,000 cpm. The binding studies were performed in duplicate 10-X 75-cm plastic tubes (Falcon Labware 2038, Div. of Becton, Dickinson & Co., Oxnard, Calif.) prerinsed with PBS-BSA-Az. The reaction mixtures contained 100 μl of an 0.8% vol/vol StaphA preparation and 100 μl of the trace radiolabeled IgG preparations. IgG concentration, incubation time, and temperature are indicated in the text. The binding reaction was stopped by transferring the tubes to an ice bath and adding 1.0 ml prechilled PBS-Az containing 5 mg/ml IgG that effectively stops ongoing binding and elution of the radiolabel (Figs. 1 and 2). The tubes were centrifuged, 200 μl of supernate was transferred to another tube, radioactivity in both tubes was determined, and the percentage bound was calculated. 100 μl of an 0.8% vol/vol suspension of StaphA had a binding capacity of 5 μg of IgG. Four lots of StaphA including one commercial lot (Pansorbin, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) had similar binding capacities.

SBA. To duplicate 10 × 75-mm plastic tubes (Falcon Labware 2039) is added 150 μl EDTA buffer, 50 μl of test serum, and 1.0 ml 6% PEG. The tubes are incubated overnight at 4°C and centrifuged at 1,000 *g* for 20 min. The supernate is aspirated and the pellet is resuspended in 5% PEG and centrifuged again. The supernate is completely aspirated, and the pellet, which is often only barely visible, is suspended in 200 μl PBS-Az and allowed to sit at room temperature for 15–20 min with agitation as required to dissolve the precipitate. 100 μl of the solubilized precipitate is transferred to a 10-× 75-mm tube, that was prerinsed with PBS-BSA-Az, and contains 100 μl of an 0.8% vol/vol suspension of StaphA in PBS-BSA-Az. After incubation in a 37°C water bath for 2 h, 0.5 ml PBS-BSA-Az is added, and the tubes are centrifuged at 1,000 *g* for 10 min. The supernate is aspirated, and the pellet is resuspended in

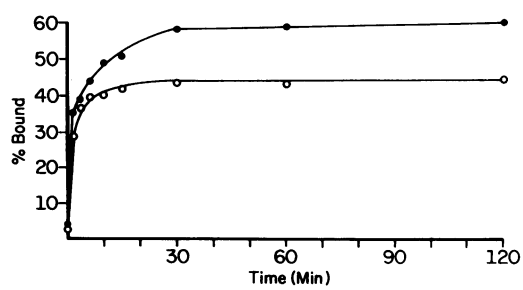

FIGURE 1 Kinetics of Mon-IgG and Agg-IgG binding to StaphA. 10 μg radiolabeled Agg-IgG (●) or 10 μg Mon-IgG (○) in 100 μl were added to 100 μl of an 0.8% vol/vol StaphA preparation (5 μg IgG binding capacity) and incubated at 37°C. At the indicated times, the reaction was stopped by adding excess unlabelled IgG at 0°C and percentage bound determined.

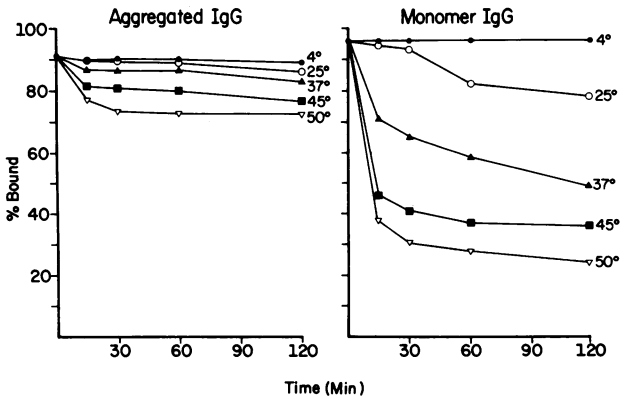

FIGURE 2 Kinetics of release of Mon-IgG and Agg-IgG from StaphA. 100 μl of an 0.8% vol/vol suspension of StaphA was incubated with 4 μg radiolabeled Agg-IgG or Mon-IgG in a 200-μl final volume for 2 h at 37°C and placed in an ice bath. At zero time, 5 mg unlabeled IgG in 1 ml was added, the tubes incubated at the indicated temperatures, and release of radiolabel into the supernate determined at the times indicated.

1.0 ml PBS-Az containing 5 mg/ml rabbit IgG. After another 2 h incubation at 37°C with frequent agitation, the tubes are centrifuged at 1,000 *g* for 10 min. From this point on, the tubes and reagents are kept in an ice bath or at 4°C. The StaphA pellets are washed twice by centrifugation with 2–3 ml PBS-Az and resuspended in 100 μl PBS-Az containing 5 mg/ml rabbit IgG. A radiolabeled rabbit anti-human IgG preparation (see below) is centrifuged at 1,000 *g* before use, and 100 μl is added to the StaphA tubes. The tubes are incubated overnight at 4°C, 0.5 ml PBS-BSA-Az is added, and the tubes are centrifuged at 1,000 *g* for 10 min. After three more washes with PBS-Az, radioactivity remaining in the tubes is counted in a gamma counter.

When Agg-IgG preparations are assayed for use as standards or when chromatography or sucrose density gradient fractions are assayed, the initial 200 μl vol in the SBA is made up of 50 μl of Agg-IgG sample, 50 μl of normal human serum (NHS), and 100 μl of EDTA buffer. The Agg-IgG standard is used at concentrations between 25 and 400 μg/ml for constructing the standard curve. The rabbit IgG solution (5 mg/ml) for elution and antisera dilution was centrifuged at 18,000 *g* for 20 min before use in the SBA. Initially in these experiments, the rabbit IgG was chromatographed on Sephacryl S-200 the night before it was used to remove aggregates, but because the detection of Agg-IgG in NHS was not found to be influenced by the preliminary step, it was omitted.

Radiolabeled antisera. Antisera to human IgG, IgM, IgA, and the Fab fragment of IgG were produced in rabbits. Antisera were rendered specific for IgG, IgM, or IgA by absorption with IgG (Cohn fractions II), IgAκ, IgAλ, or IgMκ paraproteins that had been insolubilized on Sepharose 4B (16). The anti-Fab antiserum was not adsorbed and was considered to be a polyvalent anti-human immunoglobulin reagent by virtue of light chain reactivity.

The IgG fraction of the antisera was obtained by DEAE cellulose chromatography (DE-52, Whatman Ltd, Maidstone, Kent, England). DE-52 and the antisera were equilibrated with 0.02 M phosphate buffer, pH 8.0. Material not retained on the column was made 0.15 M in NaCl and stored at 3–5 mg/ml at −20°C. The IgG preparations were radiolabeled by the lactoperoxidase technique (17) (0.16–0.2 μCi/μg sp act) and were mixed 1:4 or 1:5 with the unlabeled IgG preparation of the antisera to reduce the net specific activity by four- to five-fold. This mixture was then diluted 1:4 in PBS-Az containing 5 mg/ml rabbit IgG for use in the SBA. This dilution was predetermined by titration experiments done under the conditions used in the SBA and was in excess of that required to bind maximally to a StaphA preparation that had been saturated with human IgG.

Purified IgM rheumatoid factors. The washed cryoglobulin fraction of sera from two patients with monoclonal IgMκ paraproteins that had rheumatoid factor activity and the euglobulin fraction of serum with a high titer rheumatoid factor from a patient with rheumatoid arthritis were used as a source of IgM rheumatoid factor (RF). IgM RF was purified by repeated chromatography in low pH buffer and by immunoadsorption as described in detail (18).

Adsorption of antiglobulin activity from sera. Human IgG and BSA were coupled to cyanogen bromide-activated Sepharose 4B (16). A 50% vol/vol slurry (0.3 ml) of each Sepharose preparation in EDTA buffer was incubated with an equal volume of serum. Adsorption was carried out at 37°C for 1 h and 4°C overnight.

Precipitin curve. Human antiserum was obtained from a subject immunized and boosted with keyhole limpet hemocyanin (KLH, Pacific Bio-Marine Laboratories Inc., Venice, Calif.). Increasing amounts of KLH in PBS-Az were added to 0.5 ml of antiserum in 3-ml glass centrifuge tubes in a 1.0-ml final volume. Tubes were incubated at 37°C for 1 h and at 4°C overnight. The tubes were centrifuged at 1,500 *g* for 1 h. The supernate was removed and stored at −70°C until assayed. The precipitate was washed three times with PBS-Az at 4°C, and the protein content was determined by the Folin-Ciocalteau method (19).

Physical methods. Sucrose density gradient ultracentrifugation was performed as described in detail (18).

Other assays. Protein concentrations were determined by the Folin-Ciocalteau reaction with human IgG as a standard (19). The Raji cell radioimmunoassay for IC was performed as described by Theofilopoulos et al. (3) using the radiolabeled reagent and Agg-IgG standard described in this paper. The modified ^{125}I-Clq binding assay (ClqBA) was done as described by Zubler et al. (20). Clq was purified from human serum by the method of Yonemasu and Stroud (21) as modified by Zubler et al. (20) and radiolabeled as described by Heusser et al. (22). Radioimmunoassays for IgM RF and IgG RF were performed as described by Carson et al. (23). Radioimmune assays for hepatitis B surface antigen (HB_sAg) and antibody (anti-HB_s) were done with a commercially available kit according to the manufacturer's instructions (Aus-RIA, Aus-Ab, Abbott Diagnostics, North Chicago, Ill.). Reagents from the Abbott kit were used to detect HB_sAG or antibody in IC isolated on StaphA (Results).

RESULTS

Kinetics of IgG binding to StaphA. Uptake of trace radiolabeled Mon-IgG and Agg-IgG at 37°C was determined at intervals (Fig. 1). There was no appreciable difference in the rate of binding between Agg-IgG and Mon-IgG under these conditions or at several other concentrations of IgG.

To determine the rate of release of IgG from StaphA, StaphA was preincubated with radiolabeled Agg-IgG or Mon-IgG (90–95% bound). At zero time, excess unlabeled IgG was added to prevent rebinding of radiolabeled IgG, and the release of radiolabel into the supernate was determined at intervals (Fig. 2). The rate

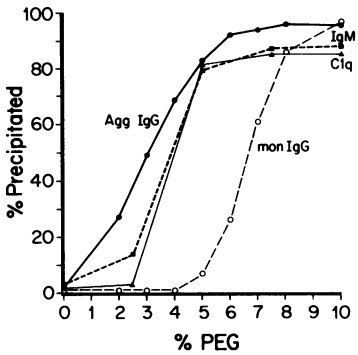

FIGURE 3 PEG precipitation. ^{125}I-labeled Agg-IgG (●), Mon-IgG (○), IgM (■), and Clq (▲) were added to NHS and precipitated with PEG under conditions used in the SBA.

of release of Agg-IgG was much slower than that of Mon-IgG. Use of rabbit IgG rather than human IgG to prevent rebinding resulted in an identical elution pattern.

Inhibition studies indicated that compared to Mon-IgG binding, Agg-IgG binding is less effectively inhibited by excess IgG. To inhibit binding by 50%, 10- to 100-fold more IgG is required to inhibit Agg-IgG binding than to inhibit Mon-IgG binding. However, with a large excess of IgG (1,000-fold), the inhibition of Agg-IgG and Mon-IgG binding were equivalent. Such a situation (Mon-IgG excess) would exist in sera containing IC. Addition of NHS to labeled Agg-IgG or Mon-IgG and incubation with StaphA under various conditions resulted in equivalent binding (or in differences too small to be reliably detected).

PEG precipitation as a preliminary step. Because Mon-IgG when present in large excess effectively competes with Agg-IgG for binding sites on StaphA, a preliminary step was introduced to separate most of the Mon-IgG from Agg-IgG in serum. PEG precipitation, which precipitates proteins largely on the basis of size (24), was used. Fig. 3 shows the precipitation pattern of radiolabeled Agg-IgG, Mon-IgG, IgM, and Clq added to NHS under the conditions used in the SBA.

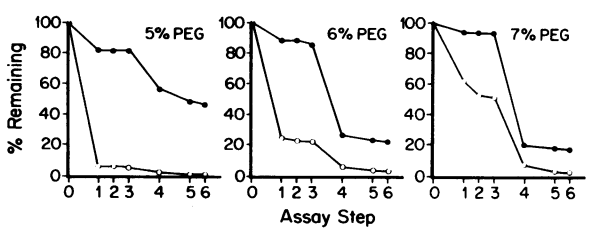

FIGURE 4 Retention of Mon-IgG and Agg-IgG in the SBA. ^{125}I-labeled Agg-IgG (●) or Mon-IgG (○) was added to NHS and the SBA procedure was performed using different PEG concentrations in the initial precipitation. After each step in the assay, radioactivity in the tube was determined. Assay steps: (*a*) removal of PEG supernate; (*b*) removal of PEG wash; (*c*) solubilization of the PEG precipitate and transfer to StaphA tube (previous points are corrected because only half of solubilized precipitate is transferred); (*d*) 2 h incubation, centrifuge, and aspirate supernate; (*e*) 2 h incubation with rabbit IgG, centrifuge, and aspirate supernate: (*f*) StaphA pellets washed twice with PBS-Az.

Radiolabeled Agg-IgG and Mon-IgG were added to NHS, precipitated with several concentrations of PEG, after which the redissolved precipitates were run through the SBA procedure. A concentration of 5% PEG was selected because Mon-IgG was effectively eliminated during the SBA procedure with substantial retention of Agg-IgG (Fig. 4).

SBA. As finally devised (Methods), the SBA involved precipitating serum with 5% PEG, solubilizing the precipitate, and incubating with StaphA. Unbound material was removed by centrifugation and rabbit IgG was added to elute monomeric IgG and block the remaining Fc receptors. Subsequently, the ^{125}I-labeled IgG fraction of a rabbit anti-human IgG was used to detect human IgG remaining bound to the StaphA. Known amounts of Agg-IgG added to NHS were used as standards. Quantification of IC in sera is then expressed in Agg-IgG equivalents per milliliter relative to the standard curve (Fig. 5A).

Sera obtained from various patients with rheumatic diseases were assayed for IC by the SBA (Fig. 6). 14 normal sera had a mean of 2.6±3.9 Agg-IgG equivalents per milliliter. Tests were run in duplicate and had an average range of 4.6% of the mean. 20 positive sera were assayed twice on separate occasions giving an average intertest difference of 17 Agg-IgG equivalents per milliliter or ±7.7% of the means of the two determinations. 90% of rheumatoid arthritis (RA) sera and 75% of systemic lupus erythematosus (SLE) sera had levels of IC in excess of 25 μg Agg-IgG equivalents per milliliter. The ClqBA gave positive tests (>5% Clq precipitated) in 77% of RA sera and 58% of SLE sera. In the limited number of SLE sera tested, there was no correlation between the SBA and hemolytic complement levels, immunoglobulin levels, antinative DNA antibodies, or the presence of clinically evident renal disease, but there was a correlation with the results of the ClqBA ($r = 0.61, P < 0.01$). In the RA sera, there were positive correlations between the results of the SBA and the ClqBA ($r = 0.84$, $P < 0.001$), IgM RF levels ($r = 0.7$, $P < 0.001$) and IgG RF levels ($r = 0.79, P < 0.001$). No correlation with hemolytic complement levels or immunoglobulin concentrations and the SBA or ClqBA was found in RA sera. Paired sera and synovial fluids from RA patients were also examined by the SBA (Fig. 7). The higher levels of IC in synovial fluids relative to sera was accompanied by parallel increases in IgG RF levels, whereas IgM RF levels tended not to be higher in synovial fluids.

Effect of various serum manipulations on the SBA. A number of manipulations of normal serum, with and

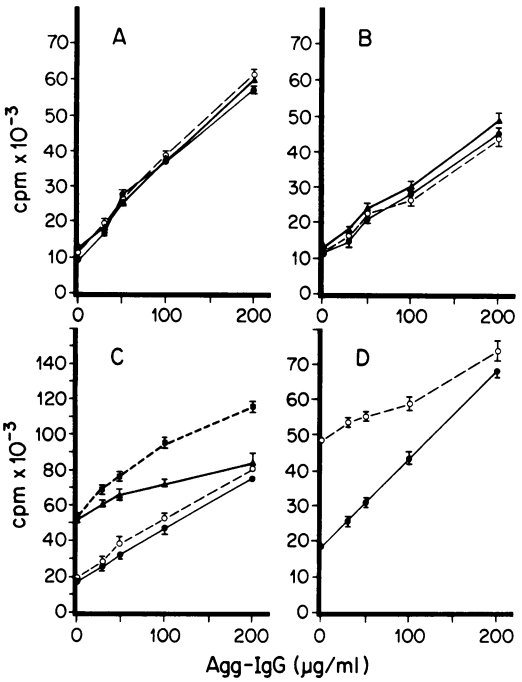

FIGURE 5 SBA standard curves. Agg-IgG was added to NHS and the SBA procedure performed using ^{125}I-labeled rabbit anti-IgG to detect residual IgG present on the StaphA. Radioactivity and range of duplicate determinations are plotted against μg/ml Agg-IgG added to NHS. (A) Standard curves comparing NHS diluted in EDTA buffer (●) and NHS diluted in PBS-Az (▲), NHS heated at 56°C for 30 min (○). (B) Standard curves comparing NHS (●), plasma (○), and NHS to which had been added 232 μg/ml purified Clq (▲). (C) NHS standard curve (●); NHS plus 329 μg/ml polyclonal IgM RF resulting in an RF latex titer of 1:800 (○); NHS plus 131 μg/ml monoclonal IgM RF resulting in an RF latex titer of 1:1600 (▲); NHS plus 109 μg/ml monoclonal IgM RF resulting in an RF latex titer of 1:3200 (■). (D) Standard curve obtained using IgG-specific reagent (●); or polyvalent anti-immunoglobulin reagent (○).

without added Agg-IgG, were done to determine their effect on the standard curve (Fig. 5). These included a comparison of serum heated at 56°C for 30 min with serum diluted in PBS-Az or EDTA buffer, addition of exogenous Clq, and the use of plasma (10 U heparin/ml of blood) rather than serum. No significant differences were found (Fig. 5A and B). Preadsorption of serum with the Wood 46 strain of *Staphylococcus* (a strain lacking in the *Staphylococcus* protein A) to eliminate anti-*Staphylococcus* antibodies did not alter the standard curve. Comparison of the standard curve obtained with a polyvalent anti-human immunoglobulin reagent rather than the IgG-specific reagent showed a lower slope and higher relative background radioactivity (Fig. 5D). This would indicate that other non-IgG classes of immunoglobulin are bound to the StaphA during the SBA procedure. Addition of a twofold excess Mon-IgG or purified IgM to NHS did not effect the detection of Agg-IgG. Sera from various patients with paraproteinemias were assayed in the SBA using polyvalent and class-specific reagents (Table I). Two of the three sera from patients with IgM paraproteins and one of the two sera from patients with IgA paraproteins showed increased uptake of the radiolabeled polyvalent anti-immunoglobulin and class-specific reagents, although there was no influence on IgG detection. Sera from six patients with IgG paraproteins showed slightly higher uptake of the IgG specific reagent, but in only one would this uptake have been recorded as an IC level >25 μg/ml Agg-IgG equivalents on the standard curve.

Effect of RF on the SBA. Two purified monoclonal IgM RF preparations and a purified polyclonal IgM RF were added to NHS with and without Agg-IgG. The two monoclonal IgM RF preparations and, to a lesser extent, the polyclonal IgM RF preparation resulted in a significant overestimation of Agg-IgG equivalents in NHS (Fig. 5C). IgM itself is not detected by the anti-IgG reagent although the IgM RF preparations do bind to StaphA.

Antiglobulin activity in RF-positive sera from patients with RA was adsorbed with insolubilized IgG and tested in the SBA (Table II). The adsorption variably reduced the levels of IC detected in the RA sera. Parallel adsorption procedures done with RF-negative sera (SLE sera and NHS) did not affect the assay results (Table II).

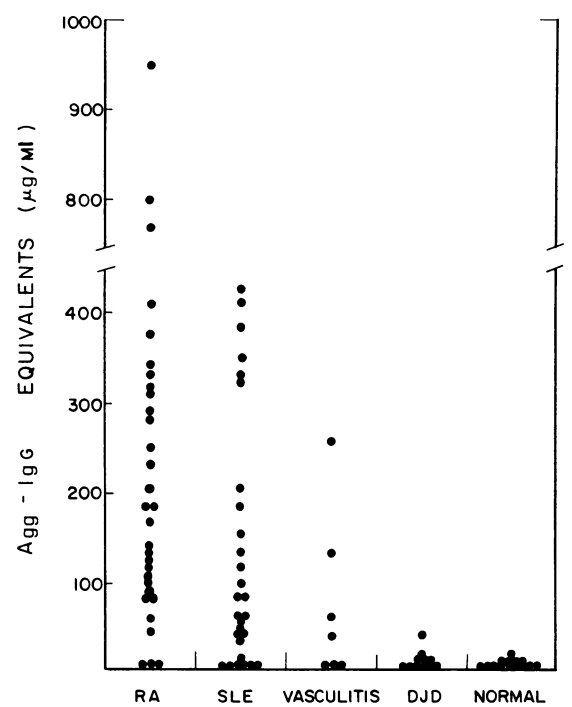

FIGURE 6 IC detected by SBA in patients with rheumatic diseases. Sera from individuals with RA, SLE, miscellaneous vasculitides, degenerative joint disease (DJD), and sera from health controls were assayed for IC by the SBA.

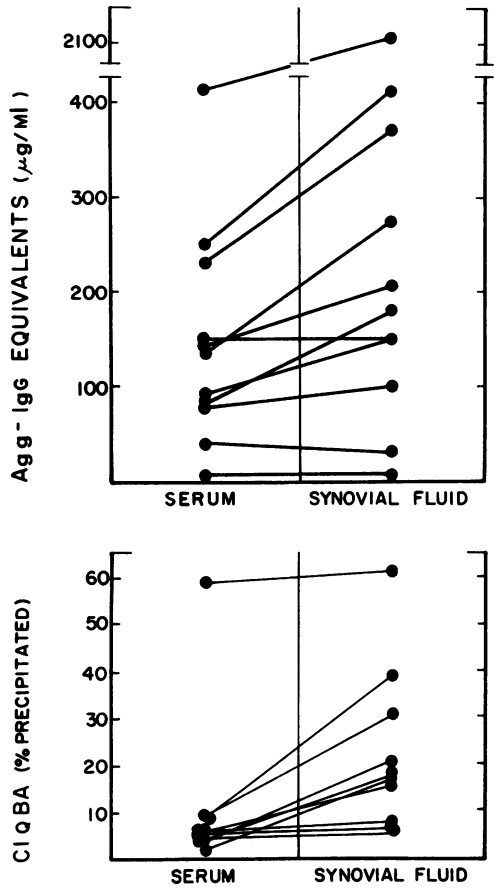

FIGURE 7 IC detected by the SBA (upper panel) or ClqBA (lower panel) in paired sera and synovial fluid from patients with RA.

Comparison of sensitivity of the SBA, ClqBA, and Raji cells assays for IC. Soluble IC were artificially prepared by reacting a human anti-KLH serum with KLH. The precipitin curve and results of supernatant tests for IC with three assays are shown in Fig. 8. The SBA, ClqBA, and Raji cell assays detected IC at equivalence and in antigen excess. IC material detected by the SBA declined somewhat in great (up to eightfold) antigen excess but the general curve showed less dependence on antigen:antibody ratio than did either the ClqBA or Raji cell assay (Fig. 8).

As an estimate of the minimal size of IC detected, IgG was heat aggregated and fractionated by sucrose density ultracentrifugation. Agg-IgG equivalents were determined for the three tests using the same Agg-IgG standard and compared to the actual protein concentration present in the fractions (Fig. 9). All three tests overestimate large aggregates and underestimate small aggregates.

IgG was heat aggregated and chromatographed on a calibrated Ultrogel AcA-22 column (LKB Instruments, Inc., Rockville, Md.). Fractions corresponding to various molecular weight ranges were pooled, serially diluted in NHS, and assayed for IC. The lowest concentration of Agg-IgG that differed substantially from the NHS control (i.e., a difference three times the average range of duplicates) was determined for each assay. This was arbitrarily chosen as the lower limit of sensitivity for a given pool of Agg-IgG. The results are shown in Table III. All three tests are less sensitive in detecting small sized aggregates than larger sized aggregates.

Use of the SBA to detect antigen in IC material. Two patients with polyarteritis associated with HB_sAg had a positive SBA for IC. These patients did not have antibody to HB_sAg by radioimmunoassay. To determine whether the HB_sAg was associated with IC, the experiment shown in Table IV was done. These two sera, NHS, buffer controls, and sera from two patients with acute hepatitis (which contained HB_sAg but no detectable anti-HB_s) were processed in the SBA. At the last step of the SBA, instead of incubating the StaphA pellets

TABLE I
Relative Binding of Immunoglobulin Classes in the SBA by Sera from Patients with Monoclonal Gammopathies

Sera tested	Relative uptake of ^{125}I-labeled antisera*			
	anti-Fab	anti-γ	anti-μ	anti-α
NHS	1.00‡	1.00§	1.00‖	1.00¶
NHS + 200 μg/ml Agg-IgG	1.80**	4.91	1.09	0.96
NHS + 400 μg/ml Agg-IgG	2.46	8.21	1.17	1.10
IgM – Waldenströms	1.54	1.12	1.43	0.72
IgM – Waldenströms	2.14	1.14	2.76	0.78
IgM – Waldenströms	1.14	1.04	1.07	0.85
IgA – Myeloma	0.88	0.83	0.64	1.07
IgA – Myeloma	1.73	0.94	1.09	2.26
IgG – Myeloma	0.76	1.08	0.96	0.93
IgG – Myeloma	1.15	1.10	0.86	0.82
IgG – Myeloma	1.15	1.14	1.19	1.18
IgG – Myeloma	1.64	1.57	1.75	1.15
IgG – Myeloma	1.16	1.13	0.79	0.91
IgG – Myeloma	0.96	1.19	0.87	0.89

* In the last step of the SBA, ^{125}I-labeled antisera with the specificities noted were reacted with the StaphA and, after washing, the relative uptake of radio-label expressed as: counts per min test sera/counts per min NHS.

‡ Radioactive uptake of ^{125}I-anti-Fab (polyvalent anti-Ig) by NHS reacted StaphA, 31,853 cpm.

§ Radioactive uptake of ^{125}I-anti-γ by NHS-reacted StaphA, 11,826 cpm.

‖ Radioactive uptake of ^{125}I-anti-μ by NHS-reacted StaphA, 11,032 cpm.

¶ Radioactive uptake of ^{125}I-anti-α by NHS-reacted StaphA, 1,119 cpm.

** Relative uptakes >1.2 are underlined.

with radiolabeled anti-IgG, the following reagents were added: ^{125}I-labeled anti-HB_sAg to detect surface antigen or ^{125}I-labeled HB_sAg to detect antibody. In addition, a 3 M NaSCN eluate from the StaphA, after dialysis against PBS, was analyzed for HB_sAg. All sera were processed in parallel using the Wood 46 strain of *Staphylococcus* (which lacks IgG receptor) as a control for nonspecific adsorption of antigenic material. HB_sAg was associated with the StaphA and eluted from it with thiocyanate using sera from the two arteritis patients, but was not found when the two acute hepatitis sera, NHS, or buffer controls were examined. With the reagents used, antibody to HB_sAg was not associated with StaphA, nor, as mentioned, was it detected in whole serum.

DISCUSSION

These experiments were done to examine the interaction of the StaphA receptor with human IgG to determine conditions in which complexed IgG is preferentially bound, and ultimately to apply such conditions to developing an assay for IC. Immunochemical considerations predict that the interaction of receptors on StaphA with IC would be favored in terms of binding energy and association constant because of the potential for multipoint attachment.

The equivalent forward rates and the distinction in reverse rate of binding between Agg-IgG and Mon-IgG (Figs. 1 and 2) suggests that, given sufficient time, Agg-IgG would be preferentially bound. Kinetic studies, mixture experiments, and 10- to 100-fold differences in concentration of IgG to obtain equivalent inhibition of binding confirmed this prediction. However, the forward rate of reaction, being diffusion limited, predicts that at any given time the probability that an unoccupied receptor site will come into contact with Agg-IgG or Mon-IgG is a function of their relative concentrations and, therefore, when Mon-IgG is present in marked excess, it would take an impractical length of time to reach equilibrium. Because sera containing IC would be expected to contain Mon-IgG in great excess of any IgG present as a complex, PEG precipitation of sera was introduced to reduce the ratio of Mon-IgG to complexed IgG. The optimal concentration of PEG was determined empirically as that concentration which maximized Agg-IgG retention and minimized Mon-IgG retention under the test conditions of the SBA (Fig. 4). The strategy was to incubate the solubilized PEG precipitate with StaphA and then add excess rabbit IgG to elute and

TABLE II
Adsorption of Anti-Gammaglobulin Activity from Sera: Effect on SBA

Sera	Agg-IgG equivalents sera preadsorbed with*		Reduction‡
	Sepharose-BSA	Sepharose IgG	
	μg/ml		%
RA	195	20	90
RA	135	20	85
RA	175	40	77
RA	295	165	44
RA	290	245	16
RA	265	275	−4
SLE	170	160	6
SLE	170	165	3
SLE	145	120	17
NHS	0	0	0

* Before assay, sera were adsorbed with BSA (control adsorption) or IgG covalently coupled to Sepharose 4B (see text).

‡ Percentage of reduction in Agg-IgG equivalents detected as a result of adsorption with IgG: Sepharose-BSA adsorption − Sepharose-IgG adsorption/Sepharose-BSA adsorption × 100.

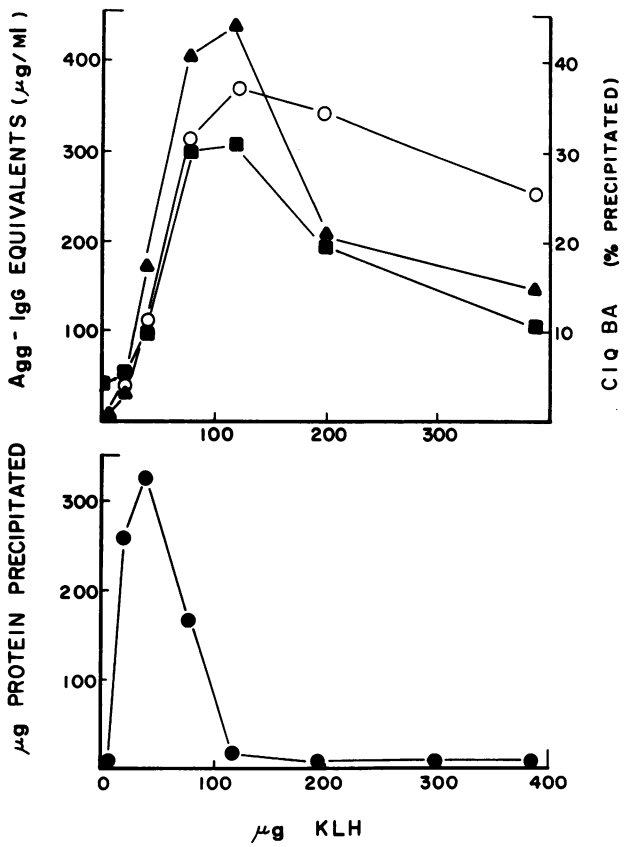

FIGURE 8 KLH anti-KLH precipitin curve and detection of IC in supernates. Lower graph indicates the total protein precipitated by increasing amounts of KLH added to a human anti-KLH antiserum. Supernatant material was analyzed by the SBA (○) and Raji cell assays (▲) and results expressed as μg/ml of Agg-IgG equivalents. The ClqBA results are expressed as percentage of Clq precipitated (■).

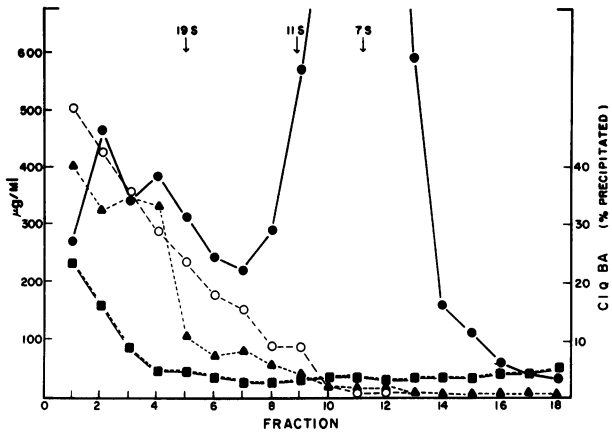

FIGURE 9 Detection of Agg-IgG in sucrose density gradient fractions. IgG was heat aggregated and fractionated by sucrose density gradient ultracentrifugation. Fractions were added to an equal volume of NHS and assayed by the SBA (○), Raji cell assay (▲), and ClqBA (■). Quantitative results with the SBA and Raji cell assay are obtained by reference to the Agg-IgG standard and compared to the actual protein concentrations present in the fractions (●). ClqBA results are expressed as percentage of Clq precipitated. Arrows indicate location of radiolabeled marker proteins run in parallel gradients.

replace Mon-IgG bound, leaving complexed IgG relatively intact. Residual IgG present on the StaphA was then detected using radiolabeled rabbit anti-human IgG.

That these manipulations result in an assay capable of detecting complexed IgG in NHS was confirmed by adding increasing amounts of Agg-IgG to NHS and testing in the SBA. A linear increase in uptake of radiolabeled antisera resulted (with an acceptable low level of uptake in normal sera) (Fig. 5), and this served as a standard curve for indirectly quantitating IC present in clinical material as Agg-IgG equivalents per milliliter (Figs. 6 and 7).

TABLE III

Minimal Concentration of Agg-IgG of Different Sizes Present in Human Serum which can be Detected by IC Assays

Molecular weight range of Agg-IgG*	Lowest concentration of Agg-IgG resulting in a positive test result		
	SBA	Raji	Clq BA
		μg/ml	
100,000–200,000	>1449‡	>1449‡	>1449‡
200,000–800,000	32	126	>252‡
800,000–1,200,000	<6§	<6§	75
>1,200,000	<6§	<6§	55
Agg-IgG standard	<6§	<6§	82

* Pooled fractions from Ultrogel AcA-22 chromatography of Agg-IgG (see text).

‡ Highest concentration tested.

§ Lowest concentration tested.

Various factors were examined for potential interference with the SBA. EDTA buffer was initially chosen as the serum diluent on theoretical grounds of dissociating the Cl complex and avoiding any influence of complement fixation by Agg-IgG in either the PEG precipitation or binding to StaphA. However, standard curves constructed with serum diluted in EDTA buffer, serum diluted in PBS, heat-inactivated serum, or serum with additional Clq did not substantially alter the standard curve (Fig. 5). IgG anti-*Staphylococcus* antibodies were not a problem; as monomeric immunoglobulins they are largely removed at the PEG precipitation step. Plasma and sera reacted equivalently (Fig. 5B) which gives the SBA a decided advantage over IC tests dependent on Clq fixation with heparinized specimens.

The role of IgG levels and other non-IgG classes of immunoglobulins was examined in several ways. IgM and IgA present in NHS would be expected to precipitate in 5% PEG and some purified preparations of IgM and IgA have been reported to bind to StaphA (10, 11). Assay of sera from patients with paraproteinemias (Table I) revealed that some IgA and IgM paraproteins were retained on the StaphA during the procedure, but they did not influence the detection of Agg-IgG using the IgG-specific reagent.

The amount of IgG precipitated from sera at a given concentration of PEG is affected by the dilution of serum used, the pH, and, to a lesser extent, by the ionic strength and amount of IgG initially present (24). All but the latter are easily controlled. There was no correlation between immunoglobulin concentrations and the results of the SBA in clinical specimens nor did the addition of a twofold excess Mon-IgG to NHS affect the detection of Agg-IgG in the SBA. With very high IgG levels such as that found in IgG myeloma sera, significant interference in tests that rely solely on PEG precipitation can result (25). In the SBA, the elution of Mon-IgG with rabbit IgG further minimizes the influence of IgG concentration and only one IgG myeloma sera tested in the SBA showed a positive result. This serum contained IgM RF that may have influenced the test.

RF factors are at least partially responsible for the IC detected in RA patients. There was a positive correlation between the levels of IC detected by the SBA and both IgM RF and IgG RF levels. Purified IgM RF when added to NHS results in a positive SBA test (Fig. 5C). Although IgM per se is not detected in the SBA, IgM RF may react as an IC with native IgG and facilitate the latter's precipitation in PEG or binding to StaphA. Comparison of paired synovial fluids and serum samples from RA patients showed a parallel increase in IgG RF and Agg-IgG equivalents detected in the SBA, whereas IgM RF did not fluctuate in parallel (Fig. 7) suggesting that fluctuations in IgG RF levels affect the levels of IC detected in the SBA. Preadsorption of antiglobulin ac-

TABLE IV

*Detection of HB_sAg in IC Isolated using the StaphA Binding Assay**

	Sera					
	Vasculitis (HB_sAg positive)		Acute hepatitis (HB_sAg positive)		NHS (HB_sAg negative)	Buffer
	Patient 1	Patient 2	Patient 1	Patient 2		
IC detected by SBA (Agg-IgG equivalents), *μg/ml*	275	260	55	6	0	0
Detection of antibody to HB_sAg using ^{125}I-HB_s antigen, *cpm*	54	48	25	ND‡	47	54
Detection of HB_sAg using ^{125}I-anti-HB_s, *cpm*	1,128	1,849	334	182	271	232
Detection of HB_sAg in 3 M NaSCN eluate from StaphA, *cpm*	1,954	4,924	159	194	56	146

* Sera processed in the SBA (Methods) but in addition to using ^{125}I anti-IgG reagent for detecting I Complexes, samples were incubated with ^{125}I-labeled HB_sAg or ^{125}I-labeled antibody to HB_sAg or a 3 M NaSCN eluate from the StaphA was analyzed for HB_sAg by radioimmune assay. All tests are means of duplicate determinations.

‡ ND, not done.

tivity from RF-positive sera reduced but did not abolish the detection of IC is most RA sera (Table II). At the same time, the adsorption procedure reduced but did not abolish RF activity (particularly IgG RF activity). There was not a clear correlation between the percentage of reduction in IC and the percentage of reduction in IgM or IgG RF. Therefore, whether IgG RF and IgM RF are solely responsible for the IC levels found in RA patients remains to be determined.

The SBA was compared with two other assays, the ClqBA and Raji cell assay, in terms of detection of IC in different antigen:antibody ratios (Fig. 8), the size complexes detected (Fig. 9), and sensitivity (Table III). The SBA was perhaps less affected by antigen:antibody ratios than were the other two assays. The SBA, and to a lesser extent, the Raji cell assay detected 11–18s Agg-IgG, whereas the ClqBA was negative in this range. All three tests tended to overestimate large sized complexes and underestimate small-sized complexes which is reflected in different sensitivity limits for different sized Agg-IgG.

The hepatitis B polyarteritis syndrome has many features of an IC disease and the SBA confirmed the presence of circulating complexes in two such patients. Radioimmunoassay analysis of the complexes isolated on and eluted from StaphA demonstrated that HB_sAg was a constituent of the complex (Table IV). However, we were not able to demonstrate the isolation of anti-HB_s antibody on the StaphA even though the results and appropriate controls indicate that the antigen was isolated by virtue of an immunoglobulin-reactive bridge. Anti-HB_s was also not detectable in sera from these patients, although antibody to the hepatitis B core antigen was found. It is conceivable that the detection of antibody is blocked by the presence of antigen or that its specificity is irrelevant to the antigen used in the anti-HB_s radioimmune assay. The SBA should have potential application to a number of disease states for isolating IC material on an insoluble substrate and for characterizing antigenic and immunoglobulin components.

The clinical value of the SBA relative to the myriad other tests for immune complexes remains to be determined. It represents a complement independent assay based on physicochemical principles that take advantage of the fact that IC are larger and contain more Fc sites than noncomplexed IgG. Unlike other tests that rely on cell receptor interactions, in the SBA active cellular processes are not involved and viable intact cells are not required. The test in quantitative, reproducible, and relatively unaffected by factors such as heparin, native antibodies, and immunoglobulin concentrations. The assay has the potential for isolating IC material on an insoluble substrate relatively free of other human serum components for antigen and immunoglobulin characterization of IC material.

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