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Research Article

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Isolation of Circulating Immune Complexes Using Raji Cells

SEPARATION OF ANTIGENS FROM IMMUNE COMPLEXES AND PRODUCTION OF ANTISERUM

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ABSTRACT Raji cells were used for the isolation of complement-fixing antigen-antibody complexes from serum. Immune complexes bound to these cells were radiolabeled at the cell surface with lactoperoxidase. The complexes were then eluted from the cells with isotonic citrate buffer pH 3.2 or recovered by immunoprecipitation of cell lysates. The antigen and antibody moieties of the complexes were isolated by dissociating sucrose density gradient centrifugation or by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A variety of preformed immune complexes were successfully isolated from serum with this approach. In addition, these techniques were used to isolate and identify the antigens in immune complexes in the serum of rabbits with chronic serum sickness and rats with Moloney virus-induced sarcomas.

Methods were also developed for the production of antisera against the antigenic moiety of immune complexes isolated from serum. Repeated challenge of rabbits with whole Raji cells with bound complexes or eluates from such cells resulted in antibody production against the antigens of the immune complexes, although reactivity against cellular and serum components was also elicited. Monospecific antisera against the antigens in immune complexes were produced by immunizing rabbits with the alum-precipitated antigen isolated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These techniques may be useful in isolating antigens in immune complex-associated diseases of unknown etiology.

INTRODUCTION

Immune complex (IC)[1] formation results from the sequence in which antibody combines with antigen during normal immune responses. Although this process usually benefits the host, ICs may cause phlogogenic reactions or interfere with immunologic mechanisms (1–4). With the refinement of the necessary techniques and the use of appropriate experimental models, the pathogenic role of circulating ICs in diseases of animals and man is becoming increasingly clear (5–11). Exogenous as well as endogenous antigens can trigger pathogenic immune responses (6), although in many IC diseases the responsible antigens are not identified. One means of identifying and characterizing these antigens would be the isolation of ICs. Once the ICs are isolated, the antigens might be identified directly or after the dissociation of antigens from antibody and the subsequent isolation of the antigenic moiety of the complex. The best sources from which to isolate ICs then are: (*a*) tissues with immune deposits, (*b*) serum with circulating ICs, and (*c*) mononuclear cells with ICs absorbed in vivo via surface Fc and C receptors. ICs deposited in tissues, notably in the kidneys, have been eluted with a variety of dissociating agents (7, 12–14), and their contents

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[1] *Abbreviations used in this paper*: BN, Brown-Norway rats; BSA, bovine serum albumin; C, complement; FITC, fluorescein isothiocyanate; HSA, human serum albumin; IC, immune complexes; MEM, Eagle's minimal essential medium; NHS, normal human serum; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate.

of both antigens and antibodies have been recovered, quantitated, and characterized in many diseases. However, because tissues are not always available and because ICs in serum do not always deposit in tissues, the alternative sources—serum and leukocytes—may provide a more convenient material for isolation of ICs.

This paper describes a method of isolating ICs from the serum with Raji cells that bind C-fixing ICs (15–17). After isolation, the ICs can be concentrated and characterized via appropriate immunochemical and electrophoretic techniques. Such preparations can then be used to raise antisera against the antigenic moiety of the IC.

METHODS

Cell lines. Raji cells (18) were cultured in Eagle's minimal essential medium (MEM) as has been described (15). Cell viability was determined by trypan blue exclusion. Previously, uptake experiments on synchronized cell cultures have indicated that receptors for IgG Fc and C on these cells are expressed equally well throughout the cell cycle (16). Wil2WT cultured cells are derived from the spleen of a patient with hereditary spherocytic anemia and are devoid of receptors for IgG Fc or human C.

Antigens and antisera. Bovine serum albumin (BSA) and human serum albumin (HSA) were purchased from Miles Laboratories Inc. (Elkhart, Ind.). The envelope glycoprotein gp70 and the major internal protein p30 from Rauscher murine leukemia virus were purified as described (19). Purified tetanus toxoid (lot 8086, 2,083 Lf/ml, 1,197 Lf/mg protein N) was prepared at the Department of Public Health, Division of Disease Control and Laboratory Services, State of Michigan, and supplied by Dr. G. R. Anderson. Human IgG, rabbit IgG, and human C3b were prepared as described (15, 16). Solutions of these proteins were prepared in phosphate-buffered saline (PBS).

Antiserum to purified Moloney virus p30 was prepared in Brown-Norway (BN) rats and supplied to us together with the antigen p30 by Dr. J. Jones (Scripps Clinic and Research Foundation, La Jolla, Calif.). Goat antisera to Moloney virus gp70 and to Rauscher virus p30 were obtained from the National Cancer Institute (National Institutes of Health, Bethesda, Md.). Tetanus immune human globulin (lot 14) was a gift from Dr. G. R. Anderson. Antiserum to BSA, to human IgG, and to goat gamma globulin were produced in rabbits, antiserum to rabbit IgG in sheep, and antiserum to HSA in mice. Monospecific antisera to purified C1q, C4, C3, C5, C6, C7, C8, C9, factor B, and properdin were prepared in rabbits or goats and provided by Dr. C. Arroyave (Scripps Clinic and Research Foundation). The IgG fraction of these antisera was used in some experiments and was isolated by DEAE-52-cellulose column chromatography (16). The IgG fraction of the rabbit anti-BSA serum and anti-C3 serum and of the sheep anti-rabbit IgG serum was conjugated with fluorescein isothiocyanate (FITC) as described (20).

Preparation of soluble ICs. Quantitative precipitin curves were constructed for the above antigen-antibody systems. A constant amount of antigen was mixed with trace amounts of ^{125}I-radiolabeled antigen and then reacted (37°C, 30 min and 4°C, overnight) with increasing amounts of the corresponding antiserum. The precipitates obtained after centrifugation were washed twice with cold physiologic saline and counted in a gamma counter. Subsequently, the precipitates were dissolved in 1 ml of 0.1 N NaOH, and the protein was quantitated by OD reading at 280 nm. The supernates containing soluble ICs at moderate antigen excess (see Results) were used in subsequent experiments. To assess the amount of soluble ^{125}I-BSA complexed to antibody at four times the antigen excess, precipitation with 50% ammonium sulfate was performed.

Labeling protein. Soluble proteins were labeled with ^{125}I or ^{131}I according to the procedure of McConahey and Dixon (21).

Uptake of ICs by cells. Binding of BSA-anti-BSA-C complexes to Raji cells was assessed as follows: the antigen was prepared by mixing trace amounts (2–5 μg) of ^{125}I-BSA with unlabeled BSA in physiologic saline. Then an adequate amount of antiserum to BSA was added to form ICs at four times the antigen excess. The mixture was incubated for 30 min at 37°C and 1 h at 4°C and centrifuged (3,000 rpm, 15 min). Thereafter, 3×10^7 Raji cells washed with Spinner's medium (MEM without Ca^{++} or protein) were incubated (37°C, 45 min) with increasing amounts of the supernate containing soluble ^{125}I-BSA-anti-BSA complexes in 200 μl of fresh normal human serum (NHS) (complement [C] source) and washed three times. Then radioactivity in the cell pellets was counted. ICs were incubated with C at 37°C for 30 min before being added to cells. In other experiments, increasing numbers of Raji cells (5×10^6–1×10^8) were incubated with a constant amount of ^{125}I-BSA-anti-BSA-C complexes, and uptake was determined. All determinations were performed in triplicate. Controls consisted of mixtures containing ^{125}I-BSA-anti-BSA-C but no cells, ^{125}I-BSA-anti-BSA in heated (56°C, 30 min) serum as well as cells incubated with ^{125}I-BSA mixed with normal nonimmune rabbit serum and C. The amount of antigen specifically bound to cells was determined after subtracting the nonspecific background counts observed in the controls.

Iodination of cells. Raji cells with or without bound ICs were iodinated by the lactoperoxidase (Calbiochem, San Diego, Calif.) cell-surface labeling technique of Phillips and Morrison (22), as modified by Kennel et al. (23). In this procedure peroxide and enzyme concentrations were adjusted to maximize incorporation of iodide while maintaining optimum cell viability. 3×10^7 cells were washed four times with Spinner's medium and incubated (37°C, 45 min) with Spinner's medium, pathological sera, normal sera, or sera supplemented with preformed ICs. Thereafter, cells were washed three times with Spinner's medium and twice with Earle's balanced salt solution without phenol red (Flow Laboratories, Inc., Rockville, Md.) supplemented with 10 μM Kl and finally adjusted to 3×10^7 cells/ml of Earle's-Kl solution. Lactoperoxidase was added from the stock solution (33 μM, 6 mg/ml) to a final concentration of 0.33 μM. After addition of an appropriate amount (500–700 μCi) of ^{125}I (50 mCi/ml; New England Nuclear, Boston, Mass.), the reaction was initiated by addition of 0.03% H_2O_2 to a final concentration of 44 μM. After 1 min, the same amount of H_2O_2 was added again. 1 min later the reaction was essentially terminated by dilution with 5 ml cold MEM and centrifugation. The cells were then washed in MEM five times to remove free iodine and enzyme. Cells carrying BSA-anti-BSA-C complexes and stained with FITC anti-BSA antibody before and after lactoperoxidase labeling showed the same intensity of immunofluorescence.

Recovery of cell-bound ICs. Two techniques were employed: (*a*) 3×10^7 Raji cells with bound ICs were incubated (37°C, 10 min) with 300 μl of freshly prepared isotonic citrate buffer, pH 3.2, supplemented with rabbit IgG (1 mg/ml). The ICs had been prepared with ^{125}I-labeled antigens or were labeled with ^{125}I at the cell surface by the lactoperoxidase technique. At the end of the incubation period, the tubes

were centrifuged, and the radioactivity associated with both cell pellet and supernate was determined. Controls included cells bearing ICs and incubated with MEM. The percent antigen released from cells incubated with ICs prepared with ^{125}I-antigen was calculated as follows: % antigen released = counts per minute in supernate/counts per minute in supernate + counts per minute in cell pellet × 100. To determine protein-associated counts in supernates, TCA precipitation was performed. (*b*) 3×10^7 Raji cells with bound radioiodinated ICs were solubilized with 1 ml of 0.05% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.) in 0.05 M Tris-HCl, 0.15 M NaCl, 10 mM EDTA, pH 8.0, for 1 h at room temperature. The supernates obtained after centrifugation (3,000 rpm, 20 min) were dialyzed overnight against 0.05 Tris-HCl, 0.15 M NaCl, 10 mM EDTA buffer, and divided into two equal portions. One equal portion was mixed with carrier nonimmune IgG (50 μg) homologous to that used as antibody in the ICs. The other aliquot was the control and was supplemented with heterologous IgG (50 μg). Both aliquots were then treated at equivalence with the respective anti-carrier IgG sera. The precipitates obtained after incubation for 2 h at 37°C and overnight at 4°C were washed twice with PBS and three times with H_2O and lyophilized.

Separation of antigens from antibodies. Three techniques were employed: (*a*) Technique I: ^{125}I-surface-iodinated Raji cells with bound ICs were eluted with isotonic citrate buffer supplemented with rabbit IgG. The eluates then were dialyzed overnight against citrate buffer, supplemented with trace ^{131}I-labeled marker proteins, and fractionated in a linear 10–25% sucrose density gradient in dissociating citrate buffer for 15 h at 135,000 *g*. 10-drop fractions were collected and counted in a two-channel gamma counter. In some experiments, fractions corresponding to radioactive peaks were pooled and dialyzed overnight against PBS. Pools containing antibody or antigen were applied separately or after recombination and incubation (30 min at 37°C and 1 h at 4°C) to a 10–37% linear sucrose density gradient in PBS and centrifuged for 16 h at 81,800 *g*. (*b*) Technique II: ^{125}I-Surface-iodinated Raji cells with bound ICs were treated with isotonic citrate buffer as in technique I. The eluates were dialyzed for 6 h against 8 M urea-1% sodium dodecyl sulfate (SDS), supplemented with ^{131}I-labeled marker proteins and heated at 100°C for 2 min. Samples were then electrophoresed according to the method of Laemmli (24) on 7.5% polyacrylamide gels in SDS. (*c*) Technique III: Lyophilized immunoprecipitates obtained from lysates of ^{125}I-surface-iodinated Raji cells with bound ICs were solubilized in 300 μl of 8 M urea-1% SDS solution, supplemented with ^{131}I-labeled marker proteins, and subjected to SDS-polyacrylamide electrophoresis (PAGE) analysis. In techniques II and III, gels were sectioned into 1-mm slices with an automatic slicer (Loyce Loebl gel slicer, The Mickle Laboratory Engeen, Inc., Eng.) and counted in a two-channel gamma counter. In some experiments radioactive gel slices were incubated overnight in 0.2 ml H_2O, and counts released were determined.

Pathological sera. Sera from rabbits with experimentally induced chronic serum sickness to BSA were obtained before and 10 min after the daily injection of BSA. These sera were kindly supplied by Dr. C. B. Wilson (Scripps Clinic and Research Foundation). The amount of BSA injected into these rabbits was predetermined on the basis of their anti-BSA antibody titers (25).

Sera from BN rats receiving 2×10^7 syngeneic Moloney-induced sarcoma tumor cells were obtained before and at different times after tumor transplantation. The rat sera were provided by Dr. C. Jennette (Scripps Clinic and Research Foundation) and assessed for levels of ICs (expressed as micrograms aggregated rat IgG equivalent per milliliter serum) by a modification of the Raji cell assay (26).

Production of antisera against antigens in ICs. Three methods were used to produce antisera against antigens in isolated ICs. (*a*) Technique I: 4×10^7 Raji cells were incubated with 200 μl NHS containing 150 μg tetanus toxoid complexed with human antitetanus toxoid serum or 184 μg BSA complexed with rabbit anti-BSA serum at four times the antigen excess. Thereafter, cells were washed eight times with physiologic saline, resuspended in 1 ml saline, and injected i.v. into adult New Zealand white rabbits. This procedure was repeated twice at 2-wk intervals, and animals were bled 1 wk after the last injection. Rabbits to receive Raji cells with bound tetanus toxoid-human antitetanus toxoid-C complexes were first rendered tolerant to human IgG by two i.v. injections 3 wk apart with 10 mg deaggregated human IgG. Tolerance was confirmed by the rate at which the animals cleared i.v.-injected radiolabeled IgG 15 days after the last injection (27). The same dose of deaggregated IgG was given i.v. at the time of immunization with cell-bound ICs. (*b*) Technique II: 4×10^7 Raji cells were incubated with tetanus toxoid-antitetanus toxoid-C complexes or BSA-anti-BSA-C complexes as above. Thereafter, cells were washed eight times and cell-bound ICs were eluted with isotonic citrate buffer supplemented with rabbit IgG. The eluates were then dialyzed against saline and mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Rabbits were injected every 2 wk alternately in the front and back footpads and in the back on three occasions. 1 wk after the last immunization, animals were bled. Rabbits receiving tetanus toxoid-antitetanus toxoid-C complexes were rendered tolerant to human IgG before immunization. (*c*) Technique III: 4×10^7 Raji cells were incubated with 280μg HSA complexed at four times the antigen excess with mouse anti-HSA in 100 μl normal mouse (Swiss) serum as a source of C. Cells were then washed, labeled with ^{125}I by the lactoperoxidase method, washed again, and eluted with citrate buffer; then dialyzed eluates were subjected to SDS-PAGE. Subsequently, gel slices containing the radioactivity corresponding to HSA were eluted by overnight incubation with 200 μl H_2O. Eluates obtained from three such gels were pooled, supplemented with rabbit IgG (1 mg/ml), and precipitated with a 1% final solution of aluminum chloride at pH 7.8.[2] The precipitates were resuspended in 1 ml saline, and injected as above in the footpads and backs of rabbits twice at 2-wk intervals. 1 wk after the last immunization the animals were bled.

RESULTS

Binding of immune complexes with fixed C to Raji cells. Binding of soluble ^{125}I-BSA-anti-BSA-C complexes formed at four times the antigen excess and added in increasing amounts to a constant number (3×10^7) of Raji cells is expressed as micrograms of the added BSA that specifically bound to the washed cells (Fig. 1). These results show that at saturation approximately 6 μg antigen were bound per 3×10^7 cells. Over 98% of the cells were viable as determined by trypan blue exclusion. All cells were strongly immunofluorescence positive when stained with FITC anti-BSA antibody. Cells incubated with ^{125}I-BSA-

[2] Lazarides, E. Personal communication.

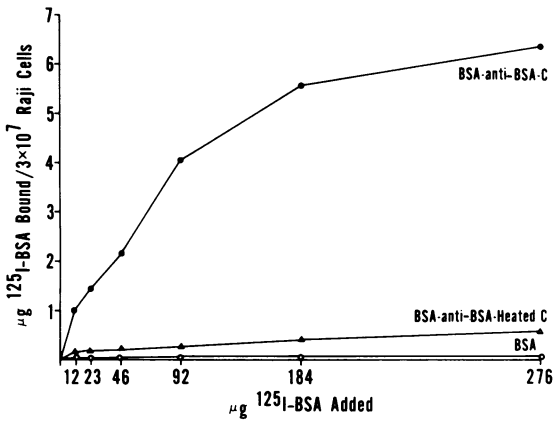

FIGURE 1 Binding of ^{125}I-BSA-anti-BSA-C complexes to Raji cells. 3×10^7 cells were incubated (37°C, 30 min) with increasing amounts of ^{125}I-BSA-anti-BSA soluble complexes at four times the antigen excess which had first been incubated (37°C, 30 min) with 200 μl of fresh (● —— ●) or heated (△ —— △) NHS. Cells were also incubated with the antigen BSA alone (○ —— ○).

anti-BSA in heated (56°C, 30 min) serum showed minimal uptake and were immunofluorescence negative. BSA alone did not bind to cells.

When 5×10^6–8×10^7 cells were incubated with ^{125}I-BSA-anti-BSA-C soluble complexes containing 184 μg of antigen (four times the antigen excess), a linear relationship was found between the number of cells present in the incubation mixture and the amount of labeled BSA specifically bound to them (Fig. 2). In addition 70% of the soluble antigen was found by ammonium sulfate precipitation to be complexed with antibody and 12% of the antigen could be precipitated

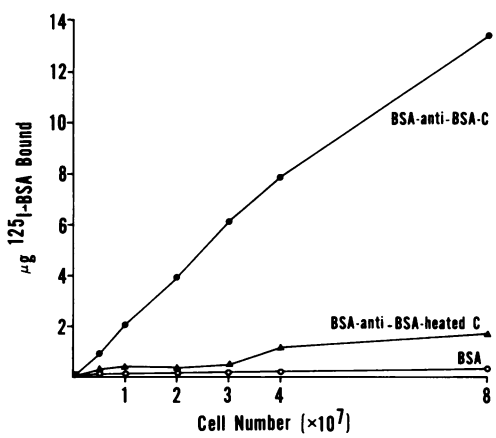

FIGURE 2 Binding of ^{125}I-BSA-anti-BSA-C complexes to Raji cells. 5×10^6–8×10^7 cells were incubated (37°C, 30 min) with 184 μg ^{125}I-BSA-anti-BSA in 200 μl of fresh (● —— ●) or heated (△ —— △) NHS. Cells were also incubated with 184 μg ^{125}I-BSA alone (○ —— ○).

with excess rabbit antihuman C3 antibody. From these findings and the uptake experiment shown in Fig. 2, we concluded that even without reaching plateau, > 50% of the ICs that fix C bind to 8×10^7 Raji cells.

Recovery of Raji cell-bound ICs. To recover Raji cell-bound ICs, we used citrate buffer elution or immunoprecipitation. As shown in Fig. 3, upon incubation of Raji cells with bound ^{125}I-BSA-anti-BSA-C complexes with isotonic citrate buffer for 10 min at 37°C, >60% of the cell-bound antigen was released. Trypan blue stained < 5% of the treated cells. Longer incubation with the buffer did not result in further release, but resulted in significant cell death. After overnight dialysis of the eluate against PBS and subsequent treatment with 50% ammonium sulfate, 99.8% of the ^{125}I-BSA was precipitated. Upon lactoperoxidase surface iodination of Raji cells with bound BSA-anti-BSA-C complexes and subsequent treatment with citrate buffer, 4.5% of the cell-associated radioactivity was released. After overnight dialysis against PBS, 32% of the released radioactivity was retained. This nondialyzable material was >90% precipitable by either 10% TCA or 50% ammonium sulfate. In contrast, when Raji cells were incubated in Spinner's medium alone, surface labeled, and eluted with citrate buffer, 4% of the cell-associated radioactivity was released, but of this only 3% was not dialyzable. Raji cells eluted of bound BSA-anti-BSA-C complexes were immunofluorescence negative when stained with FITC anti-

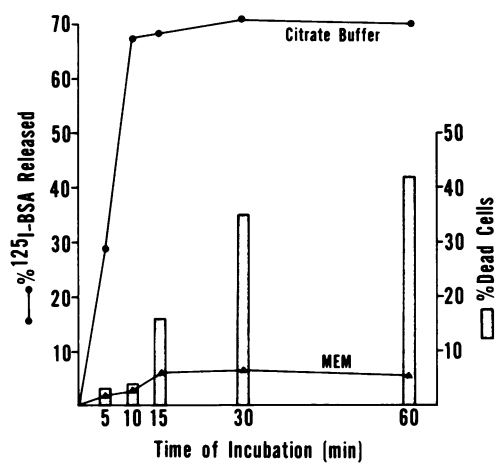

FIGURE 3 Release of cell-bound ^{125}I-BSA-anti-BSA-C complexes by isotonic citrate buffer. 3×10^7 cells were incubated with 184 μg ^{125}I-BSA-anti-BSA-C complexes. The washed cells were then treated for varying times at room temperature with 300 μl of isotonic citrate buffer pH 3.2 (● —— ●) or 300 μl of MEM (▲ —— ▲). Supernates obtained after centrifugation were counted, and percent antigen released was determined. The cell pellets were resuspended in medium, and viability was determined by trypan blue exclusion (shaded column).

BSA or FITC anti-rabbit IgG. These findings indicate the efficiency of a brief treatment with a low pH buffer in removing cell-bound ICs without removing cell-membrane macromolecular structures.

To ascertain whether citrate buffer treatment also results in the release of IC-associated cell-bound C3b or C3d, two types of experiments were performed. In the first, Raji cells were incubated (37°C, 30 min) with 50 μg ^{125}I-radiolabeled purified C3b, washed, and treated with MEM or with citrate buffer for 10 min at 37°C. Measurement of radioactivity in the supernate indicated that only 1.3% of the cell-bound C3b had been released. In the second experiment, cells were incubated with BSA-anti-BSA-C complexes, washed, and treated with MEM or citrate buffer. Subsequently, the washed cells were incubated (4°C, 30 min) with FITC anti-C3 antibody. The cells treated with either MEM or with citrate buffer were equally immunofluorescence positive. However, the same citrate buffer-treated cells, in contrast to MEM-treated cells, were immunofluorescence negative when stained with FITC anti-BSA antibody or FITC anti-rabbit IgG. These results indicate that treatment with citrate buffer sufficient to release cell-bound ICs does not affect cell membrane receptor-bound C3.

For the recovery of Raji cell-bound ICs an immunoprecipitation technique was also employed. Cells with bound ^{125}I-BSA-rabbit anti-BSA-C complexes were washed and solubilized with Triton X-100. After centrifugation, the soluble material was dialyzed, supplemented with carrier rabbit IgG, and treated with sheep anti-rabbit IgG at equivalence. Greater than 91% of the cell-bound antigen was recovered in the precipitate. Incubation of a similar cell lysate with carrier human IgG followed by rabbit antihuman IgG resulted in precipitation of only 8% of the radiolabeled antigen. Specific rabbit IgG immunoprecipitates obtained from lysates of lactoperoxidase surface-iodinated Raji cells with bound BSA-anti-BSA-C complexes contained 2.8% of the total cell lysate-associated radioactivity, whereas nonspecific human IgG precipitates contained 0.2% of the total cell lysate-associated radioactivity.

Separation of antigens from antibodies. We employed dissociating sucrose density gradients and SDS-PAGE to separate antigens from their antibodies in the ICs released from Raji cells. In technique I, cells were incubated with serum containing preformed ICs, radiolabeled with ^{125}I by the lactoperoxidase method and subsequently eluted with isotonic citrate buffer. The dialyzed eluates were then ultracentrifuged through a 10–25% sucrose density gradient in citrate buffer. The results obtained using this technique with two antigen-antibody systems (BSA-anti-BSA-C, p30-anti-p30-C) are shown in Fig. 4. Only peaks of ^{125}I radioactivity cosedimenting with ^{131}I-internal marker

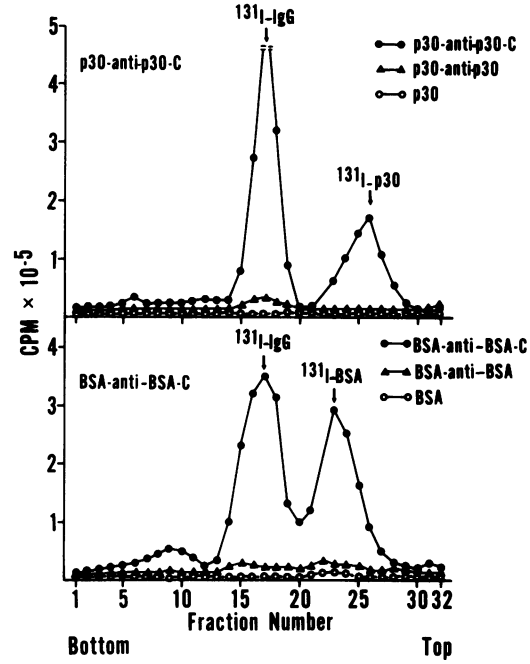

FIGURE 4 Fractionation of ICs eluted from Raji cells by dissociating sucrose density gradients. 3 × 10^7 Raji cells were incubated with 100 μg p30-goat anti-p30 complexes at twice the antigen excess or 184 μg BSA-anti-BSA complexes at four times the antigen excess in 200 μl of fresh NHS (● —— ●). Control cells were incubated with the same amounts of complexes without serum (△ —— △) or with antigen alone (○ —— ○). Cells were washed, surface labeled with ^{125}I by the lactoperoxidase method, washed again, and eluted with 300 μl of citrate buffer. The eluates were dialyzed against citrate buffer, supplemented with ^{131}I-marker antigen and IgG, applied on a 10–25% sucrose gradient in citrate buffer, and ultracentrifuged (15 h, 135,000 *g*). 10-drop fractions were collected from the bottom and counted in a two-channel gamma counter. The position of the ^{131}I markers is indicated by the arrows.

antigens and antibodies are observed. By incubating cells with serum containing various amounts of BSA-anti-BSA complexes, it was found that at least 100 μg BSA complexed with antibody per milliliter serum (i.e. 20 μg added to cells) were necessary to produce distinct peaks of radioactivity. From 46–63% of the released antigen was recovered after centrifugation of ^{125}I-BSA-anti-BSA-C complexes. Upon recovery of the antigen and antibody from the gradient and their subsequent neutralization, recombination, and ultracentrifugation in neutral sucrose density gradient, radioactivity shifted toward the bottom of the gradient associated with disappearance of the radioactive peaks that represented the uncombined antigen and antibody (Fig. 5). This finding indicates the capacity of the recovered reactants to recombine and form ICs.

To improve the separation of antigen and antibody bound as IC to Raji cells, other techniques were developed using SDS-PAGE. Two types of samples were

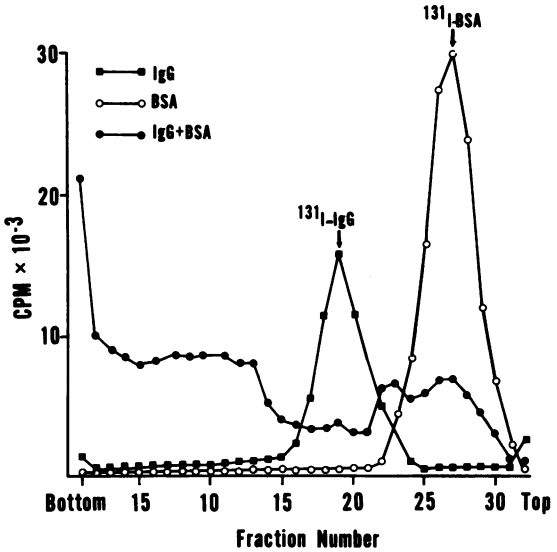

FIGURE 5 Recombination of antigen and antibody eluted with citrate buffer from Raji cells. Cells were incubated with 184 μg BSA-anti-BSA-C complexes, washed, surface iodinated, and eluted with citrate buffer. The eluate was dialyzed against citrate buffer and fractionated into antigen and antibody by ultracentrifugation in a dissociating sucrose density gradient. Antigen and antibody recovered from the gradient were dialyzed against PBS pH 7.2. Subsequently, the antigen (○ —— ○) and antibody (■ —— ■), alone or in combination (● —— ●), were applied to a neutral (pH 7.2) 10–37% sucrose density gradient and ultracentrifuged (16 h, 81,800 *g*). 10-drop fractions were collected and counted. Note the shift of the radioactivity toward the bottom of the gradient associated with the disappearance of the radioactive peaks that represented the uncombined antigen and antibody. The position of the ^{131}I markers is indicated by the arrows.

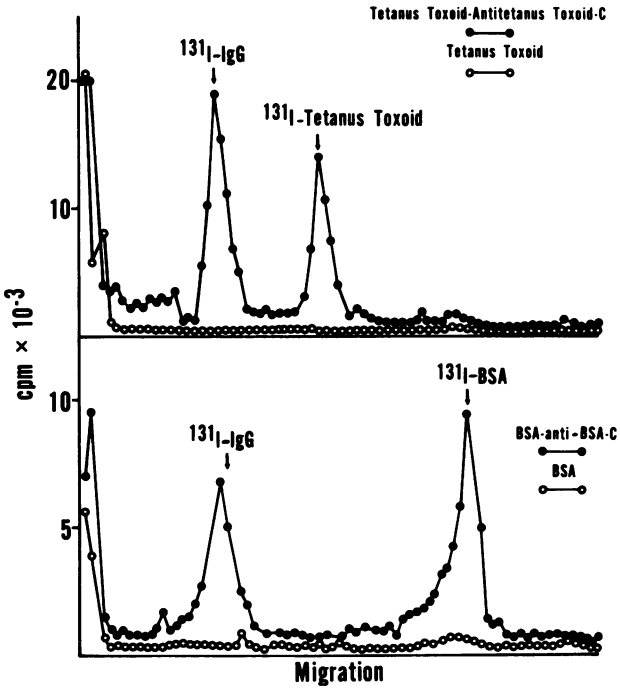

FIGURE 6 Fractionation of ICs eluted with citrate buffer from Raji cells by SDS-PAGE. 3×10^7 Raji cells were incubated with 100 μg tetanus toxoid-antitetanus toxoid-C complexes or 184 μg BSA-anti-BSA-C complexes at four times the antigen excess. The washed cells were surface radioiodinated with ^{125}I by the lactoperoxidase method and washed again. The cells were then eluted with 300 μl of citrate buffer. Eluates were dialyzed against 8 M urea-1% SDS, supplemented with ^{131}I-labeled markers antigen and IgG, and subjected to SDS-PAGE analysis (● —— ●). Control eluates were obtained from cells that had been incubated with the antigen alone (○ —— ○). Gels were sectioned and counted in a two-channel gamma counter. Arrows indicate the position of the ^{131}I markers.

assessed; citrate buffer eluates obtained from surface-iodinated IC-bearing Raji cells (technique II) and immunoprecipitates obtained from lysates of such cells (technique III). The results obtained with these techniques with Raji cells that had absorbed BSA-anti-BSA and tetanus toxoid-antitetanus toxoid complexes from NHS are presented in Figs. 6 and 7. As indicated by both methods, peaks of ^{125}I radioactivity comigrating with ^{131}I-labeled IgG and antigen markers were observed with excellent resolution. Control eluates or immunoprecipitates obtained from cells incubated with ICs in heated serum or antigen alone did not demonstrate ^{125}I peaks, nor did immunoprecipitates obtained from lysates of IC-bearing cells treated with a heterologous IgG-anti-IgG system, indicating little or no nonspecific entrapment of antigens (Fig. 7). Addition of varying amounts of BSA-anti-BSA-C or HSA-anti-HSA-C complexes to 3×10^7 Raji cells indicated that under the conditions employed a minimum of 25 μg of complexed antigens per milliliter serum (5 μg added to cells) were needed to identify peaks of radioactive antigens and antibody.

To determine the efficacy of the SDS-PAGE in recovering antigen, ^{125}I-labeled BSA was reacted with antibody and C and bound to the surfaces of Raji cells. These cells were then processed as in the lactoperoxidase labeling method but without the addition of further ^{125}I. After elution and SDS-PAGE, from 67–73% of the released antigen was recovered, whereas after solubilization-immunoprecipitation and SDS-PAGE, from 79–85% of the immunoprecipitated antigen was recovered in the gels. Greater than 95% of the polyacrylamide gel-associated antigen was eluted in H_2O.

Isolation and characterization of antigens in IC absorbed to Raji cells from pathological sera. The techniques used to isolate preformed in vitro ICs in sera were next applied to isolating ICs from pathological animal sera. We first examined sera from three rabbits with BSA-induced chronic serum sickness. Fig. 8 pictures the profile of citrate buffer eluates obtained from Raji cells incubated in sera of a representative one of these rabbits. Peaks of ^{125}I radioactivity

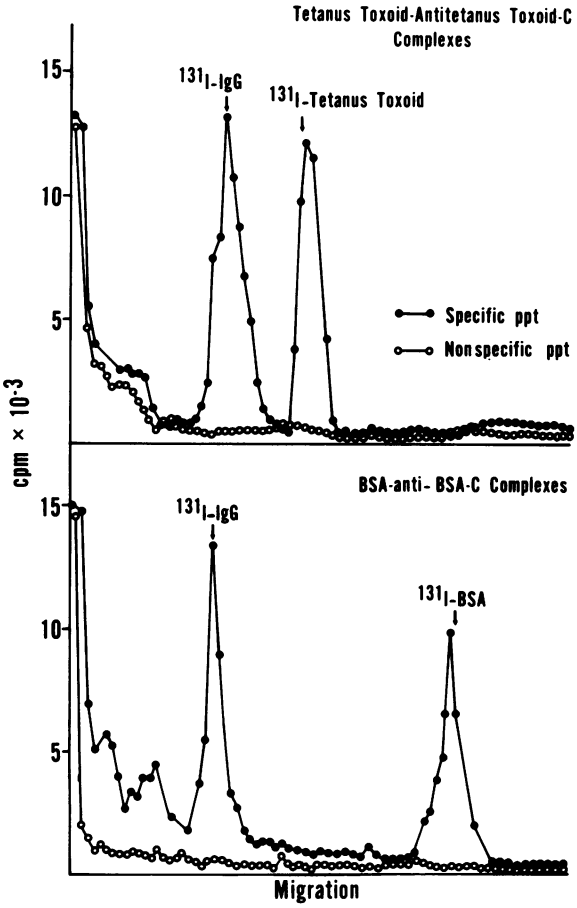

FIGURE 7 Fractionation of ICs immunoprecipitated from Raji cell lysates by SDS-PAGE. 3×10^7 Raji cells were incubated with tetanus-antitetanus toxoid-C or BSA-anti-BSA-C complexes. The washed cells were surface radioiodinated with ^{125}I by the lactoperoxidase method and washed again. Subsequently, the cells were solubilized with Triton X-100. The supernates obtained after centrifugation were dialyzed and divided into two equal portions. One equal portion was mixed with carrier nonimmune IgG (50 μg) homologous to that used as antibody in the complex (● —— ●). The other equal portion was the control and was supplemented with heterologous IgG (50 μg) (○ —— ○). Both equal portions were then treated at equivalence with the respective anti-IgG sera. The immunoprecipitates (ppt) were lyophilized, resuspended in 300 μl 8 M urea-1% SDS, supplemented with ^{131}I markers antigen and IgG, and subjected to SDS-PAGE analysis. Gels were sectioned and counted in a two-channel gamma counter. The position of the markers is indicated by the arrows.

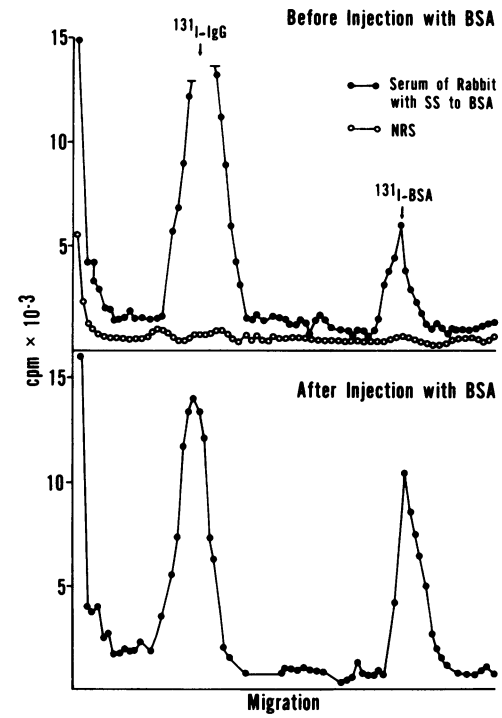

FIGURE 8 Isolation of ICs from sera of rabbits with BSA-induced chronic serum sickness (SS). 3×10^7 Raji cells were incubated with 200 μl of rabbit serum obtained immediately before and 10 min after the daily injection with BSA (● —— ●). Control cells were incubated with normal rabbit serum (NRS) (○ —— ○). Cells were washed and labeled with ^{125}I by the lactoperoxidase method and washed again. These cells were then eluted with citrate buffer, eluates were dialyzed vs. 8 M urea-1% SDS, supplemented with ^{131}I markers, and subjected to SDS-PAGE analysis. The position of the markers is indicated by the arrows.

corresponding to ^{131}I-IgG and BSA markers are apparent. Eluates from cells incubated in sera obtained either before or after an injection of BSA demonstrate the antigen peak, which is more pronounced in the latter. Control eluates derived from surface-iodinated cells incubated with MEM, BSA, or normal rabbit serum in addition to serum from a normal rabbit injected with BSA did not show similar peaks of radioactivity. Eluates derived from iodinated Raji cells first incubated with the IgG fraction of a rabbit anti-BSA serum showed a minor peak corresponding to the marker IgG (Fc receptor binding) without a peak corresponding to the BSA marker.

Sera obtained serially from four BN rats receiving syngeneic Moloney sarcoma tumors were similarly assessed. Fig. 9 shows a typical representative example. Before tumor transplantation this rat's serum was negative for ICs, and no peaks of radioactivity were observed upon SDS-PAGE analysis of the Raji cell citrate buffer eluates. However, analysis of the eluate derived from cells incubated with an IC-positive serum sample obtained from the same animal 27 days postinoculation with tumor cells showed four major peaks of ^{125}I radioactivity. Peak I comigrated with the ^{131}I marker IgG, peak II corresponded to a mol wt of 115,000 daltons, peak III comigrated with the marker gp70,

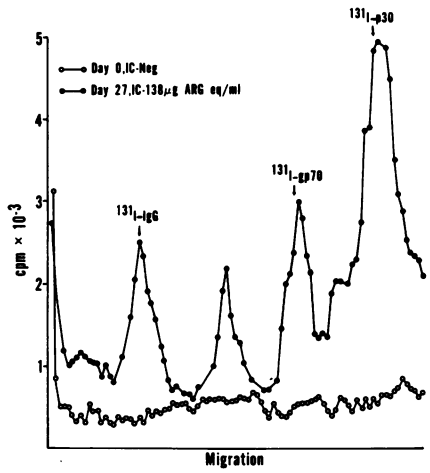

FIGURE 9 Isolation of ICs from sera of BN rats with syngeneic Moloney-induced sarcomas. 3×10^7 Raji cells were incubated with 200 μl of rat serum obtained before (day 0, IC-neg; O —— O) and 27 days after (IC-138 μg aggregated rat IgG eq/ml; ● —— ●) tumor transplantation. Cells were washed, labeled with ^{125}I by the lactoperoxidase method, washed again, and eluted with citrate buffer. Eluates were dialyzed vs. 8 M urea-1% SDS, supplemented with ^{131}I markers and subjected to SDS-PAGE analysis. The position of the markers is indicated by the arrows.

and peak IV comigrated with the marker p30. The ^{125}I peaks became more pronounced at 40 days post-inoculation when the animal had a large tumor and higher levels of circulating ICs.

To demonstrate that the ^{125}I peaks comigrating with the ^{131}I-radiolabeled gp70 and p30 markers were related to Moloney virus gp70 and p30 the following procedure was performed. Gel slices corresponding to gp70 and p30 markers were eluted with H_2O; eluates were supplemented with rabbit IgG (1 mg/ml) and dialyzed against PBS for 6 h. Subsequently, the eluates were divided into two equal portions; one was treated with goat anti-Moloney gp70 or goat anti-Rauscher p30 and rabbit anti-goat antibody at equivalence and the other with normal goat serum and rabbit anti-goat IgG antibody. 38% of the ^{125}I counts eluted from the slices corresponding to the gp70 marker and 53% of the ^{125}I counts eluted from the slices corresponding to p30 marker coprecipitated with the corresponding goat antisera as opposed to 6.7 and 1.3% with the normal goat serum. This finding indicated that the ^{125}I peaks III and IV represented Moloney virus envelope gp70 and core p30 proteins. To demonstrate that Raji-cell bound gp70 and p30 were complexed with IgG, the citrate buffer eluate obtained from surface-iodinated Raji cells, which had been incubated with an IC-containing rat serum, was neutralized by overnight dialysis against PBS and treated with 50% ammonium sulfate. The supernate obtained after removing the precipitate by centrifugation was dialyzed against 8 M urea-1% SDS, supplemented with ^{131}I-marker proteins and subjected to SDS-PAGE analysis. In contrast to the nonammonium sulfate-treated eluate obtained from surface-iodinated Raji cells incubated with the same serum, the ammonium sulfate-treated eluate did not show ^{125}I-labeled peaks of gp70 and p30 upon SDS-PAGE analysis. Only 15% of noncomplexed isolated ^{125}I-gp70 and ^{125}I-p30 in PBS were precipitated after treatment with 50% ammonium sulfate. In addition, eluates obtained from surface-iodinated Raji cells incubated in 5 μg purified gp70 or 5 μg purified p30 demonstrated no peaks of radioactivity, indicating that these isolated proteins did not bind by themselves to Raji cells. The nature of peak II was not determined.

Production of antisera against antigens in Raji cell-bound ICs. The reactivity of tetanus toxoid against serum obtained from a rabbit immunized i.v. with whole Raji cells with bound tetanus toxoid-anti-tetanus toxoid-C complexes (technique I) is illustrated in Fig. 10. In the same figure the reactivity of tetanus toxoid against serum obtained from a rabbit immunized with complete Freund's adjuvant mixed with saline-dialyzed citrate buffer eluate derived from Raji cells with bound complexes (technique II) is also shown. The antiserum against whole IC-bearing Raji cells was more potent (1:128 antibody titer) than the antiserum against the eluate (1:8 antibody titer) as determined by Ouchterlony analysis. Additionally, the antiserum against whole IC-bearing Raji cells contained anti-Raji cell antibodies demonstrated by a complement-dependent cytotoxicity test. Antiserum raised against the eluate lacked reactivity with Raji cells. Neither antisera reacted with isolated human IgG indicating that the rabbits were tolerant to human IgG. However, both antisera produced two precipitin lines upon double immunodiffusion with NHS. The first of the two lines was caused by antibodies to HSA as shown by identity with an anti-HSA serum upon double immunodiffusion against NHS. The second, thinner line showed non-identity with IgM, IgA, and most C components and could not be identified further. However, both lines could completely be removed by one absorption of the antisera with glutaraldehyde-insolubilized NHS. Control animals injected i.v. with washed Raji cells incubated with tetanus toxoid alone or tetanus toxoid-antitetanus toxoid in heated serum did not produce antibodies to the antigen.

Antiserum to BSA was obtained similarly by injecting rabbits i.v. with whole BSA-anti-BSA-C bearing Raji cells or with citrate buffer eluates obtained from Raji cells bearing BSA-anti-BSA-C complexes. Control animals injected with washed Raji cells incubated in

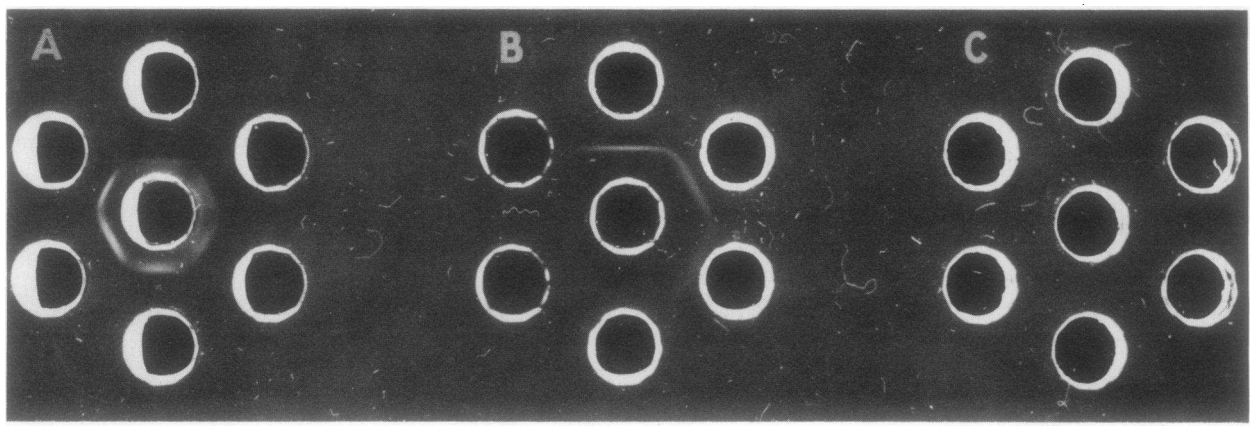

FIGURE 10 Production of antibodies to antigens in isolated immune complexes. Sera were obtained from rabbits tolerant to human IgG and that had been injected with either whole Raji cells carrying tetanus toxoid-human antitetanus toxoid-C complexes (A), with citrate buffer eluates from Raji cells carrying the complexes (B), or with whole Raji cells incubated with tetanus toxoid alone (C). Peripheral wells contain serial twofold dilutions of the respective rabbit antisera, and central wells contain tetanus toxoid (1 mg/ml).

Spinner's medium, BSA alone, or BSA-anti-BSA in heated serum failed to produce anti-BSA antibodies. Moreover, animals injected with washed Wil2WT cells (devoid of Fc and C receptors) incubated with BSA-anti-BSA-C complexes did not produce anti-BSA antibodies.

To avoid antigenic competition and to produce antisera that would react only with the antigen in the IC, another approach was utilized (technique III). In this method, eluates obtained from surface-iodinated Raji cells bearing HSA-anti-HSA-C complexes were first electrophoresed on SDS-polyacrylamide gels. The ^{125}I radioactivity corresponding to the trace ^{131}I-HSA marker was eluted, and the eluates from three such gels were combined and precipitated with alum. The precipitates were solubilized in saline and injected into rabbits. An antiserum was produced that reacted only with HSA but not with the cells or any component in normal mouse serum used as a source of antibody and C.

DISCUSSION

We have developed procedures by which antigens in ICs can be isolated from serum. First, C-fixing ICs were separated from serum and concentrated on C receptor-bearing Raji cells. Then, these cell-bound ICs were radiolabeled and eluted from intact cells or immunoprecipitated from cell lysates. Finally, the constituent antigens and antibodies were separated under dissociating conditions.

Past efforts to detect and isolate circulating ICs involved such physicochemical methods as gradient centrifugation, column chromatography, and precipitation in the presence of polyethylene glycol, ammonium sulfate, or low temperature. Recently, methods involving more specific interactions have been developed for the detection of ICs. These methods utilize serum proteins such as C1q (28), rheumatoid factor (9), and conglutinin (29, 30), as well as cells with Fc and C receptors (3, 16). The selective affinity of these materials for ICs makes them particularly attractive for use in attempts to isolate ICs. We have chosen Raji cells because they possess large numbers of high affinity receptors for C (15, 16) and, as seen in the present studies, they bind sufficient quantities of C-fixing ICs to allow further analysis. Of course, the uptake of ICs by Raji cells, as previously shown (15, 16), will vary according to the size of the complexes and their ability to fix C3. The purpose of this work was not only to isolate ICs but also to explore and develop techniques for IC characterization.

ICs bound to Raji cells were released from the cells by brief treatment with low pH isotonic citrate buffer. This method has previously been used to release tissue-deposited ICs (12–14), IgG cytophilically absorbed on mononuclear cells (31), and antibodies and putative ICs bound to tumor cells (32, 33). In our system we were able to remove >60% of bound ICs without cell death. In addition, our study with surface-iodinated IC-bearing Raji cells indicated that little cellular material was released along with the ICs. For example, control eluates obtained from surface-iodinated Raji cells that had not been exposed to complexes showed no peaks of radioactivity upon SDS-PAGE. Nor were peaks of radioactivity other than those due to antigen and antibody observed in the SDS-PAGE analysis of eluates from surface-iodinated Raji cells with bound ICs.

No detailed studies are available to date as to the

binding of C components on soluble complexes. It is of interest that Raji cell-bound soluble C3b and IC-fixed C3b were not released by the low pH buffer treatment of these cells. The strong affinity of C3b for the immune adherence receptor on lymphoid cells has been previously documented (15). Therefore, we may conclude that C fragments were not detected on the SDS-PAGE analysis of ICs eluted from Raji cells either because they remain firmly attached on the Raji cell surface or because they are present in insufficient quantities to be detected by our procedures.

ICs bound to Raji cells were also recovered after solubilizing the cells with detergent and precipitating ICs by anti-IgG antibody. The cell solubilization-immunoprecipitation technique, although technically more complicated, appears to be superior to citrate buffer elution in recovering the antigen in ICs. Moreover, the SDS-PAGE technique gave better separation of antigens from their antibodies in ICs than the sucrose density gradient technique. However, the reactants recovered after dissociating sucrose density gradient fractionation retained their capacity to recombine and form ICs. This permitted us to demonstrate that materials absorbed on Raji cells from serum are indeed ICs.

In our SDS-PAGE system we used 7.5% gels, with an expected exclusion limit of 180,000 daltons. This presented no difficulty because the antigens used to prepare in vitro models of ICs were <180,000 daltons in molecular weight. However, these gels could fail to demonstrate large unknown antigens in IC-containing sera. On the other hand, we might expect many pathological ICs to contain small antigens because isolated viral and cellular antigens have been found in general to have a mol wt of 100,000 daltons or less. It could be considered that for initial screening purposes use of dissociating sucrose density gradients or 4–10% gradient SDS-polyacrylamide gels may be preferable.

The methods developed in this study were used to isolate ICs from sera of rabbits with serum sickness and from rats with syngeneic Moloney sarcoma tumors, both models of human diseases for which these procedures should be useful. In the serum sickness model it was possible to isolate BSA-anti-BSA complexes and to separate the antigen from the antibody. The demonstration of ICs in eluates derived from Raji cells incubated with rabbit sera obtained before and after the daily injection of the antigen indicates the efficacy of these cells in absorbing ICs at both antibody and antigen excess.

Jennette and Feldman (26) and Jones et al. (34), using Moloney sarcoma virus-induced tumors in rats, have developed an excellent model of IC-associated malignancy. In our study of the sera of such rats, we have indicated that gp70 and p30 are present in the circulating ICs. Thus, SDS-PAGE analysis of Raji cell-eluted material showed 70,000 and 30,000 mol wt peaks that are partially immunoprecipitated by antisera to gp70 and p30, respectively. These peaks are absent if the eluates are precipitated with ammonium sulfate before electrophoresis. In addition, we demonstrated that neither gp70 nor p30 alone bound to Raji cells. Of course, our present demonstration of gp70-anti-gp70 and p30-anti-p30 ICs in these sera does not exclude the possible presence in low levels of other IC systems composed of alloantigens or tumor-specific antigens and their corresponding antibodies.

Although Raji cells are obviously useful in recovering ICs from sera, it would be difficult by this technique to isolate large quantities of ICs for detailed studies. Therefore, we considered it important to develop techniques for producing antisera against antigens in isolated ICs. With whole IC-bearing Raji cells or eluates from these cells, antisera were produced against both a relatively poor immunogenic material (BSA) and a strongly immunogenic one (tetanus toxoid). The production of antiserum against BSA with small quantities ($\cong$6–7 μg) of cell-bound or soluble complexed antigen is in accord with other studies which indicate that complexed antigens can be more immunogenic than free antigens (35). In the case of free BSA, injection of $\cong$100 μg of antigen has been found to be necessary for induction of antibodies (36). Although only two rabbits were looked at with each technique, the cell-bound complexed antigens appear to be superior to eluted complexed antigens in the production of antisera. This may be the result of different handling or of the presence of higher amounts of antigen on cells than in eluates. However, injection of whole cells or eluates is complicated by the formation of antibodies against other serum proteins and Raji cells. Monospecific antiserum to antigens in isolated ICs from serum was prepared by injecting alum-precipitated antigens recovered by elution of polyacrylamide gels in which isolated ICs released from Raji cells had been electrophoresed. Other workers have previously shown successful immunization with antigen in SDS solution (37, 38) or antigen eluted from SDS-polyacrylamide gels (39).

Evidence for circulating ICs has now been obtained in a wide variety of diseases of animals and humans. However, in many of these diseases virtually nothing is known about the identity of antigens in these ICs. Isolation of ICs from sera of patients could help in identification of etiologic agents. The principles and techniques described for isolating ICs using Raji cells could be adopted to other substances which bind ICs, e.g. C1q, rheumatoid factor, staphylococcal protein A, and conglutinin. However, Raji cells may be superior for this purpose because they specifically bind ICs, whereas substances such as C1q, rheuma-

toid factor, and staphylococcal A protein are known to interact with non-IC substances such as endotoxin, DNA, monomeric Ig, viral proteins, etc. Moreover, similar techniques may be employed for the direct demonstration and isolation of ICs bound to mononuclear cells or other cells in vivo.[3] Purified antigens, even though incompletely identified, could be used diagnostically to assess the presence of specific antibodies in sera, to induce cutaneous delayed-type hypersensitivity or lymphocyte stimulation. In addition, they might be used therapeutically to stimulate a weak immune response. Furthermore, antisera raised against the isolated antigens may be useful in developing reproducible and sensitive techniques for detecting the corresponding antigens either in serum or in tissues of patients. Moreover, the availability of such antisera will allow the use of immunochemical techniques, such as affinity chromatography, to purify large quantities of respective antigens.

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