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 immnunizing 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) 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

1Abbreviations 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.

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of both antigens and antibodies have been recovered, quantitated, and characterized in many diseases. How-
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 antiserum against the antigenic moity 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. Pre-
viously, 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 pa-

tient with hereditary spherocytemia and are devoid of
receptors for IgG Fc or human C.

Antigens and antiserum. 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). Puri-
fied tetanus toxoid (lot 8086, 2,083 LF/ml, 1,197 LF/mg pro-
tein 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 phos-
phate-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 antiserum 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 antiserum 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
antiserum 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 125I-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 super-
nates containing soluble ICs at moderate antigen excess
(see Results) were used in subsequent experiments. To assess
the amount of soluble 125I-BSA complexes to antibody at
four times the antigen excess, precipitation with 50% am-
onium sulfate was performed.

Labeling protein. Soluble proteins were labeled with
125I or 131I 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 125I-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 × 10⁷ Raji cells washed with Spinner’s medium
(MEM without Ca²⁺ or protein) were incubated (37°C, 45 min)
with increasing amounts of the supernate containing soluble
125I-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
to added cells. In other experiments, increasing numbers of
Raji cells (5 × 10⁶–1 × 10⁷) were incubated with a constant
amount of 125I-BSA-anti-BSA-C complexes, and uptake was
determined. All determinations were performed in triplicate.
Controls consisted of mixtures containing 125I-BSA-anti-
BSA-C but no cells. 125I-BSA-anti-BSA in heated (56°C, 30 min)
saline as well as cells incubated with 131I-BSA mixed with
normal nonimmune rabbit serum and C. The amount of anti-
gen specifically bound to cells was determined after sub-
tracting the nonspecific background counts observed in the
controls.

Iodination of cells. Raji cells with or without bound ICs
were iodinated by the lantoperoxidase (Calbiochem, San
Diego, Calif.) cell-surface labeling technique of Phillips and
Morrison (22), as modified by Kennedy et al. (23). In this
procedure peroxide and enzyme concentrations were adjusted
to minimize incorporation of iodine while maintaining soluble
ICs. 3 × 10⁷ cells were washed four times with Spinner’s medium and incubated (37°C, 45 min) with
Spinners medium, pathological sera, normal sera, or sera
supplemented with preformed ICs. Thereafter, cells were
washed three times with Spinners medium and twice with
Earles balanced salt solution without phenol red (Flow
Laboratories, Inc., Rockville, Md.) supplemented with 10 μM
Kl and finally adjusted to 3 × 10⁷ cells/ml of Earles’Kl solution.
Lactoperoxidase was added from the stock solution
(55 μM, 6 mg/ml) to a final concentration of 0.33 μM. After
addition of an appropriate amount (500–700 μCi) of 125I
(50 mCi/ml). After 1 min, the same amount of H2O2
was added again. 1 min later the reaction was essentially
terminated by dilution with 5 ml cold MEM and centrifuga-
tion. The cells were then washed in MEM five times to remove
free iodine and enzyme. Cells carrying BSA-anti-BSA-C com-
plexes and stained with FITC anti-BSA antibody before and
during lantoperoxidase labeling showed the same intensity
of immunofluorescence.

Recovery of cell-bound ICs. Two techniques were employed:
(a) 3 × 10⁷ 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 125I-labeled antigens or
were labeled with 131I at the cell surface by the lantoperox-
Idase technique. At the end of the incubation period, the tubes

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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 antibodies was calculated as follows: percent antigen released = (activity per minute in supernate - activity per minute in supernate + activity per minute in cell pellet) × 100. To determine protein-associated counts in supernates, TCA precipitation was performed. 

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 194 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 280 μg 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 ^121I by the lactoperoxidase method, washed again, and eluted with citrate buffer; then dialyzed eluates, subjected to SDS-PAGE. Subsequently, gel slices containing the radioactive protein corresponding to HSA were eluted by overnight incubation with 200 μl H2O. Eluates obtained from three such gel slices were pooled, supplemented with rabbit IgG (1 mg/ml), and precipitated with 1% final solution of aluminum chloride at pH 7.8. 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 ^121I-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 was 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 ^121I-BSA-

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1 Lazarides, E. Personal communication.
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 x 10⁶ - 8 x 10⁷ cells were incubated with 125I-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 complexes 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 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 x 10⁷ 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 125I-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 125I-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 1** Binding of 125I-BSA-anti-BSA-C complexes to Raji cells. 3 x 10⁷ cells were incubated (37°C, 30 min) with increasing amounts of 125I-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 (□).}

**Figure 2** Binding of 125I-BSA-anti-BSA-C complexes to Raji cells. 5 x 10⁶ - 8 x 10⁷ cells were incubated (37°C, 30 min) with 184 μg 125I-BSA-anti-BSA in 200 μl of fresh (○) or heated (△) NHS. Cells were also incubated with 184 μg 125I-BSA alone (□).

**Figure 3** Release of cell-bound 125I-BSA-anti-BSA-C complexes by isotonic citrate buffer. 3 x 10⁷ cells were incubated with 184 μg 125I-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 125I-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 125I-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 anti-human 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 125I 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 125I radioactivity cosedimenting with 125I-internal marker 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 125I-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
assessed; citrate buffer eluates obtained from surfaceiodinated 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 $^{131}$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 $^{131}$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 $3 \times 10^7$ Raji cells indicated that under the conditions employed a minimum of 25 $\mu$g of complexed antigens per milliliter serum (5 $\mu$g added to cells) were needed to identify peaks of radioactive antigens and antibody.

To determine the efficacy of the SDS-PAGE in recovering antigen, $^{131}$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 $^{131}$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$_2$O.

**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 $^{131}$I radioactivity

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**FIGURE 5** Recombination of antigen and antibody eluted with citrate buffer from Raji cells. Cells were incubated with 184 $\mu$g 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 (O — O) 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. $3 \times 10^7$ Raji cells were incubated with 100 $\mu$g tetanus toxoid-antitetanus toxoid-C complexes or 184 $\mu$g BSA-anti-BSA-C complexes at four times the antigen excess. The washed cells were surface radioiodinated with $^{131}$I by the lactoperoxidase method and washed again. The cells were then eluted with 300 $\mu$l 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 (O — O). Gels were sectioned and counted in a two-channel gamma counter. Arrows indicate the position of the $^{131}$I markers.
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 post-inoculation with tumor cells showed four major peaks of $^{131}$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,
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 larger tumor and higher levels of circulating ICs.

To demonstrate that the $^{125}$I peaks comigrating with the $^{125}$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 $\text{H}_2\text{O}$; 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 $^{125}$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 $\mu$g purified gp70 or 5 $\mu$g 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-antitetanus 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 antisera against whole IC-bearing Raji cells was more potent (1:128 antibody titer) than the antisera against the eluate (1:8 antibody titer) as determined by Ouchterlony analysis. Additionally, the antisera 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 glibutaraldehyde-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
Spinor’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 Fe 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 radioactivity corresponding to the trace 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 antisera 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 Fe 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 (≈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 ≈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. Clq, 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 Clq, 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 immunochromatographic techniques, such as affinity chromatography, to purify large quantities of respective antigens.

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