Recovery of Immunologically Reactive Antibodies and Antigens from Breast Cancer Immune Complexes by Preparative Isoelectric Focusing

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ABSTRACT

A new technique for the recovery of immunologically reactive antibodies and putative antigens from breast cancer-associated immune complexes was described. Soluble immune complexes were isolated from extracellular fluids by 2.5% polyethylene glycol fractionation and affinity chromatography on Protein A-Sepharose CL-4B. The immune complexes bound to immobilized Protein A were then subjected to preparative isoelectric focusing. The dissociated immunoglobulins and putative antigens were recovered from the appropriate pH regions of the preparative isoelectric focusing gel. G-type immunoglobulins recovered from immune complexes isolated from a breast cancer patient bound to the recovered putative antigen(s) and three breast cancer cell culture lines, but these immunoglobulins did not bind to carcinoembryonic antigen or other cancer cell culture lines originating in colon, pancreas, prostate, or lymphoblast. The recovered putative antigens had isoelectric points between pH 3.0 and 5.0 and molecular weights of 20,000 and 42,000.

INTRODUCTION

The study and characterization of immune complexes are important to the understanding of their role in the pathogenesis of immune complex-associated diseases. Immune complexes have been detected in various malignant and nonmalignant diseases (22, 28, 29). Immune complex levels have been correlated with prognosis of breast cancer patients (11, 24), and increased levels may be associated with disseminated disease (10). Data also indicate that immune complex measurements may be useful in the differential diagnosis of benign and malignant breast diseases (20). Immune complexes have been implicated as one of the possible factors involved in "blocking" or "inhibition" of cell-mediated immunity in cancer patients (3, 23, 26) and immunoregulation (9, 12, 18).

Evidence has been provided to show that immune complexes may contain tumor-associated antigens or antibodies (2, 19, 29). The existence of tumor-associated immune complexes has not been unequivocally established, and the identity of the antigen component(s) of such immune complexes remains obscure in most instances (1, 25).

Therefore, the isolation and characterization of the components of immune complexes may lead to the elucidation of the role of immune complexes in human pathology, the development of new concepts in the pathogenesis of immune complex-associated diseases, and the identification of new tumor-related products which may be candidates as markers for diagnosis and monitoring.

Recently, a technique using analytical IEF3 was developed which dissociated antigen-antibody complexes and separated the antigen and antibody components, based upon differences in isoelectric points (16). Furthermore, the dissociated immune complex components were recovered, identified, and found to retain immunological or biological reactivity. Subsequently, this IEF technique was used to examine immune complexes isolated from extracellular fluids of cancer patients. The immune complexes were purified by a combination of PEG fractionation and Protein A-Sepharose CL-4B affinity chromatography. IgG and putative antigens were detected in clinical specimens obtained from cancer patients (17). As a continuation of this study, preparative IEF has been used for the isolation of antigen and antibody components from immune complexes purified from the extracellular fluid of a breast cancer patient.

MATERIALS AND METHODS

Purification of Immune Complexes from Clinical Specimens. Pleural fluids were obtained aseptically from a breast cancer patient by thoracentesis. Cytological examination confirmed the presence of malignant cells in the specimens.

Purification of immune complexes from pleural fluid has been summarized previously (17) and is described as follows. Briefly, pleural fluid was brought to 0.02 M disodium EDTA (Eastman Kodak Co., Rochester, N. Y.) with solid EDTA at room temperature for 2 hr and was clarified by centrifugation at 3000 × g for 30 min at room temperature. Ten ml of 25% (w/v) PEG (M.W. 6000; Sigma Chemical Co., St. Louis, Mo.) in 0.05 M TBS (pH 8.4) were mixed with 90 ml of EDTA; pleural fluid solution and incubated with continuous stirring for 90 min at room temperature. After centrifugation at 2000 × g for 10 min at room temperature, the precipitate was washed with 2.5% PEG in TBS (pH 8.4) and then centrifuged at 2000 × g for 10 min. The supernatant was discarded. The precipitate was redissolved in 15 ml of 0.05 M TBS (pH 7.5) and dialyzed against

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3 The abbreviations used are: IEF, isoelectric focusing; PEG, polyethylene glycol; TBS, 0.05 M Tris:0.14 M NaCl:0.1% NaN3, pH 7.5 unless otherwise noted; PEG-PPT, polyethylene glycol-precipitated materials; Protein A, purified Staphylococcus aureus Protein A; PrepAb, putative antibodies recovered by preparative isoelectric focusing at pH 6.0 to 8.0-region; Ag3-5, putative antigens recovered by preparative isoelectric focusing at pH 3.0 to 5.0-region; TBS, 0.05 M Tris:0.024 M Na2HPO4:0.14 M NaCl; pH 7.5; IE, immunoelectrophoresis; PBS, 0.05 M NaH2PO4:Na2HPO4:0.14 M NaCl pH 7.0; BSA, bovine serum albumin; CEA, carcinoembryonic antigen; RPMI Medium 1640, Roswell Park Memorial Institute Medium 1640; SDS-PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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TBS with several changes of buffer. The dialyzed solution, PEG-PPT, was clarified by centrifugation at 3000 × g for 30 min. Ten ml of Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) in 50% TBS (v/v) stock suspension were mixed with 15 ml of PEG-PPT at room temperature for 2 hr and then at 4°C for 18 hr. Nonbinding materials were removed by repeated washing of the immobilized Protein A with TBS until no protein was detected in the 2000 × g supernatant, as determined spectrophotometrically at 280 nm. This preparation of immune complexes bound to immobilized Protein A was used for preparative IEF.

Preparative Flat-Bed IEF of Immune Complexes Bound to Protein A. Granular gel bed preparation and sample application were performed by modifying the procedures outlined in LKB Application Note 198 (30). The granular gel bed (11 × 24.5 cm) was prepared with 5% IEF Sephadex (w/v) with 2% ampholites (pH 3 to 10). A sample application trough was prepared by placing the 9.5- × 2.5-cm sample applicator template 1.5 cm away from the parallel to the anode. The gel included within the sample applicator template was then removed. Five ml of Protein A-Sepharose CL-4B with bound immune complexes in 85% TBS (v/v) were placed in the sample applicator template. The template was removed, and the gel bed was allowed to equilibrate hydrostatically for 5 min. IEF was performed using a Buchler Model 3-1500 power unit set at 2 watts, constant power, for 40 to 42 hr at 4°C. Anode buffer was 1 M H3PO4, and cathode buffer was n NaOH. A 30-zone fractionating grid was pressed into the gel bed at the cessation of the run. The pH gradient was determined using Bio-Lyte Gel Pro-pHiler (Bio-Rad Laboratories, Richmond, Calif.) and a Fisher Accument Model 140 pH meter.

Recovery of Isoelectrically Focused Material. Isofocused materials were separated from the gel media by filtration through Whatman no. 1 filter paper. The materials were washed from the gel by applying approximately 2 bed volumes of TBS to the gel. The materials recovered from the pH 6.0 to 8.0 region of the gel bed (prepAb) and the materials from the pH 3.0 to 5.0 region of the gel bed (Ag3-5) were each dialyzed against distilled water at 4°C with several changes of distilled water. The diazoyzed materials were then lyophilized.

Gel Filtration on Sephadex G-200. PEG-PPT or PrepAb reconstituted with TBS were chromatographed over a Sephadex G-200 (Pharmacia) packed column (0.9 x 60 cm) equilibrated with TBS; 1-ml fractions were eluted with TBS, collected, and monitored for absorbance at 280 nm. Fractions were also analyzed for IgG by immunodiffusion against rabbit anti-human IgG (γ chain-specific) antiserum (Behring Diagnostics, Somerville, N. J.) in 1% agarose (Sigma type 1) in 0.08 M TTB, at room temperature for 24 hr. Agarose gels were washed extensively in 0.14 M NaCl and then distilled H2O, pressed dry, stained with crocein scarlet (5), and destained in 0.3% acetic acid.

IEP. IEP was performed using 1% agarose in TTB buffer. Samples (5 µl) were placed in sample wells and electrophoresed at 4°C with 150 V constant voltage. Electrophoresis was monitored with bromophenol blue-dye marker. The anode and cathode buffers were TTB. Eighty µl of rabbit anti-human IgG (γ chain-specific) antiserum or rabbit anti-human serum antiserum (DAKO, Copenhagen, Denmark) were transferred by pipet into a 6-mm-long trough cut 5 mm away from the sample and parallel to the direction of electrophoretic migration. The IEP plate was incubated at room temperature for 24 hr, washed, and destained as described for the immunodiffusion agarose gels.

Radioiodination of Ag3-5 and Protein A. Approximately 50 µg Ag3-5 were reconstituted with 100 µl 0.05 M PBS, mixed with 0.3 mCi dried 125I as Bolton and Hunter reagent for protein iodination (Amersham/Searle, Arlington Heights, Ill.) in a glass test tube (1 x 10 cm), and incubated at 4°C for 18 to 20 hr. One hundred µl purified Protein A (Pharmacia) were reconstituted with 100 µl PBS, mixed with 1 mCi dried 125I as Bolton and Hunter reagent in a glass test tube (1 x 10 cm), and incubated at room temperature for 15 min. Five hundred µl of 0.2 M glycine in PBS were added to each tube to block unreacted reagents. Free 125I was removed by desalting the mixtures separately over a PD-10 (Pharmacia) column (1.5 × 5 cm) equilibrated with 0.05 M sodium phosphate:0.5 M NaCl, pH 7.2, or PBS:3% BSA (Sigma). Fractions (1 ml) were collected and monitored for 125I by using a Packard Auto-Gamma scintillation spectrometer. The fractions corresponding to the void volume were pooled. 125I-labeled Protein A was diluted with PBS:3% BSA, aliquoted, and stored at −20°C until use. The specific activity was approximately 8 µCi/µg. Radiolabeled Ag3-5 was mixed with Pansorbin (Calbiochem-Behring, La Jolla, Calif.) to remove nonspecifically binding materials, incubated for 18 to 20 hr at 4°C, and centrifuged at 3000 × g for 10 min. The supernatant was used for the immunological recombination assay.

Immunological Recombination Assay. The PrepAb was reconstituted in TBS: 1% BSA (Sigma) to a concentration of 2.5 µg PrepAb per ml. A 2.5-µg solution of human IgG per ml (monomeric 7S preparation) in TBS:1% BSA was prepared. Thirty µl 125I-labeled Ag3-5, 30 µl TBS:1% BSA, and 30 µl PrepAb diluted with TBS:1% BSA (1:1; 1:10; or 1:100) were mixed in a polypropylene conical test tube and vortexed. After 3 hr of incubation at room temperature, 30 µl of Pansorbin were added to each tube, vortexed, incubated for 1 hr at room temperature, centrifuged at 3000 × g for 10 min, and washed twice with 2 ml TBS:1% BSA:0.5% Triton X-100 (Sigma). Nonspecific binding to normal human IgG was measured by substituting the same concentrations of human IgG in TBS:1% BSA for PrepAb in TBS:1% BSA. Also, 125I-labeled CEA (Roche Diagnostics, Nutley, N. J.) was substituted for radiolabeled Ag3-5 to measure nonspecific binding. After the last centrifugation, the supernatant was aspirated and discarded, and the tubes were counted in a Packard Auto-Gamma spectrometer. Specific binding was calculated as follows: (PrepAb cpm) − (human IgG cpm). Both PrepAb and the human IgG were the same concentration. The specific binding was calculated versus PrepAb dilution.

Cell Culture. Human cell cultures were grown, unless otherwise indicated, in plastic culture flasks under standard conditions in PRMI Medium 1640 supplemented with 15% fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.) and 1-fold solution of insulin, minimum essential medium with nonessential amino acids, and glutamine (Grand Island Biological Co.; supplied as 100-fold solution). Cell lines of human adenocarcinoma origin included BT-20 (14), MCF-7 (27), SK-BR-3 (from Dr. J. Fogh, Sloan-Kettering Memorial Institute for Cancer Research), CaPan-2 (from Dr. J. Fogh), PC-3 (13), HT-29 (6), and a human lymphoblastoid cell line, Raji (21).
**125I-labeled Protein A Assay for Cell Surface Antigens.** The assay was performed in triplicate as modified from the procedure described by Brown et al. (4). Adenocarcinoma cells were tested as monolayers in flat-bottomed Microtest 2 tissue culture plates (Falcon Plastics, Oxnard, Calif.). Monolayers were prepared by adding the appropriate cells (5 x 10^5) suspended in RPMI Medium 1640 with 15% bovine serum to each well and incubating at 37°C in a humid incubator with 5% CO2 in air. The assay was performed the next day after cells had formed a confluent monolayer.

At the start of the assay, the culture medium was removed by aspiration, and the cells were washed twice with fresh RPMI Medium 1640:1% BSA. One hundred µl of the appropriate antibody test solution, PrepAb (250 µg/ml), human IgG (250 µg/ml), or buffer (RPMI Medium 1640:1% BSA) were added to the wells. After incubation at room temperature for 2 hr, the antibody test solution was removed, and the cells were washed with RPMI Medium 1640:1% BSA. One hundred µl 125I-labeled Protein A (10^5 cpm) were then added to each test well. After a 1-hr incubation at room temperature, the reagent was removed, and the cells were washed twice with 200 µl RPMI Medium 1640:1% BSA. The cells were then dissolved in 100 µl of 2 N NaOH and transferred to polypropylene tubes for 125I determination in a Packard Auto-Gamma scintillation spectrometer. The assay was modified for the Raji cells. Raji cells were incubated with the appropriate reagents in polypropylene conical test tubes in suspension, washed repeatedly by centrifugation at 1000 x g for 10 min, and then aspirated. The final pellet of cells was counted in a Packard Auto-Gamma scintillation spectrometer for 125I determination. A specific binding index was then calculated by the following formula:

\[
\frac{(\text{PrepAb cpm} - \text{buffer cpm}) - (\text{human IgG cpm} - \text{buffer cpm})}{(\text{PrepAb cpm} - \text{buffer cpm})}
\]

**SDS-PAGE of 125I-labeled Ag3-5.** Ninety µl of 125I-labeled Ag3-5 absorbed previously with Pansorbin, 90 µl of TBS:1% BSA, and 90 µl of PrepAb (2.5 µg/ml) were mixed in a polypropylene conical test tube and vortexed. After a 3-hr incubation at room temperature, 90 µl of Pansorbin were added to the tube to precipitate IgG and IgG-bound 125I-labeled Ag3-5, vortexed, incubated for 1 hr at room temperature, washed with TBS:1% BSA:0.5% Triton X-100, and centrifuged until the supernatant contained no detectable 125I radioactivity. The Pansorbin precipitate was mixed with 100 µl 1% SDS:0.05 M Tris:0.38 M glycine, pH 8.4, and incubated for 18 hr at room temperature. Polyacrylamide gels with 7.5% acrylamide (total concentration, 7.5%; cross-linking agent, 2.5%) and 0.05 M Tris:0.1% SDS were prepared (16). The Pansorbin precipitate was mixed with 100 µl glycerol with bromophenol blue dye marker and applied to the top of 0.5 x 10-cm gels. The sample was overlaid with running buffer (0.1% SDS:0.05 M Tris:0.38 M glycine, pH 8.4) and electrophoresed using LKB Model 2103 power unit with 2 ma/tube constant current at 22°C until the dye indicator migrated to within 5 mm of the bottom of the gel. The gel was removed from the glass tube and sliced into 2-mm segments. Each 2-mm gel segment was placed in a separate tube and counted in a Packard Auto-Gamma scintillation spectrometer for 125I determination.

**Rheumatoid Factor and Erythrocyte Agglutination Tests.** PrepAb was tested for rheumatoid factor activity using a latex agglutination test (Rapi/tex-RF kit; Behring Diagnostics) and tested for hemagglutination activity with freshly obtained type A and type B human erythrocytes in a tube hemagglutination assay (7).

**Isolation of Human IgG.** Monomeric human IgG was isolated from normal human serum by ammonium sulfate precipitation followed by a DEAE anion exchange chromatography as described previously (15). Purity of isolated IgG preparation was assessed by Sephadex G-200 gel filtration and by IEP with rabbit antisera to human IgG (γ chain specific) and rabbit antisera to human serum protein (DAKO) and further confirmed by ultracentrifugation (8). No aggregated IgG was detected.

**RESULTS**

**Isolation of Immunoglobulins.** Fractionation of the pleural fluid from a breast cancer patient by 2.5% PEG precipitation resulted in the isolation of high-molecular-weight (greater than 232,000) proteins as shown by Sephadex G-200 gel filtration chromatography. High-molecular-weight IgG, presumably in the form of immune complexes, was detected in the fractions corresponding to the void volume of the Sephadex G-200 column, and no IgG (M.W. 150,000) was detected. The materials containing high-molecular-weight IgG were allowed to react with immobilized Protein A and then subjected to preparative IEF (pH 3 to 10). The proteins (PrepAb) recovered from the immunoglobulin region (pH 6.0 to 8.0) of the gel were characterized by IEP and Sephadex G-200 gel filtration chromatography. The recovered PrepAb was shown to be human IgG as determined by IEP with antisera against human IgG and antisera against normal human serum (Fig. 1). By gel filtration chromatography, the recovered PrepAb eluted as a single symmetrical protein peak corresponding to a molecular weight of 158,000 (Chart 1). The amount of PrepAb recovered represented approximately 0.08% of the total protein present in the original pleural fluid (3,750 mg total protein).

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**Fig. 1.** Immunoelectrophoretic analysis of isolated IgG (PrepAb). Proteins (PrepAb) recovered from the pH 6.0- to 8.0-region of the preparative IEF gel were analyzed by IEP as described in "Materials and Methods." Well 1, human serum albumin; Well 2, PrepAb, Trough A, rabbit anti-normal human serum protein antisera; Trough B, rabbit anti-human IgG (γ chain-specific) antisera.
No rheumatoid factor activity or agglutination of type A or type B human erythrocytes was detected when the PrepAb was analyzed by the rheumatoid factor latex agglutination test or hemagglutination tube assay.

Immunological Recombination of Recovered Immunoglobulin and Putative Antigen. In order to show recombination of the recovered PrepAb and the recovered putative antigen, the PrepAb was mixed with radiolabeled Ag3-5. Pansorbin was then added to the mixture to precipitate IgG and IgG-bound 125I-labeled Ag3-5. Nonspecific binding to human normal IgG was measured by substituting human IgG for the PrepAb at the same concentration. Binding of 125I-labeled CEA to PrepAb and human IgG was also determined. The recovered PrepAb and 125I-radiolabeled recovered antigen (125I-labeled Ag3-5) were shown to recombine (Chart 2). It was shown that the specific binding of 125I-labeled Ag3-5 to PrepAb decreased with increasing PrepAb dilution. No significant binding of 125I-labeled CEA to PrepAb was observed.

SDS-PAGE Analysis of Putative Antigens. SDS-PAGE was used to obtain approximate molecular weights of immunologically reactive radiolabeled putative antigens. 125I-labeled Ag3-5 was incubated with PrepAb, and the resultant complexes were precipitated with Pansorbin. The Pansorbin precipitate was analyzed by SDS-PAGE. The SDS-PAGE gel was sliced into 2-mm segments, and each segment was counted for 125I radioactivity. Two 125I-labeled Ag3-5 radioactivity peaks were detected; a minor radioactive peak corresponding to a molecular weight of 42,000 and a major radioactive peak corresponding to a molecular weight of 20,000 were demonstrated (Chart 3). 125I-labeled Protein A Assay for Cell Surface Antigen Detection. The recovered PrepAb was analyzed for binding specificity to various malignant cultured cells. Malignant cells were grown to confluence on flat-bottomed Microtest 2 plates. Cells were incubated with PrepAb, human IgG, or buffer (RPMI Medium 1640:1% BSA) and washed. The cells were then incubated with 125I-labeled Protein A to detect cell surface-bound IgG. A specific binding index was calculated as described in "Materials and Methods." A high specific binding index of PrepAb was observed with 3 breast cancer cell lines (MCF-7, BT-20, and SK-BR-3) (Table 1). A low specific binding index of PrepAb was calculated when HT-29 colon cancer, CaPan-2 pancreas cancer, PC-3 prostate cancer, or Raji lymphoblastoid cell lines were used.

DISCUSSION

Immune complexes purified by 2.5% PEG fractionation and Protein A:Sepharose CL-4B affinity chromatography are dissociated and separated by IEF (17). The present data show that immunologically reactive immune complex components can be recovered from preparative IEF of immune complexes purified from the pleural fluid of a breast cancer patient. Immune complex components (antibody and antigen) are recovered and are immunologically reactive. The supportive data are as follows: (a) IgG (M.W. 150,000) is recovered from the preparative IEF of high-molecular-weight IgG obtained by 2.5% PEG fractionation and then bound to immobilized Protein A; (b) the recovered antibody (PrepAb) and the recovered antigens (125I-labeled Ag3-5) are able to recombine immunologically as

![chart1](chart1.png)

**Chart 1.** Gel filtration analysis of isolated IgG (PrepAb). PrepAb was chromatographed over a packed Sephadex G-200 column (0.9 x 60 cm). One-ml fractions were collected and analyzed for protein by absorbance at 280 nm. Markers were: catalase (M.W. 232,000); aldolase (M.W. 158,000); and BSA (M.W. 66,000). Vo, void volume; Vt, total bed volume.

![chart2](chart2.png)

**Chart 2.** Immunological recombination of PrepAb and radiolabeled Ag3-5. PrepAb was incubated with 125I-labeled Ag3-5, precipitated with Pansorbin, and washed, and bound 125I radioactivity was counted. The nonspecific binding to human IgG was determined by the identical experiment substituting human IgG for PrepAb. Specific binding (PrepAb cpm – human IgG cpm) was calculated for 125I-labeled Ag3-5 and 125I-labeled CEA at various dilutions of PrepAb and human IgG.

![chart3](chart3.png)

**Chart 3.** SDS-PAGE analysis of immunoreactive radiolabeled Ag3-5; 125I-labeled Ag3-5 was incubated with PrepAb. PrepAb with bound 125I-labeled Ag3-5 was then precipitated with Pansorbin. The Pansorbin precipitate was analyzed by SDS-PAGE. After electrophoresis, the gel was sliced into 2-mm segments, and each segment was counted for 125I radioactivity. 125I cpm were plotted versus cm distance of migration into the SDS-PAGE gel. Arrows, markers: BSA (M.W. 66,000); ovalbumin (M.W. 44,000); and RNase (M.W. 13,700).
were incubated with radiolabeled Protein A, washed, and counted. The specific binding determined by the recombination assay; and (c) the recovered from the pleural effusion of a breast cancer patient show IgG, or buffer and then washed. In order to detect cell surface-bound IgG, cells presented in this report provide evidence to substantiate the antibody (PrepAb) binds cell surface antigens associated with active putative antigen(s) recovered exhibits isoelectric focus cell surface antigen(s) is tumor associated or organ specific colon, or lymphoid origin. Whether this reactive breast cancer BT-20, and Sk-Br-3 breast cancer tissue cell lines, and they MARCH 1981 to the identification of new breast tumor-related products. gen moieties with breast cancer is the subject of continuing study. Additional characterization of these moieties may lead Reactivity of isolated IgG (PrepAb) with cell surface antigens of human tumor cells

<table>
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<th>Cell line</th>
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<td>BT-20</td>
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<td>Human IgG 527 179 146</td>
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<tr>
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<td>Buffer 4896 867 183</td>
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* Specific binding index

In conclusion, the preparative IEF of immune complexes isolated by 2.5% PEG precipitation and Protein A:Sepharose affinity chromatography results in the recovery of immunologically active antibody and putative antigen components. The isolated antibody and putative antigen components can be characterized physicochemically and immunologically. Further, the isolated antibody can be used as a reagent for the detection and isolation of antigens, and the isolated antigen can be used for the production of heteroantisera or hybridoma antibodies that may be useful in the development of specific immunoasays for diagnosis and monitoring of immune complex-associated antigens.

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