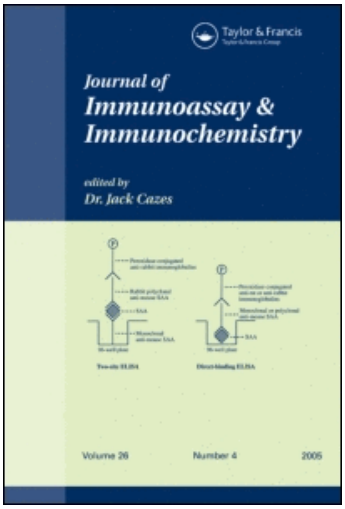

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RAPID ASSAY FOR IMMUNE COMPLEX BOUND ANTIGENS FROM URINE OF CANCER PATIENTS

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(KEY WORDS: immune complex, tumor-associated antigen, autoantigen, immunoblot, urinary antigen)

ABSTRACT

Concentrated urine from patients with various types of cancer was fractionated by S-500 gel filtration chromatography to yield high molecular weight (mw) immune complexes (IC) and lower mw immunoglobulin (Ig) fractions. Column fractions were assayed for the presence of IgM and IgG by immunoblot probing using anti-human IgM and anti-human IgG alkaline phosphatase conjugates. These results were used to define IC fractions (those from high mw S-500 fractions which were positive for immunoglobulin) and Ig fractions (those from medium mw S-500 fractions which were positive for immunoglobulin). Antigen components of high mw S-500 IC fractions were then determined by immunoblot probe using medium mw S-500 Ig fractions as the antibody probe (i.e. autoantibody). This method for identification of immune complex antigens has the potential to probe for tumor-associated antigens, autoantigens, or foreign antigens from starting material which contains both immune complex and free immunoglobulin.

INTRODUCTION

Elevated levels of immune complexes (composed of varying stoichiometries of immunoglobulin, antigen, and sometimes complement) have been associated with a variety of diseases, including certain forms of cancer (e.g. 1-5) as well as several autoimmune diseases (e.g. 6-7). Normally, immune complexes (ICs) are present in low levels as circulating antibody and antigen associate and dissociate, and it is thought that ICs play a role in regulation of immune function via complement fixation and immunosuppression (reviewed in 2). Although the mechanisms are not completely clear, IC levels increase dramatically at certain stages of autoimmune diseases and in some cancers as measured in various body fluids, e.g. serum/plasma, urine and CSF. It is assumed that most, if not all, of these ICs contain autoantigens (in the case of autoimmune diseases) or tumor-associated antigens (in the case of certain cancers), making the isolation of ICs and analysis of their antigenic components a valuable tool in the study of these diseases.

The isolation and analysis of ICs is complicated by the fact that immune complexes vary in size (2, 5, 8) and immunoglobulin isotype (5), not to mention antigen composition. Thus, each of the most commonly used methods of IC isolation have certain limitations based

on the IC size or composition/function being selected. For example, polyethylene glycol (PEG) precipitation of ICs will isolate ICs of various sizes depending on the percent PEG used (8). Affinity purification is also selective; ICs containing IgG are obtained with protein A or DEAE Affigel Blue whereas C1q is used to obtain complement fixing ICs and would favor IgM-rich ICs. Perhaps the most elegant work in IC purification and analysis has been done by Phillips et al (2) and Queen et al (5) in which they were able to isolate and classify ICs on the basis of their size and composition, with a view to identifying tumor-associated antigens (TAAs) and the nature of the ICs in which TAAs are found. Not surprisingly, such extensive information is somewhat time consuming to obtain. Our work has focussed on the identification of antigen and immunoglobulin in the urine of cancer patients. We have chosen a scheme that would be specific for putative TAA but would not exclude certain ICs on the basis of their size, immunoglobulin isotype, or complement content.

Sephacryl 500 gel filtration resolves molecules or aggregates in a molecular weight range from approximately 10K to more than 1000K (9). The largest and most prevalent ICs elute in the void volume, while smaller ICs elute prior to the prominent immunoglobulin peak. Thus, ICs of all conceivable sizes are retained, eliminating the need for affinity methods which would

select for ICs of certain immunoglobulin and/or complement composition. Antigen and immunoglobulin can be visualized by immunoblot assay which consists of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, and the strategic use of immunoprobes. Immunoglobulin (whether free or in the form of immune complex) is detected directly by the use of anti-human IgM and IgG alkaline phosphatase (AP) conjugates while antigens (potential TAAs) are identified indirectly by their ability to bind the patient's own immunoglobulin (S-500 medium mw fractions shown to contain immunoglobulin), detectable after addition of secondary anti-human IgM and IgG AP conjugates. Thus, we report a rapid method for the identification of immune complex components such as tumor associated antigens, autoantigens or foreign antigens.

MATERIALS AND METHODS

Concentration of Urine

Urine samples from cancer patients and normal volunteers were concentrated 100-fold using a S1Y10 spiral cartridge (Amicon) for volumes greater than 1 L and a 180 ml or 350 ml capacity stirred cell with YM10 (10K mw cutoff, Amicon) for smaller volumes. Concentrated urine was diluted 50-fold into Dulbecco's

PBS + 0.1% NaN_3 and reconcentrated to 1% (100-fold concentration) of the original volume.

S-500 Fractionation

1 ml aliquots of concentrated urine were precleared in a Fisher model 59A microfuge at 9,300 x g for 1 min and fractionated on a 100 x 1.6 cm S-500 (Sephacryl-500, Pharmacia) gel filtration column using 10% PBS as elution buffer. 3 ml fractions were collected at a flow rate of 1 ml/min and concentrated 10X using a speed vac (Savant).

Immunoblot Assay

A. Sodium Dodeyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE): 15 µl concentrated S-500 fractions were mixed with an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) B-mercaptoethanol, 0.0025% (w/v) bromophenol blue) and heated at 95^0 C for 5 min. Samples were then separated on a 11% acrylamide gel using the Laemmli buffer system (10) and the Mini-Protean II Dual Slab Cell (Bio-Rad) at constant voltage = 200 V for 30 - 40 min.

B. Western Transfer: Protein bands from SDS-PAGE gels were electrically transferred onto 0.45 µ nitrocellulose (Bio-Rad) using the method of Towbin et al (11). Transfer buffer consisted of 25 mM Tris, 192

mM glycine, pH 8.3, 20% methanol. The Mini Trans-Blot cell (Bio-Rad) was used at constant voltage = 100 V, upper limit 250 mA for 1 hr.

C. Assay for Immunoglobulin: Nitrocellulose sheets (typically 4) were blocked with approximately 50 ml 3% powdered milk (Carnation) in 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.05% Tween 20 (TBST) by shaking for 30 min. at room temp. Blocking solution was removed and nitrocellulose sheets were rinsed very briefly with TBST or deionized water until milky color had been removed. Nitrocellulose sheets were then reacted with 50 ml of a combination of goat anti-human IgM*AP (μ-chain specific, Sigma or Southern Biotechnology Associates, Inc.) at 1:1000 and goat anti-human IgG*AP (γ-chain specific, Promega) at 1:7500 in TBST and shaken for 30 min. at room temp. Sheets were rinsed for 3 X 5 min in at least 50 ml TBST before reaction with freshly prepared substrate solution [330 ll 50 mg/ml NBT (nitro blue tetrazolium, Promega) diluted into 50 ml AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2) followed by addition of 165 μl 50 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, Promega)]. Immunoglobulin bands were detected as purple bands within 15 to 30 min. of the addition of substrate.

D. Assay for Antigen: Nitrocellulose sheets which had been assayed previously for immunoglobulin (part C) were cut into individual lanes and then each lane

subdivided to test under control (-) and self immunoglobulin (+) conditions. Each S-500 fraction (or pooled fractions) thus prepared as an antigen strip was then probed with S-500 fractions containing the patient's own immunoglobulin (+) and TBST (part C) was used as a negative control (-). Antigen strips were incubated with test immunoglobulin diluted 1:2 - 1:10 in TBST (0.5 ml per lane) for 3 hours at room temp. with shaking in a Transtar-96 12-channel disposable reservoir liner (Costar). Strips were then washed with TBST and probed with conjugate (goat anti-human IgM*AP + goat anti-human IgG*AP) as described in the immunoglobulin assay (part C).

RESULTS

S-500 Gel Filtration Separates Immune Complexes From Free Immunoglobulin

The solid lines in Figure 1 represent the S-500 elution profile (OD 280/310) of concentrated urine from a breast cancer patient (A) and a sarcoma patient (B) into immune complex-enriched and immunoglobulin-enriched fractions. Migration of molecular weight standards is indicated below the elution profile (V_O, 670K, 158K, 44K, 17K). Above the elution profile are the results of immunoblot assays for the presence of immunoglobulin in individual fractions. Selected S-500 fractions were

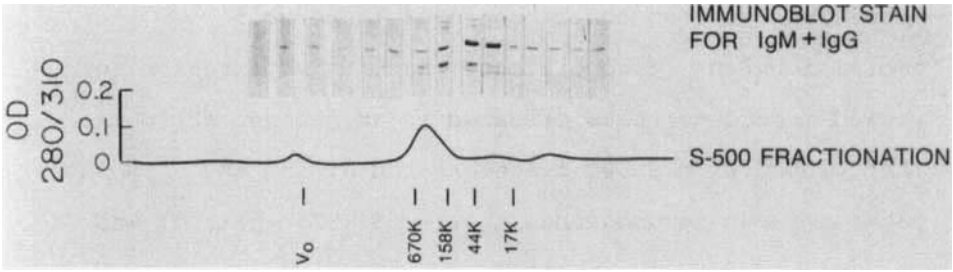

FIGURE 1. 1 ml of 100X concentrated urine from a breast cancer patient (Figure 1A) and a sarcoma patient (Figure 1B) was fractionated on a 100 x 1.6 cm Sephacryl S-500 (Pharmacia) gel filtration column using 10% Dulbecco's PBS, pH 7.2 as elution buffer and monitoring continuously at OD 280/310 nm (solid line) as described in Materials and Methods. Position of Bio Rad gel filtration standards thyroglobulin (670K mw), gamma globulin (158K mw), ovalbumin (44K mw), myoglobin (17K mw), and cyanocobalamin (1.35K) is indicated below the elution profile.

Individual fractions were concentrated 10X, separated into individual protein bands on 11% Laemmli SDS-PAGE minigels (Bio Rad), and transferred to 0.45 u nitrocellulose membranes at 100V for 1 hr at 4 C. Immunoblot assay for urinary IgM and IgG was performed by blocking nitrocellulose with 3% milk in 10 mM Tris-HCl, pH 8.0. 0.15 M NaCl, 0.05% Tween 20 (TBST), probing with goat anti-human IgM*AP (Sigma) 1:1000 in TBST + goat anti-human IgG*AP (Promega) 1:7500 in TBST, and rinsing membranes with TBST prior to addition of AP substrate (0.33 mg/ml NBT + 0.165 mg/ml BCIP in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl2).

The V_o peak represents an exclusion limit of approximately 2,000K mw according to the manufacturer and is enriched for immunoglobulin in the form of immune complex. High levels of immunoglobulin elute trailing the major peak in the same position as the gamma globulin mw standard.

assayed by immunoblot for IgM and IgG by running SDS-PAGE, transferring to nitrocellulose and probing with goat anti-human IgM and goat anti-human IgG alkaline phosphatase (AP) conjugates. Immunoglobulin bands were

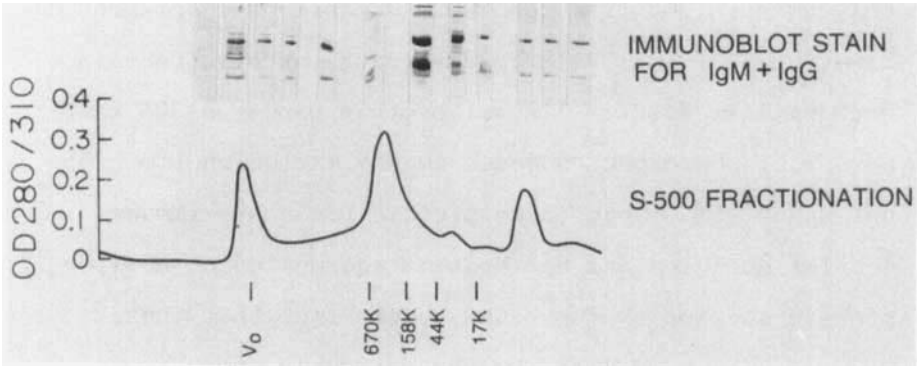

FIGURE 1B

then visualized by the addition of alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in the presence of nitro blue tetrazolium (NBT).

Void volume fractions (designated V_o in Fig. 1) contain molecules or aggregates with a molecular weight much greater than 1,000K mw based on conservative estimates of the manufacturer's S-500 exclusion limit for globular proteins (9). The presence of immunoglobulin molecules (158K mw) in void volume fractions during S-500 gel filtration indicates very high mw aggregates of immunoglobulin. The following observations indicate that these aggregates are immune complexes of immunoglobulin and antigen. 1) When staining separately for IgM and IgG, the prevalent isotype in void volume fractions is IgG, not the 790K mw pentameric form of IgM. 2) Earlier studies found that

only void volume fractions were capable of complement fixation (12). 3) Void volume fractions also contain much smaller (250K - 10K mw) protein bands on SDS-PAGE than would be expected based on the exclusion limit of the S-500 gel. That these protein bands are immune complex antigens and not high mw aggregates of smaller protein species is suggested by the fact that these bands can be recognized by the patient's own immunoglobulin (Fig. 2 below).

Immunoglobulin peaks appear in the same position as the gamma globulin (158K) gel filtration standard as shown by the heavy banding seen in Fig. 1A & B immunoblots of fractions just trailing the major OD 280/310 nm peak. Immunoglobulin fractions are recognized by goat anti-human IgM and IgG alkaline phosphatase conjugates and demonstrate cross-reactivity with S-500 immune complex antigens as described below. Bands produced by this assay are not due to endogenous alkaline phosphatase activity in urine since immunoblots of human and E. coli alkaline phosphatase gave no detectable signal.

Immunoblot for IC Antigens

Nitrocellulose sheets containing void volume IC fractions are first stained for immunoglobulin with goat anti-human IgM*AP and goat anti-human IgG*AP conjugates as described. Detection of immunoglobulin (IgM and/or

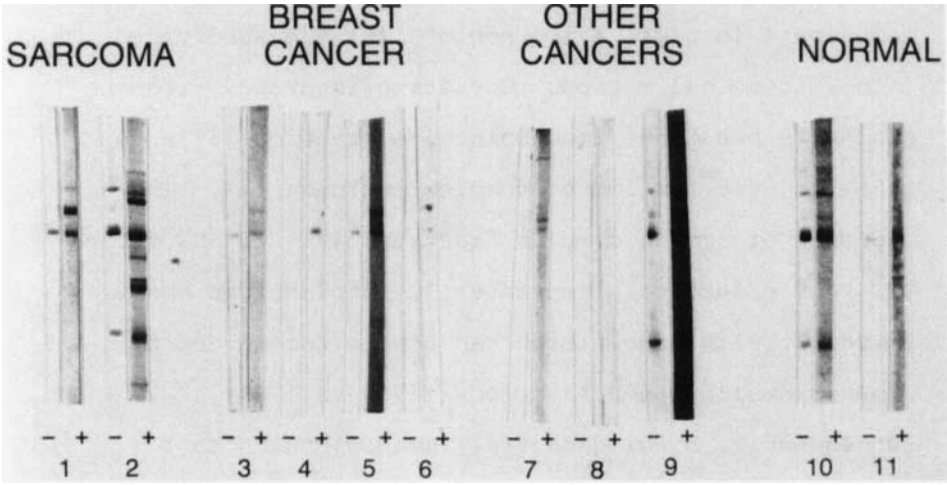

FIGURE 2. V_o fractions or pooled peaks containing immune complex from S-500 fractionation of several patient urines were separated by SDS-PAGE, transferred to nitrocellulose, and assayed for the presence of immunoglobulin IgM + IgG (indicated with (-) in the figure) as described in Materials and Methods and Figure 1. Individual immune complex (IC) lanes were subdivided into smaller strips, blocked with 3% milk in 10 mM Tris-HCl, pH 8.0. 0.15 M NaCl, 0.05% Tween 20 (TBST), and incubated with one or more S-500 fractions of the patient's own free immunoglobulin (Ig) diluted 1:2 - 1:10 in TBST to test for IC antigens recognized by free immunoglobulin (+); incubation in TBST alone (-) served as control. Probing with anti-human IgM and IgG alkaline phosphatase conjugates was used to detect binding of self immunoglobulins to IC antigens (+) and presence of IgM and IgG in IC (-). 1) Sarcoma patient PAC; 2) sarcoma patient WES; 3) sarcoma patient CIL; 4) breast cancer patient THO; 5) breast cancer patient PEN; 6) breast cancer patient STR; 7) colon cancer patient WHI; 8) hepatocellular cancer patient FUL; 9) pancreatic cancer patient CAV; 10) normal volunteer TAY; 11) noncancer patient MON.

IgG) in void volume fractions indicates the presence of immune complex, since free immunoglobulin elutes much later. Lanes of V_o fractions containing immune complex components (antigen + immunoglobulin) are subdivided into 2 identical strips. One strip is probed with the patient's own S-500 immunoglobulin fraction (670K to 158K) to test for immune complex antigens (+). The exposure of immune complex fractions (V_o) to SDS during SDS-PAGE effectively separates IC into antigen and immunoglobulin bands which can now be recognized by immunoglobulin S-500 fractions (670K to 158K). Subsequently, binding of free immunoglobulin to IC antigens is detected by anti-human IgM and IgG alkaline phosphatase conjugates. This technique is based on the assumption that medium mw S-500 immunoglobulin fractions have the same antigen specificity as immunoglobulins bound to antigen in immune complex (V_o) fractions. A second strip serves as a negative control (-) to indicate background IC immunoglobulin bands for comparison with the (+) strip's IC antigen bands added to the IC immunoglobulin bands already stained.

The results of immunoblot assays to identify potential immune complex antigens from several patients are shown in Figure 2. In some cases only one or a few antigen bands (+) are observed (patient 1, 3, 4, 5, 6, 7, 8, 9, 11) when compared to their corresponding negative control strip. Sarcoma patient 2, however,

shows multiple antigen bands. Although urine from normal volunteer 10 contains V_O immunoglobulin, there is no recognition of antigen by the volunteer's own immunoglobulin. This result is in contrast with urine from a noncancer hospital patient (patient 11) who does demonstrate IC antigen by this assay.

DISCUSSION

We report a general method for identification of IC antigens by immunoblot assay following 1) separation of high molecular weight immune complexes from medium molecular weight immunoglobulin by S-500 gel filtration and 2) localization of immunoglobulin-rich fractions. Immunoglobulin-rich fractions, both immune complex and immunoglobulin, are localized by Western transfer of S-500 fractions and probing with anti-immunoglobulin alkaline phosphatase conjugates. Subsequent probing of Western blotted IC fractions (V_O) with the patient's own immunoglobulin (obtained in the S-500 fractionation step 670K to 158K) is then used to identify IC antigens.

This technique is subject to the following limitations. First, there must be sufficient immunoglobulin in a given fraction (either immune complex or immunoglobulin) for detection by the anti-Ig conjugate. Both the anti-IgM and the anti-IgG conjugates we have used can detect as little as 1-10 ng of immunoglobulin in an immunoblot format. Second, by

inference, there must be at least 1 ng of immunoglobulin with the same specificity for immune complex antigen as that of immune complex immunoglobulin. Third, as mentioned above, we have found it necessary to use not just 158K fractions but the higher molecular weight fractions just preceding 158K in order to get sufficient signal for IC antigen band detection. Immunoglobulin present in these higher molecular weight fractions, i.e. 670K to 158K, is probably in aggregate form. This explanation is consistent with the enhanced sensitivity of our assay when these fractions are used; for every antigen bound by aggregated immunoglobulin, 2 or more pairs of heavy chains (as opposed to 1 pair) may be recognized by heavy chain specific alkaline phosphatase conjugates. However the likelihood that aggregated immunoglobulin is involved in IC antigen detection raises the question of whether immunoglobulin in the 670K to 158K molecular weight range is itself in the form of small immune complexes. If 670K to 158K immunoglobulin fractions represent small immune complexes, this defines a minimum IC size, i.e. not less than about 700K, for which this method is effective. Fourth, it is possible, though not likely, that IC antigen(s) could comigrate with immunoglobulin if their molecular weight is identical to that of immunoglobulin bands; if so, detection of IC antigens would be

obscured. Finally, the inherent limitations of SDS-PAGE must be mentioned: 1) the molecular weight range of molecules effectively resolved is a function of the % acrylamide (for 11% acrylamide this range is approximately 5K to 250K), and 2) nonlinear epitopes sensitive to either SDS denaturation or B-mercaptoethanol reduction will remain undetected.

There are several advantages to this method. First, immunoblot assays which detect a) immunoglobulin (using anti-IgM and anti-IgG probes) and b) IC antigen (using the patient's own immunoglobulin) involve highly specific interactions and do not require purification of immune complex, IC antigen or free immunoglobulin prior to analysis. The S-500 sizing step serves merely to enrich for immune complex and immunoglobulin prior to immunoblotting analysis. Second, the elimination of further purification steps expedites antibody and antigen identification and obviates the need for prior knowledge of the nature of the antigen(s). Third, dissociation and reassociation of immune complex (where optimum conditions vary with the nature of the antigen and may affect Ig viability) is not necessary. Because antigen and antibody are automatically dissociated by SDS treatment prior to electrophoresis and additional antibody is available to probe, there is no need to recover IC immunoglobulin as long as there is antibody

excess. Finally, dissociation of IC antigen and antibody by SDS-PAGE in the immunoblot format also has a distinct advantage over the more common ELISA format in which immune complex remains in aggregate form. IC antigen and antibody cannot always be "seen" by the appropriate probe in an ELISA when recognition sites are hidden within the immune complex.

This assay was developed as a means to identify potential tumor-associated antigens whose presence was suggested by less direct methods (13-16). Currently it is thought that certain TAAs may appear frequently in patients with a given form of cancer while other TAAs may be unique to one or a few individuals with that form of cancer. The method described here is especially valuable for the identification of the latter class of TAAs since it tests each patient's excreted antigen(s) against his/her own immunoglobulin rather than against specific monoclonal or polyclonal antibodies which may not recognize individual TAAs.

The interaction between V_O antigens and medium molecular weight antibody demonstrated by this method is not direct proof that these antigens are associated with immune complex. For example, it is possible that a given antigen band has been dissociated from a very high mw multimer, mixed multimer, or aggregate which co-elutes with high mw immune complexes but is not bound to

immunoglobulin in any way. However, any of these phenomena would also require that antigen be recognized by S-500 medium molecular weight immunoglobulin fractions but not IC-associated immunoglobulin. Direct proof of IC-association for a given antigen requires purification of immune complex and subsequent purification and identification of antigen from IC such as the schemes devised by Phillips et al (2) and Qiemm et al (5). The method described here is designed as a rapid means to identify potential immune complex antigens and as such can be a powerful first step in the identification of tumor-associated antigens, autoantigens, or foreign (bacterial or viral) antigens from a variety of body fluids, in vitro systems or cell fractions.

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REFERENCES

1. Rossen, R.D., Reisberg, M.A., Hersh, E.M., and Gutterman, J.U. The C1q binding test for soluble immune complexes: clinical correlations obtained in patients with cancer. J. Natl. Cancer Inst. 1977; 58:1205-15.

2. Phillips, T.M., Queen, W.D., and Lewis, M.G. The significance of circulating immune complexes in patients with malignant melanoma. In: Reisfeld, R.A and Ferrone, S. (Eds.), Melanoma, Antigens, and Antibodies. Plenum Press, New York, 1989:289-316.

3. Singhal, A.K., Singhal, M.C, Nudelman, E., Hakomori, S-i., Balint, J.P., Grant, C.K. and Snyder, Jr., H.W. Presence of fucolipid antigen with mono- and dimeric X determinant (Lex) in the circulating immune complexes of patients with adenocarcinoma. Cancer Res. 1987; 47:5566-71.

4. Gupta, R. K. Circulating immune complexes in malignant melanoma. Dis. Markers 1988; 6:81-95.

5. Queen, W.D., Bharwani, N., Phillips, E.A., Ruether, B.A., Phillips, T.M., and Jerry, L.M. Biochemical classification of circulating immune complexes in human malignant melanoma and hematologic neoplasms. Oncology 1989; 46:14-25.

6. Inman, R.D., Hamilton, N.C., Redecha, P.B., and Hochhauser, D.M. Electrophoretic transfer blotting analysis of immune complexes in rheumatoid arthritis. Clin. Exp. Immunol. 1986; 63:32-40.

7. Meryhew, N.L., Westling, P.R., Eerdmans, M.E., Janecek, E.J., and Runquist, O.A. A kinetic study of erythrocyte-DNA/anti-DNA immune complex association and dissociation reactions in systemic lupus erythematosus. Biochim. Biophys. Acta 1989; 991:470-6.

8. Imai, H., Chen, A., Wyatt, R.J., and Rifai, A. Composition of IgA immune complexes precipitated with polyethylene glycol. J. Immunol. Methods 1987; 103:239-45.

9. Pharmacia Fine Chemicals. Sephacryl S-400, S-500, S-1000 Superfine for the separation of very large molecules and small particles. Vastra Aros Tryckeri, Sweden, 1981; S451.

10. Laemmli, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227:680-5.

11. Towbin, H., Staehelm, T. and Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Nat. Acad. Sci. USA 1979; 76:4350-4.

12. Huth, J.F., Saxton, R.E, Morton, D.L., and Irie, R.F. A human monoclonal antibody produced by in vitro sensitization of human lymphocytes with an antigen from urine of a sarcoma patient. J. Surg. Res. 1987; 42:591-6.

13. Huth, J.F., Gupta, R.K., and Morton, D.L. Development of an enzyme immunoassay to detect and quantitate tumor-associated antigens in the urine of sarcoma patients. Cancer 1981a; 47:2856-61.

14. Huth, J.F., Gupta, R.K., and Morton, D.L. Assessment of in vivo effectiveness of tumoricidal chemotherapy and radiation therapy by serial analysis of tumor-associated urinary antigen titers in patients with sarcoma. Cancer Treatment Reports 1981b; 65:1037-42.

15. Huth, J.F., Gupta, R.K., and Morton, D.L. Relationship between circulating immune complexes and urinary antigen in human malignancy. Cancer 1982; 49:1150-7.

16. Huth, J.F., Gupta, R.K., Eilber, F.R., and Morton, D.L. A prospective postoperative evaluation of urinary tumor-associated antigens in sarcoma patients. Cancer 1984; 53:1306-10.

17. Lloyd, K.O. and Old, L.J. Human monoclonal antibodies to glycolipids and other carbohydrate antigens: Dissection of the humoral response in cancer patients. Cancer Res. 1989; 49:3362-9.