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(54) Title: ASSAY FOR IDENTIFYING θ2u-GLOBULIN-MEDIATED NEPHROPATHY

(57) Abstract: An immunochemical assay kit and associated methodology for identifying α2u-globulin-mediated nephropathy in rodent toxicity/carcinogenicity studies including the following contents: a primary antibody for binding to α2u-globulin; an enzyme-conjugated secondary antibody for binding to the primary antibody; a chemical substrate for creating a detectable change when contacted with the enzyme-conjugated secondary antibody; a first reservoir for contacting α2u-globulin with the primary antibody; a second reservoir for contacting the primary antibody, the enzyme-conjugated secondary antibody, and the substrate with one another; positive and negative controls samples; and purified α2u-globulin. Also included are methods for preparing buffers and reagents; preparing control samples and unknown samples; performing total protein assays and reagent dilution assays; running a competitive inhibition enzyme-linked immunosorbent assay (ELISA) in control samples and unknown samples; and calculating results.

ASSAY FOR IDENTIFYING α_{2u}-GLOBULIN-MEDIATED NEPHROPATHY

BACKGROUND OF THE INVENTION

The present invention relates to an immunochemical assay kit and associated methods for identification and assessment of α_{2u}-globulin-mediated nephropathy in rodent toxicity/carcinogenicity studies.

Safety studies investigating the potential toxicity and carcinogenicity of certain chemicals as part of human risk assessment, require testing suspect chemicals in at least two mammalian species. Due to their hardiness, longevity, genetic stability, background lesion incidence, and sensitivity to tumor induction, rats are the species most commonly subjected to such testing. Consequently, the scientific literature contains much information regarding the incidence of background lesions and responses to certain test chemicals in various species of rats. Furthermore, rats have been found to exhibit a unique susceptibility to renal tumorgenicity following exposure to certain organic chemicals which are collectively termed "Chemicals that Induce α_{2u}-Globulin Accumulation" or "CIGA" compounds. CIGA compounds include petroleum-based and synthetic fuels, military aviation propellants, and solvents.

Kidney disease, or "nephropathy" arises as a result of the formation of hyaline droplets in the kidney proximal tubule epithelial cells. These droplets are the result of an excessive accumulation of the protein α_{2u}-globulin. Once present, these droplets induce a sequence of pathological events, which ultimately manifest as renal tubule neoplasia. The protein α_{2u}-globulin is found in high concentrations in the urine of male rats, and is not present in the urine of other species. α_{2u}-Globulin-induced renal nephropathy results from a conformational change in α_{2u}-globulin upon binding CIGA compounds. This event subsequently inhibits lysosomal degradation, and results in a lethal accumulation of the α_{2u}-globulin-CIGA complex. Whereas plasma half-lives of low molecular weight proteins are typically measured in terms of minutes, the half-life of the α_{2u}-globulin-CIGA complex is substantially extended, on the order of hours, due to limited catabolism of α_{2u}-globulin.

An observed increase in the incidence of renal tumors in animal

subjects during a toxicity/carcinogenicity study is highly relevant to human risk assessment involving test chemicals because risk assessment in humans generally assumes that chemicals that produce tumors in laboratory animals are potentially hazardous to humans. This extrapolation often remains appropriate even in the absence of data to the contrary; therefore, there is considerable commercial and regulatory interest associated with identifying and establishing the mechanistic basis for any nephropathy arising in rats during chemical safety studies.

Understanding the mechanistic basis of α_{2u}-globulin-mediated nephropathy permits a more accurate assessment of the toxic or carcinogenic potential of CIGA compounds in humans because histopathological events involving (i) the excessive accumulation of hyaline droplets containing α_{2u}-globulin in renal proximal tubule cells, (ii) cytotoxicity and single cell necrosis of the tubule epithelia, (iii) sustained regenerative tubule cell proliferation and hyperplasia, and ultimately, (iv) formation of renal tubule tumors, can all be traced back to events at the molecular level. Furthermore, differentiating chemically induced renal tumors in rats from tumors arising due to exposure to CIGAs during chemical safety studies is vital for human risk assessment since renal tumors arising from the aberrant accumulation of α_{2u}-globulin in rats have no significance regarding human exposure, and thus are irrelevant in hazard characterization for human risk assessment. Thus, there is a need for a reliable, quantitative immunochemical assay that accurately characterizes the excessive accumulation of α_{2u}-globulin in kidneys collected from rats in chemical safety studies.

Japanese patents 5-333025 and 6-118076 each disclose similar methods for evaluating the carcinogenicity of suspect chemicals by detecting α_{2u}-globulin nephropathy in rats exposed such chemicals. Following exposure of a suspect chemical to rats, any increase in α_{2u}-globulin is observed by purifying protein from kidney, serum, and urine samples and performing SDS-polyacrylamide gel electrophoresis to detect a protein band that of the appropriate molecular size. Prepared α_{2u}-globulin antiserum is used to confirm the presence of α_{2u}-globulin through Western blotting techniques. These

methods lack the inherent specificity provided by immunochemical detection of a target protein, and unlike immunochemical assays, are largely qualitative.

U.S. Patent No. 5,139,932 to Cederholm et al. discloses an immunoassay for diagnosing IgA nephropathy, which includes the steps of: (i) preparing a substrate capable of binding fibronectin or IgA; (ii) contacting the substrate with a sample to bind any fibronectin-IgA-complex present in the sample to the substrate; (iii) and determining the presence of the bound complex by using the reaction between the exposed part of the bound complex and an antibody which corresponds to the complex. A kit version of this assay is also provided.

U.S. Patent Nos. 5,534,431 and 5,654,158 to McDonald disclose an immunoassay for determining the presence of nephropathy-related immunoglobulin-like protein (NRIg) in a sample of body fluid, which includes the steps of: (i) simultaneously contacting a test sample of body fluid with a first and second antibody which are specific for NRIg at different antigenic sites (the first antibody is detectably labeled and soluble in the body fluid, and the second antibody is bound to a solid carrier which is insoluble in the body fluid under conditions which allow the formation of an insoluble complex of the first antibody the NRIg, and the second antibody); (ii) separating the insoluble complex from the sample of body fluid and unreacted labeled first antibody; (iii) measuring the amount of labeled antibody bound to the insoluble complex or the amount of unreacted labeled antibody; and (iv) relating the amount of labeled antibody bound to the insoluble complex or the amount of unreacted labeled antibody to control samples and determining the presence or concentration of NRIg in the test sample.

Despite superficial similarities, the prior art discussed above does not disclose an immunological assay kit with associated methods for utilizing α_{2u}-globulin as an indicator of α_{2u}-globulin nephropathy in kidney proximal epithelial cells. The prior art directed toward detecting renal toxicity either utilizes a different technical approach (i.e., the use of NRIg as a protein marker), or offers a far less sensitive measure of detection (i.e., SDS-polyacrylamide gel electrophoresis) as opposed to the immunological format

of the present invention.

SUMMARY OF INVENTION

These and other deficiencies of the prior art are overcome by the present invention, which provides a kit and associated methods, specifically utilizing α_{2u}-globulin as an indicator of hyaline droplet nephropathy in rats during chemical safety studies.

The present invention utilizes the enzyme-linked immunosorbent assay (ELISA) format of immunochemical assays, which results in a highly sensitive, highly specific, quantitative assay. Immunochemical assays are dependent upon antibodies (polyclonal and/or monoclonal) with appropriate specificities for detection of the molecule of interest (i.e., distinct antigenic determinants on α_{2u}-globulin). Thus, the present invention is very useful for detection and assessment of α_{2u}-globulin-mediated nephropathy in toxicity and carcinogenicity studies, as well as for evaluating the carcinogenic potential of certain toxic chemicals in general when rats are used as the test subjects.

A preferred embodiment of this invention is a competitive indirect ELISA kit for detection of α_{2u}-globulin in rat kidneys. The preferred version of the kit includes: (1) mouse anti-α_{2u}-globulin monoclonal antibody for use as a primary antibody for specific binding to α_{2u}-globulin; (2) alkaline phosphatase-conjugated goat anti-mouse IgG for use as a secondary antibody for binding to mouse anti-α_{2u}-globulin for quantitation (3) 4-methylumbelliferyl phosphate (MUP) which is converted to a fluorogenic product (4-methylumbelliferone) following enzymatic cleavage by alkaline phosphatase conjugated to the secondary antibody for signal amplification; (4) a 96-well flat bottom, clear, methacrylate plate for reacting the α_{2u}-globulin with the mouse anti-α_{2u}-globulin monoclonal antibody; (5) a 96-well U-bottom, black pigmented, styrene plate for reacting the mouse anti-α_{2u}-globulin monoclonal antibody, alkaline phosphatase-conjugated goat anti-mouse IgG, and MUP with one another; (6) purified α_{2u}-globulin for preparation of assay standards and controls; (7) kidney tissue from untreated female rats for use as a matrix in calibration standards and quality control samples; (8) kidney tissue from male

rats not exposed to a chemical known to induce α_{2u}-globulin nephropathy for use as negative control samples; (9) kidney tissue from male rats exposed to a chemical known to induce α_{2u}-globulin nephropathy for use as positive control samples; and (10) a set of instructions for how to use the assay kit, how to make all necessary calculations, and how to interpret results obtained using the immunochemical assay kit.

A preferred method for detecting accumulation of α_{2u}-globulin in tissue samples by means of an ELISA, includes (1) purifying α_{2u}-globulin for use in preparation of calibration standards; (2) exposing male rats to a chemical known to induce α_{2u}-globulin nephropathy to generate control samples; (3) preparing necessary buffers and reagents; (4) preparing control samples and unknown samples by diluting the samples in the buffers; (5) performing a total protein assay to determine protein concentrations of control samples; (6) performing a reagent dilution assay for determining optimal concentrations of reagents for subsequent reactions; (7) running a competitive inhibition ELISA on the control samples and unknown samples; and (8) calculating results.

Therefore, it is an objective of the present invention to provide an immunochemical assay kit, which may be made commercially available for evaluating α_{2u}-globulin-induced nephropathy in rodent toxicity/carcinogenicity studies involving a variety of suspect chemicals.

It is an additional objective of this invention to provide instructions for preparing the various components required for completing the ELISA assay, as well as instructions for calculating quantitative results based on data gathered from performing the assay.

Further objects, advantages, and novel aspects of this invention will become apparent from a consideration of the subsequent detailed description.

DETAILED DESCRIPTION OF THE INVENTION

As stated, exposure of rats to CIGA compounds results in a dose-dependent accumulation of hyaline droplets in the proximal renal tubules due to the accumulation of a complex formed between the compound and a 18.7 kD protein, α_{2u}-globulin. This condition is referred to as hyaline droplet nephropathy. The correlation of hyaline droplet formation, accumulation of α_{2u}-globulin within the droplet, and increased cell replication at the same specific location in the proximal tubule of the kidneys indicates that chronic exposure to CIGAs will cause α_{2u}-globulin-mediated droplet formation and persistent cell proliferation as a result of unrepaired mutations resulting from enhanced DNA replication. This persistent cell proliferation may result in the formation of malignant tumors in the kidneys of the test rats.

Differentiating renal tumors occurring in rats as the results of α_{2u}-globulin-mediated nephropathy from renal tumors arising from an alternative etiology, is vital for human risk assessment. Direct measurement of α_{2u}-globulin in kidney homogenates is used to distinguish between kidney toxicity resulting from hyaline droplet formation versus other chemically induced mechanisms.

The present invention provides an immunochemical assay and kit for detection of excessive accumulation of α_{2u}-globulin in rat tissues and fluids. α_{2u}-Globulin is produced in the liver, transported through the bloodstream (serum) to the kidneys, and excreted in urine. Thus, the detection of excessive α_{2u}-globulin accumulation may include analysis of liver or kidney tissue, serum, urine or combinations thereof. By measuring α_{2u}-globulin in rat tissues and fluids, the present invention is useful in toxicity/carcinogenicity studies for identifying organic compounds with significant CIGA potential. Alternately, the present invention may be used as an early indicator of potential pathological changes the renal function of rats chronically exposed to weaker CIGA compounds.

In the context of the present invention, the terms "tissues and fluids" include any tissue or fluid containing detectable amounts of α_{2u}-globulin. Such fluids typically include kidney, liver, serum, and plasma (i.e., serum that has

not had consumable clotting factors removed). Kidney and urine samples are preferred for use in the present invention. Kidneys from rats may are only obtainable following terminal sacrifice of test animals; therefore, additional animals must be added to an ongoing toxicity/carcinogenicity study. Otherwise, α_{2u}-globulin analysis can only be conducted at the end of the study. In contrast, analysis of α_{2u}-globulin in urine samples is non-invasive and can be conducted repeatedly on the same test animals at frequent intervals during the study without the loss of animals from the cohort receiving chronic administration of the test chemical.

The present invention requires the production of polyclonal and/or monoclonal antibodies to α_{2u}- globulin. α_{2u}-Globulin is an 18.7 kD protein with unique antigenic determinants, which are useful in inducing the production of polyclonal or monoclonal antibodies. The antibodies produced by immunizing suitable host animals with purified α_{2u}-globulin bind specifically, and with very high affinity, to the antigenic determinants expressed in α_{2u}-globulin.

Polyclonal antibodies result from the production of antibodies to all recognizable sites on a substance injected into a host animal. The "polyclonal antibody response" produces a broad range of antibodies of differing affinities and specificities for a particular immunogenic substance. Multiple antibody-producing cells from multiple sites in the host's body produce antibodies to that part of the immunogenic substance for which they are activated; each antibody-producing a clone making only one type of antibody to only one antigenic epitope on the foreign substance. Serum containing polyclonal antibodies (polyclonal antiserum) may be harvested from the immunized animal for the duration of the animal's lifetime.

Monoclonal antibodies are produced by recovering specific immune lymphocytes from the immunized host animal and fusing these cells with myeloma tumor cells derived from the same animal species to form giant somatic cell hybrids. Hybrid cell lines have the capacity to grow rapidly and indefinitely in tissue culture due to the characteristics of the myeloma parent cell, and they secrete large amounts of the antibody specified by the genes of the normal antibody-secreting lymphocyte parent. After appropriate selection

and cloning, the hybridomas are propagated in tissue culture or in a genetically identical or immunocompromised animal for an indefinite period of time to continuously produce primary antibody.

Methods and techniques for immunizing host animals with α_{2u}-globulin; isolating polyclonal antibodies, or in the case of monoclonal antibodies, recovery of specifically immune lymphocytes and establishment of hybridoma cell lines for continual production of antibody to α_{2u}-globulin; and antibody characterization are generally known to those skilled in the art.

To detect anti-α_{2u}-globulin antibodies in immunochemical assays in general, a variety of alternative standard substrates are used, including: radioactive substrates such as tritium, carbon-14, phosphorus-32, and iodine-125; fluorescence substrates such as fluorescein, rhodamine, and phycoerythrin, luminescent substrates, or enzyme markers such as horseradish peroxidase, alkaline phosphatase, and β-glactosidase. The use of enzyme markers requires the addition of a substrate, which is converted to a product, which enhances signal amplification. For example, for alkaline phosphatase conjugates, preferred substrates may include 4-nitrophenyl phosphate (colorimetric), 4-methylumbelliferyl phosphate (fluorogenic), 3-[2′-(spiroadamantane)-4-methyl-4-3″-phosphoryloxyphenyl-1-2-dioxetane, disodium salt] (luminescence), and avidin-biotin. Methods and techniques for labeling antibodies with such markers, and for detecting these markers, are generally known to those skilled in the art.

Immunochemical assays utilizing enzymes are typically classified as either competitive heterogeneous or competitive homogeneous enzyme immunoassays. The competitive heterogeneous enzyme immunoassays, which include the ELISA, are based on the separation of antigen-antibody complexes formed following incubation of antigen and antibodies from free antigen and antibody. Competitive homogeneous enzyme immunoassays, in which the activity of an enzyme-ligand conjugate is reduced when the ligand is complexed to an antibody, do not involve a separation step prior to measurement. The usefulness of homogeneous enzyme immunoassays tends to be limited to the assay of low molecular weight haptens, whereas the

heterogeneous enzyme assay is generally applicable to the measurement of almost any antigen. *See generally,* Voller, A., Bartlett, A., and Bidwell, D.E., *Enzyme immunoasays with special reference to ELISA techniques,* Journal of Clinical Pathology, 1978, 31:507-520.

The present invention provides an immunochemical assay for measurement of α_{2u}-globulin. The preferred embodiment of this invention is an enzyme-linked immunosorbent assay (ELISA) comprising two major components. The first component is the immunological reaction, which occurs between an antigen and an antibody. An enzyme-labeled antibody is required, and may be either the primary antibody with specificity to the antigenic determinants in the target molecule (i.e., α_{2u}-globulin), or a secondary antibody with antigenic determinants directed against the primary antibody. The second component of the ELISA requires the attachment or immobilization of a "capturing agent" (i.e., antigen or antibody) to a solid support made of an inert material such as glass, synthetic polymers, synthetic resins, cellulose, or various suitable metals. This attachment is accomplished by covalent or non-covalent linkage, adsorption, or other process. In a preferred embodiment, the inert material to which the capturing agent is attached has an extensive, continuous form, such as a membrane or sheet, which is either flat or molded into convenient shapes such as multiwell plates (i.e., 96-well microtiter plates). In alternative embodiments, the inert material is in the form of discrete particles or beads.

The methods and techniques required for attaching these capturing agents to inert materials will vary depending upon selected agents and substrates. These methods and techniques, as well as the suitable inert materials used for immunochemical assays, are generally known to those skilled in the art.

I. Renal Toxicity Detection Kit

In a preferred embodiment of the present invention, an immunochemical assay kit for assessing α_{2u}-globulin-mediated nephropathy in carcinogenicity and toxicity studies includes: (1) mouse anti-α_{2u}-globulin monoclonal antibody for use as a primary antibody for binding to α_{2u}-globulin;

(2) alkaline phosphatase-conjugated goat anti-mouse IgG for use as a secondary antibody for binding to mouse anti-α_{2u}-globulin (3) 4-methylumbelliferyl phosphate (MUP) for creating a fluorimetric change when contacted with the alkaline phosphatase-conjugated goat anti-mouse IgG; (4) a 96-well flat bottom, clear, methacrylate plate for reacting the α_{2u}-globulin with the mouse anti-α_{2u}-globulin monoclonal antibody; (5) a 96-well U-bottom, black pigmented, styrene plate for reacting the mouse anti-α_{2u}-globulin monoclonal antibody, alkaline phosphatase-conjugated goat anti-mouse IgG, and MUP with one another; (6) purified α_{2u}-globulin for preparation of assay standards and controls; (7) kidney tissue from untreated female rats for use as a matrix in calibration standards and quality control samples; (8) kidney tissue from male rats not exposed to a chemical known to induce α_{2u}-globulin nephropathy for use as negative control samples; (9) kidney tissue from male rats exposed to a chemical known to induce α_{2u}-globulin nephropathy for use as positive control samples; and (10) a set of instructions for how to use the assay kit, how to make all necessary calculations, and how to interpret results obtained using the immunochemical assay kit.

Preferred methods of preparing the components of this kit (where necessary), and other developmental aspects of the assay are discussed in greater detail below where such details are not widely known by those skilled in the art.

Purified α_{2u}-Globulin

The present invention requires purification and characterization of α_{2u}-globulin. α_{2u}-Globulin may be purified from the urine of male rats and characterized as described in Mao et al., *Analysis of α_{2u}-globulin in Rat Urine and Kidneys by Liquid Chromatography-Electrospray Ionization Mass Spectrometry*, Chemical Research in Toxicology, 11:953-961 (1998). Additionally, isolation, purification, and characterization of α_{2u}-globulin may be accomplished by several types of separation techniques including: SDS gel electrophoresis; Western blotting, immunochemical assays; or liquid

chromatography-electrospray ionization mass spectrometry. Preferred methods for isolation, purification, and characterization are discussed below.

Urine is collected over dry ice from mature (about 10 to 12 weeks of age) male rats (Sprague-Dawley, Fischer 344/N or equivalent) and kept frozen at about minus 20°C prior to isolation of α_{2u}-globulin. Low molecular weight proteins are isolated using a combination of molecular weight cut-off filters and high performance liquid chromatography (HPLC). For example, 90 mL of rat urine is concentrated to about 9.0 mL using Centriprep-10 centrifugal concentrators (Amicon, Inc., Beverly, VA), which also eliminates low molecular weight (<10,000 daltons) materials. Aliquots (typically 500 µl) of the concentrated urine are then subjected to HPLC. Separations are performed on a Synchropak AX300 (250 mm x 10 mm) analytical column (Eichrom Technologies, Darrien, IL) or an equivalent column using a solvent system consisting of 10 mM Tris-HCl (pH 7.4) ramped to 0.5 M NaCl at 2.5 mL/min over 25 min. The HPLC system (Hewlett Packard 1050 or equivalent) is used for separation and purification. A number of UV absorbing materials are observed in the chromatographic profile, however, a peak may be identified that is found only in male rat urine (i.e., was absent in female rat urine). Once this peak is identified, successive 0.5 mL aliquots are chromatographed and material eluting at the retention time window corresponding to the peak found uniquely in the chromatogram of male rat urine is pooled and concentrated in Centriprep-10 centrifugal concentrators.

α_{2u}-Globulin is dissolved in a known volume of buffer for characterization tasks, which include assessment of the amount of protein, purified, confirmation of identity and estimation of purity. Total protein analysis may be conducted using a variety of commercially available reagents (e.g., Bicinchoninic (BCA) Protein Assay Reagent (Pierce Chemical Co., Rockford, IL). Typically, about 1.5 mg of α_{2u}-globulin is purified from about 4.0 mL of male rat urine. Identity confirmation and estimation of purity of the isolated α_{2u}-globulin fraction is accomplished using one or more of these methods: (i) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to assess the apparent molecular mass of the material, (ii) gel

electrophoresis in combination with antibodies directed against α_{2u}-globulin (i.e., Western blotting) for specific identification of α_{2u}-globulin in the gel banding pattern, or (iii) liquid chromatography-electrospray ionization mass spectrometry to assess the molecular mass of the protein. Preferred versions of methods are described in greater detail below.

SDS-PAGE is accomplished using 18% gels run on a SE 250 Mighty Small™ Vertical Slab Minigel Unit (Hoefer Scientific Instruments, San Francisco, CA) using methods described in *Protein Electrophoresis Applications Guide* (Hoefer Scientific, 1994). Gels (7 x 8 cm, 1 mm thick) are self-cast using a SE 245 Gel Caster (Hoefer Scientific Instruments, San Francisco, CA). Urine samples are lyophilized to dryness using a SpeedVac™ (Savant Instruments, Inc., Farmingdale, NY) and diluted in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 5% β- mercaptoethanol, 2.5% (w/v) SDS and heated to 95°C for five minutes. After cooling to room temperature 5 µl samples is applied to the gels and run at constant current at 30 mA using a BioRad 3000/300 Power Supply (Bio-Rad Laboratories, Hercules, CA) at ambient temperature. The gels are stained with a solution consisting of 0.025% Coomassie Brilliant Blue R 250, 40% methanol and 7% acetic acid and destained with a sequential treatment of methanol:acetic acid (40%:7%) and acetic acid:methanol (5%:7%) solutions. A prestained low molecular weight range (43, 29, 18.4, 14.3, 6.2, and 3 kDa) calibration mixture (Life Technologies, Gaithersburg, MD) is run on each gel and used to determine the molecular weight range of the proteins.

Immunoblotting of purified α_{2u}-globulin fractions with anti-α_{2u}-globulin monoclonal antibodies can be conducted to confirm identity. Purified samples from the α_{2u}-globulin fractions and commercially available low range molecular weight markers (Life Technologies, Gaithersburg, MD) concurrently are resolved by SDS-PAGE as previously described. The gel is divided and one portion of the gel is stained with Coomassie Blue as previously described, and proteins from the second portion of the gel are electrophoretically transferred to sheets of nitrocellulose (Bio-Rad Laboratories, Hercules, CA) using a semi dry blotter (Integrated Separation Systems, Natick, MA). The sheets are

washed twice with Tris-buffered saline containing 0.1% Tween-20 (TBS-Tween; 20 mM Tris-HCl (pH 7.4) 137 mM NaCl, 0.1% Tween-20) and incubated for 60 minutes at 37°C in a mixture of 5% powdered milk (fat free) in TBS-Tween in an effort to reduce non-specific binding of reagents in subsequent phases of the experiment. The sheets are then washed with TBS-Tween (once for 15 minutes and then three times at 5 minutes each) and incubated with a solution of the mouse anti-α_{2u}-globulin monoclonal antibodies (1:5000 dilution with 2% bovine serum albumin (BSA) in TBS-Tween) for 60 minutes at 37°C. The sheets are then washed with TBS-Tween (once for 15 minutes and then three times at 5 minutes each) and incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG (heavy + light chain) antibody (Pierce Chemical Co., Rockford, IL or equivalent) with 2% BSA in TBS-Tween (1:5000 dilution) for 60 minutes at 37°C. The sheets are washed with TBS-Tween (once for 15 minutes and then three times at 5 minutes each) and placed into a protective cover. Atto-Phos™ substrate reagent (JBL Scientific, Huntington, England) is used as the substrate for fluorescence development. Detection of fluorescence activity is performed on a Vistra Fluorescence FluorImager SI (or equivalent) interfaced to a Dell Dimension XPS P90 computer with Image QuaNT™ software (Molecular Dynamics, Inc. Austin, TX).

Confirmation of identity and estimation of purity of the isolated α_{2u}-globulin fractions may be accomplished by liquid chromatography-electrospray ionization mass spectrometry according to methods reported By Mao et al., *Analysis of α_{2u}-globulin in Rat Urine and Kidneys by Liquid Chromatography-Electrospray Ionization Mass Spectrometry*, Chemical Research in Toxicology, 11:953-961 (1998). In a preferred method, 90 µl of HPLC-purified α_{2u}-globulin in 1 mM Tris-HCl (pH 7.4) are desalted thrice with 1.0 mL of deionized water using sterile ultra spin cellulose triacetate 10,000 amu molecular weight cut-off filters (Alltech Assoc. Inc. Deerfield, IL). The filters are centrifuged using a Microcentrifuge (Alltech Assoc., Inc., Deerfield, IL, or equivalent) operating at 2,000 g. Material retained on the filter is resuspended in 100 µL of methanol:water (1:1) containing 1% acetic acid yielding a final concentration

of 900 µg/mL. The spectra are acquired with a Finnigan MAT TSQ 7000 (Finnigan MAT, San Jose, CA or equivalent) triple quadrupole mass spectrometer equipped with a Finnigan atmospheric pressure electrospray ionization (ESI) source. The sample is infused into the source by a syringe pump (Model 22, Harvard Apparatus, South Natick, MA) at a flow rate of approximately 2 µL/minutes. The electrospray voltage is set at 3.9 kV, the heated capillary is operated at 200°C and nitrogen is used as a sheath gas at 55 psi. The quadrupole manifold and metal ESI ion inlet capillary are heated to 70°C and 200°C. The mass spectrometer is initially tuned and calibrated using a solution of myoglobin (25 µg/mL). The quadrupoles are scanned over the mass range from 1000 to 2500 amu with a scan time of 1.9 seconds per scan. Bioworks software (Finnigan MAT, San Jose, CA) is used for deconvolution of the charge envelop. The molecular mass of α_{2u}-globulin is 18,730 daltons.

Preparation of Anti-α_{2u}-Globulin Monoclonal Antibody

Mouse (or other suitable host species) anti-α_{2u}-globulin monoclonal antibody (or polyclonal antiserum) is also required in the preferred embodiment of the present invention. Mouse anti-α_{2u}-globulin monoclonal antibody is prepared by methods known by those skilled in the art using α_{2u}-globulin extracted and purified from rat urine (see, for example, methods described in *Cellular and Molecular Immunology,* Abbas et al., 1991; *Monoclonal Antibodies: Principals and Practice,* Goding, 1996; *Purification Tools for Monoclonal Antibodies,* Gagnon, 1996. The alkaline phosphatase-conjugated goat anti-mouse IgG is a commercially available product.

Optimization of Experimental Conditions (ELISA Reagent Dilution)

Development of a competitive indirect ELISA initially requires optimization of experimental conditions using a reagent dilution assay. The reagent dilution assay, performed whenever a new preparation of antibodies or antigen is used, is conducted to determine: (i) the optimal dilution of antigen for coating wells of microtiter plates; and (ii) the dilution at which an

ELISA reading of 50% maximum value occurs for optimal interactions in subsequent competition experiments. Measurement of α_{2u}-globulin is then accomplished by a competitive inhibition experiment wherein reactions are prepared that involve a competition for antibodies between α_{2u}-globulin adsorbed to wells of microtiter plates and free α_{2u}-globulin in solution. The assay is calibrated by comparison of the ELISA reading obtained upon measurement of a sample to that obtained upon measurement of known concentrations of α_{2u}-globulin in a standard curve. The following is a description of a preferred method for conducting the required reagent dilution assay in accordance with the present invention.

A typical 96-well microtiter plate consists of eight stacked, horizontal rows of wells (labeled A-H) each row consisting of 12 individual wells organized into vertical columns. In the preferred method, the wells in rows B, C, D, E, F, and G of U-bottom shaped MicroFluor 96 well microtiter plates (Dynatech Laboratories, Inc., Chantilly, VI or equivalent) are equilibrated overnight at 4°C with 100 μL aliquots of purified α_{2u}-globulin prepared at target concentrations of 100, 250, 500, 750, 1000, and 2000 ng/mL in phosphate buffered saline (PBS, 0.14 M NaCI, 1.0 M KH_2PO_4, 8 mM Na_2HPO_4, 3 mM KCl, pH 7.4). Negative controls are established in rows A and H which are filled with 100 μL aliquots of PBS, and PBS/0.05% Tween containing 1% bovine serum albumin, PBS-Tween, respectively.

After removal of the coating solutions (accomplished by inverting the microtiter plate and shaking the contents into a waste container or by using a pipette), the plates are washed by alternately filling and emptying each well five times with PBS-Tween. The washing step is accomplished manually using a plastic squirt bottle containing PBS-Tween or an automated microtiter plate washer (i.e., Dynatech Ultrawash Plus or equivalent). Two hundred microliter aliquots of PBS-Tween containing 1% bovine serum albumin (BSA) are added to each well of the plate and allowed to equilibrate for 2 hours at 37°C in an effort to reduce non-specific binding of primary and secondary antibodies in subsequent stages of the experiment. Serial dilutions of the primary antibody (e.g., mouse anti-α_{2u}-globulin monoclonal antibody diluted from 1:2000 to

1:10^8) are prepared in PBS-Tween, and 100 μL of each diluted solution is added to the wells of columns 1 through 12 of the microtiter plate after washing the wells five times with PBS-Tween. Following a 2 hour incubation with the primary antibody solutions, 100 μL of an alkaline phosphatase conjugated goat anti-mouse IgG (heavy and light chain) secondary antibody (1:1000 dilution in PBS-Tween containing 1% BSA) is added to all wells. The secondary antibody conjugate is allowed to equilibrate at 37°C for 2 hour and then the wells of the plate are washed five times with PBS-Tween and twice with 50 mM 2-amino-2-methyl-1,3-propanediol containing 0.01% BSA. One hundred microliters of 0.1 mM 4-methylumbelliferyl phosphate (MUP) prepared in 50 mM 2-amino-2-methyl-1,3-propanediol containing 0.01% BSA is added to the wells and incubated for 30 minutes at room temperature. The fluorescence intensity is measured using a microplate reader (such as the Cytofluor II Microplate Fluorescence Reader Biosearch Instruments, Millipore Corp., Bedford, MA) using a 360 nm/460 nm excitation/emission filter pair.

The minimum concentration of α_{2u}-globulin required to coat the wells of the microtiter plates is 750 ng/mL. Lower concentrations of α_{2u}-globulin in the coating solution lead to a decrease in the maximum relative fluorescence intensity observed at various dilutions of antibody. Therefore, the preferred methods require a concentration of 2000 ng/mL to coat the wells of microtiter plates for subsequent ELISA measurements. This concentration is suitable over a wide range of antibody dilutions. Using one stock of monocloncal antibodies, the dilution of primary antibody at which an ELISA reading of 50% maximum value occurred was 1:1.5 x 10^5. This preferred dilution was chosen for subsequent competitive inhibition experiments. The time course for fluorescence substrate development is also assessed in the reagent dilution assay by recording fluorescence intensity to determine the linear range for the assay. Under a given set of experimental conditions, fluorescence measurements increased linearly over the course of at least 120 minutes, permitting measurements at any time during this period.

Selection of Concentration Ranges for Analytical Method

In the present invention, the preferred range of the analytical method is based on analysis of the available scientific literature concerning accumulation of α_{2u}-globulin in kidney samples following exposure to chemical agents such as unleaded gasoline, 2,2,4-trimethylpentane, 1,4 dichlorocbenzene, isophorone, decalin and *d*-limonene (Swenberg et al., *Toxicol. Appl. Pharmacol.,* 97: 35-46, 1989; Borghoff et al., *Toxicol. Appl. Pharmacol.,* 107: 228-238, 1991; Flamm and Lehman-McKeeman, *Regul. Toxicol. Pharmacol.,* 13: 70-86, 1991; Borghoff, *CITT Activities,* 13: 1-8, 1993, and Hard et al., *Environ. Health Perspect.,* 99: 313-349, 1993. Based on this information, the preferred method performance evaluation is designed to permit measurements of α_{2u}-globulin ranging from about 0.005 to 1.0 ng/ng total soluble protein in kidney homogenates.

Preparation of Spiked Kidney Standards, Control Samples and Blanks

The following are descriptions of preferred methods for preparation of spiked kidney standards, positive and negative control samples, and blank samples. Stock solutions are prepared by dilution of purified α_{2u}-globulin isolated from male rat urine. Solutions used in preparing spiked female rat kidney homogenate standards for development of standard curves, and spiked female rat kidney homogenate samples for use as quality control (QC) samples are prepared by further dilution of these stock solutions in PBS-Tween. Given that antibody:antigen reactions occur more favorably in a biological matrix, the present invention uses a calibration curve spiked with soluble female rat kidney homogenate when study samples are analyzed, and measures sufficient blank female kidney homogenate samples to ensure that α_{2u}-globulin concentrations in the matrix are below the detection limits of the ELISA.

Spiked Tissue Standards: Blank female rat kidney homogenates are used as a matrix in calibration standards and QC samples. For this purpose, kidneys from female rats are removed and homogenized in 4X (w/v) ice-cold

67 mM sodium/potassium phosphate buffer (pH 7.2) in a 50-mL polypropylene centrifuge tube using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH). The homogenate is centrifuged in a Beckman T6-J table top refrigerated centrifuge at a setting of 10 for 15 minutes at 4°C. Following centrifugation, the supernatant is removed and stored at –70°C. Prior to ELISA, the kidney homogenate is freshly diluted 1:50 with 67 mM sodium/potassium phosphate buffer, and total protein content is determined using Pierce BCA reagent (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA) as the standard. Aliquots containing 500 ng of female rat kidney homogenate are then dispensed into each of several clean, 5.0-mL polypropylene culture tubes. These samples are then spiked with 1.0, 2.5, 5.0, 10, 25, 50, 100, 250, 500, 1000, and 2500 ng of α_{2u}-globulin and the volume of each standard solution brought to 1.000 mL with PBS-Tween. Every other calibration standard is prepared from an alternate, independently prepared stock solution of α_{2u}-globulin.

Positive Tissue Controls: In a preferred method for generating positive control tissue samples, male Sprague-Dawley rats (Taconic Farms, Germantown, PA) 10 to 12 weeks of age are gavaged (force-fed) with either corn oil (control group), 100, or 200 mg/kg of decalin in corn oil for four consecutive days at a volume of 5 mL/kg. On day five, rats are anesthetized with carbon dioxide, exsanginated, and their kidneys immediately excised. The dissected kidneys are weighed and then immediately quick-frozen in liquid nitrogen. The kidneys are stored frozen at –70°C until homogenized. Homogenized is conducted in 2X (weight to volume) ice-cold 67 mM sodium/potassium phosphate buffer (pH 7.2) in a 50-mL polypropylene centrifuge tube using an Ultra-Turrax (Tekmar Co, Cincinnati, OH) homogenizer. Between samples, the homogenizer probe is rinsed with 3 changes each of water, ethanol, and buffer prior to the next sample. The homogenates are centrifuged in a Beckman T6-J table top refrigerated centrifuge at a setting of 10 for 15 minutes at 4°C. Following centrifugation, the supernatant is removed and stored at minus 70°C.

Prior to the ELISA, kidney homogenates are freshly diluted 1:50 with

67 mM sodium/potassium phosphate buffer and total protein content is determined using Pierce BCA reagents (Pierce Chemical Co., Rockford, IL) with bovine serum albumin (BSA) as the standard. ELISA is performed on kidney homogenates freshly diluted to 2.5 μg/mL total protein in PBS-Tween. The concentration of α_{2u}-globulin observed in kidney homogenates from male rats administered corn oil mixtures containing up to 200 mg/kg of decalin over the course of four consecutive days increases linearly as a function of decalin exposure. Kidney homogenates from male rats receiving only corn oil are found to have 0.0056 ng α_{2u}-globulin /ng soluble kidney tissue protein. In earlier studies, we noted that the level of α_{2u}-globulin increased linearly with doses of up to 200 mg/kg, but that oral administration of 400 mg/kg decalin resulted in a significantly lower concentration of α_{2u}-globulin (0.030 ng/ng protein) than observed at a dose of 200 mg/kg. Based on qualitative data obtained using SDS-PAGE, significant increases in the intensity of the protein band corresponding to α_{2u}-globulin are observed in the urine from treated male rats, indicating that α_{2u}-globulin is passed from the kidney into the urine, thus implying disruption or exfoliation of proximal tubule cells.

ELISA Competitive Inhibition Assay

A preferred method for measuring α_{2u}-globulin in standards, QC samples, and unknown samples (all analyses are conducted at least in triplicate) during the competitive inhibition experiment initially involves coating the wells of a CytoFluor flat-bottomed 96 well microtiter plate (Plate 1: Biosearch Instruments, Millipore Corp., Bedford, MA or equivalent) with 200 μL of PBS-Tween overnight at 4°C. Plate 1 is then used for incubation of free antigen with primary antibody prior to addition of this solution to a second microtiter plate which, in the preferred method is a black U-bottom shaped MicroFluor 96 well microtiter plate (Plate 2: Dynatech Laboratories, Inc., Chantilly, VA or equivalent) that is previously incubated overnight at 4°C with 2000 ng α_{2u}-globulin per mL of PBS. The optimal concentration of α_{2u}-globulin is determined from the reagent dilution assay, and several wells on each microtiter plate should be incubated with PBS alone to serve as negative

control wells for assessing non-specific binding of reagents. The following day, Plate 1 is washed three times with PBS-Tween and 60 μL aliquots of matrix-spiked kidney homogenate standards, positive and negative control samples and blanks, and either solvent standards or samples containing unknown amounts of α_{2u}-globulin, are added to designated wells of the microtiter plates. To these samples, 60 μL of the primary antibody prepared in PBS-Tween is added at a dilution which yields 50% maximum ELISA readings.

Calibration curves are performed in triplicate on each ELISA plate using a wide range of concentrations of α_{2u}-globulin to ensure that relative fluorescence measurements plateau at either extreme. Samples spiked with 1000 and 2500 ng/mL of α_{2u}-globulin tend to almost completely inhibit fluorescence activity in competition experiments. Conversely, α_{2u}-globulin concentrations below 2.5 ng/mL are insufficient to produce a fluorescence value different from blank samples. Samples in the high and low ends of the calibration curve plateau, falling off the log-linear relationship, and are thus useful as one type of assay control. More explicit negative controls include female kidney homogenates diluted to the exact concentration as are the spiked samples, wells that contain only primary antibody, male rat kidney homogenates diluted tenfold less than used for measurements, and wells not containing α_{2u}-globulin adsorbed to the microtiter plate.

In the preferred method, the optimal dilution of stock primary antibody (monoclonal antibodies or polyclonal antiserum) is made in PBS-Tween to achieve 50% of the maximum value. Positive controls should be established in several wells containing only primary antibodies, PBS-Tween buffer spiked with a diluted solution of female kidney homogenate. Plate 1 (containing the primary antibody and antigen in solution) is incubated for 2 hours at 37°C. During this same time period, the wells of Plate 2 are incubated with 200 μL of PBS-Tween containing 1% BSA in an effort to reduce non-specific binding of primary and secondary antibodies during subsequent phases of the assay. Following the 2-hour blocking step, each well of plate 2 is washed five times with PBS-Tween. For the purpose of this discussion, the washing step is

accomplished manually using a plastic squirt bottle containing PBS-Tween or an automated microtiter plate washer (i.e., Dynatech Ultrawash Plus or equivalent). Following the washing step, 100 μL from each well in Plate 1 is directly transferred to the corresponding wells in Plate 2. The competition reaction initiated in Plate 2 is then allowed to proceed at 37°C for 2 hours after which the wells are washed five times with PBS-Tween. One hundred microliters of an alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) secondary antibody (Pierce Chemical Co., Deerfield, MI or equivalent) (current batches require 1:1000 dilution in PBS-Tween containing 1% BSA) are then added to all of the wells of the microtiter plate. The secondary antibody conjugate is allowed to equilibrate at 37°C for 2 hours and then the wells of the plate are washed five times with PBS-Tween, and twice with 50 mM 2-amino-2-methyl-1,3-propanediol containing 0.01 % BSA. One hundred microliters of 0.1 mM 4-methylumbelliferyl phosphate (MUP) prepared in 50 mM 2-amino-2-methyl-1,3-propanediol containing 0.01 % BSA is added to the wells and incubated for 30 minutes at room temperature. The fluorescence intensity is measured using a microplate reader (such as the Cytofluor II Microplate Fluorescence Reader Biosearch Instruments, Millipore Corp., Bedford, MA) using a 360 nm/460 nm excitation/emission filter pair.

Calculations, Precision, Accuracy, and Detection Limits

A characteristic feature of ELISA calibration data is the log-linear relationship of the instrument response as a function of inhibitor concentration. Calibration curves of a sigmoid nature may be constructed using the entire data set or, alternatively, calibration data from either plateau region may be removed to facilitate construction of calibration curves using a logarithmic least-squares fit of the form

$y = -m\log(x) + b$, where y is the relative fluorescence, x is the concentration, m is the slope, and b is the intercept. In the latter approach, standards with responses that are obviously not in the linear range of the assay are removed prior to fitting a line through the remainder of the data. In this manner, calibration curves are achieved which exhibit a log-linear relationship with

correlation coefficients >0.98. Once a set of conditions are defined for an assay, the analyst may decide to modify the concentrations of α_{2u}-globulin in the calibration standards to achieve a set of conditions where all calibration standards are used for the ultimate generation of a standard curve. The sensitivity obtained with ELISA is not only dependent on the type and design of the assay, the enzyme used and how its activity is measured, but also to a great extent on the affinity of the antibodies used in the assay.

II. Method of Identifying α_{2u}-Globulin-Mediated Nephropathy in Rats Using the Present Invention

The measurement of α_{2u}-globulin in rat kidney homogenates using an ELISA according to the preferred embodiment of the present invention includes the following general steps (1) preparation of buffers and reagents; (2) preparation of samples; (3) completion of a total protein assay; (4) completion of a reagent dilution assay; and (5) completion of the ELISA competitive inhibition assay and calculation of results. These steps are discussed in greater detail below.

1. Preparation of Buffers and Reagents: First prepare the homogenization buffers as follows: (1) 0.106 M sodium phosphate dibasic buffer; (2) 2 M potassium phosphate monobasic buffer; and sodium/potassium phosphate buffer, pH 7. Next prepare the ELISA reagents as follows: (1) Phosphate Buffered Saline (PBS, Coating Buffer), pH 7.2; (2) PBS-Tween Washing Buffer (PBS-Tween), pH 7.2; (3) Blocking Reagent consisting of 1% BSA in PBS; (4) 4-Methylumbelliferyl phosphate (MUP) Dilution Buffer: 50 mM 2-amino-2-methyl-1,3-propanediol buffer containing 0.01% (w/v) BSA; (5) 2000 ng α_{2u}-globulin per mL of PBS Stock Antigen (for plate coating): purified stock α_{2u}-globulin; (6) secondary antibody solution: for each plate to be assayed, prepare a 1:250 dilution of goat anti-mouse IgG stock (alkaline phosphatase-conjugated goat anti-mouse IgG (heavy and light chain) secondary antibody); (7) 1.0 mM MUP Solution; (8) 0.1 mM MUP solution.

2. Sample Preparation: Kidney Homogenization: (1) thaw and weigh kidney samples; transfer samples to 50-mL polypropylene culture tubes; (2) homogenize kidney tissue in 2X weight/volume sodium/potassium phosphate buffer (pH 7.2) using an Ultra-Turrax homogenizer (Tekmar Co.); between samples, rinse the homogenizer in succession with water (changed after each sample), ethanol, and water; (3) pellet homogenized tissue in refrigerated centrifuge; (4) Following centrifugation, remove the supernatant and store the supernatant at –70°C; (5) make appropriate dilutions (e.g. 1:10, 1:50, 1:10,000, etc.).

3. Total Protein Assay: Perform a total protein determination on an appropriate dilution of the kidney homogenates (i.e. 1 :50 dilution) using the BCA assay or a similar assay.

4. Reagent Dilution Assay: The reagent dilution assay is used to determine optimal concentrations of reagents for subsequent competitive inhibition assays. The overall strategy is to optimize the concentrations of both the antigen and antibody in the reagent dilution phase. Use these results to select appropriate concentrations of antigen and antibody for competitive inhibition experiments. Follow this procedure for each new lot of primary antibody and whenever optimal reagent concentrations are in question.

Coating Solution Preparation: Prepare 4.0 mL of solutions containing α_{2u}-globulin at concentrations of 40, 100, 200, 300, 400, 800 ng/mL for coating ELISA plate wells (100 µL of appropriate solution per well) using a 2000 ng α_{2u}-globulin per mL of PBS stock antigen solution.

Primary Antibody Solution Preparation: The current version of the anti-α_{2u}-globulin monoclonal antibody (in ascites fluid) is diluted with PBS-Tween to final dilutions ranging from 1:2.5 x 10^3 to 1:1.0 x 10^8.

Day 1: (1) Add 100 μL of α_{2u}-globulin (4 ng to 80 ng per well) to designated wells of a black pigmented styrene U-Bottom shaped 96-well microtiter plate. Include negative control wells containing PBS-Tween (no α_{2u}-globulin). Let this plate sit overnight at 4°C in a covered plastic container to prevent evaporation. This step allows sufficient time for the antigen to bind non-specifically to the plastic wells.

Day 2: (2) wash each well with PBS-Tween (A Dynex Ultrawash Plus plate washer, or equivalent may be used if available). The wash cycle includes three repeats of the following sequence: wells filled with about 300 μL of PBS-Tween, soaked for 10 seconds and emptied under vacuum; (3) in the blocking step, add 200 μL of PBS-Tween containing 1% BSA to each well of the plate and incubate for 90 minutes at 37 ± 2°C. The wells in the plate are now adequately blocked with 1% BSA in an effort to reduce non-specific binding of primary and secondary antibodies; (4) repeat washing steps described in (2); (5) add 100 μl of each preparation of the serial dilution of primary antibody (range of 1:2.5x10^3 to 1:1.0 x 10^8) in PBS-Tween to the microtiter plate. Incubate microtiter plates for 90 minutes at 37 ± 2°C. This incubation step provides sufficient time for the primary antibodies to bind to the antigen; (6) repeat washing steps described in (2); (7) the secondary antibody incubation step is performed by adding 100 μL of Secondary Antibody solution to each well and incubating the microtiter plate for 90 minutes at 37 ± 2°C. An enzyme, alkaline phosphatase, is coupled to the secondary antibody that is directed toward mouse IgG. Therefore, this incubation step provides the secondary antibody with sufficient time to bind to the primary antibody; (8) Repeat washing steps described in (2); (9) Wash wells two times with the MUP dilution buffer, draining plate after each wash; (10) add 100 μL of 0.1 mM MUP solution to each well. MUP is converted to umbelliferyl phosphate (UP) by alkaline phosphatase; (11) read the initial fluorescence at excitation 360 nm and emission 460 nm using a microplate

reader (such as the Cytofluor II Microplate Fluorescence Reader Biosearch Instruments, Millipore Corp., Bedford, MA), then hold the plate at room temperature and read fluorescence at about 15 minutes, about 30 minutes and beyond if level of fluorescence continues to change significantly.

5. α_{2u}-Globulin Competitive Inhibition Assay

Solutions: (1) Female Rat Kidney Matrix: Dilute a homogenate prepared from an untreated female rat kidney to an appropriate dilution (i.e., range of 1:10 to 1:10,000 depending on the dilution of kidney tissue require for analysis of a set of experimental samples) with PBS-Tween for use as negative controls and as the blank matrix for use in preparation of calibration standards; (2) Standard Curve Preparation: Prepare a 10 mL working solution with a final concentration of 5000 ng/mL α_{2u}-globulin by diluting the appropriate volume of purified α_{2u}-globulin stock to 10.0 mL with PBS-Tween. Prepare a sufficient volume of spiked calibration standards at least at 8 concentration levels ranging from 2.5 to 2500 ng/mL containing an appropriate amount of female rat kidney matrix; (3) Primary Antibody Preparation: For each plate, prepare 6 mL of an appropriate dilution of anti-α_{2u}-globulin antibody (currently our ascites fluid containing mouse anti-α_{2u}-globulin monoclonal antibody requires a $1:1.5x10^5$ dilution in PBS-Tween)

Assay of Samples: Sample analysis is conducted on 96-well microtiter plates, with wells designated as negative controls in which no antigen will be adsorbed to the walls of the wells and wells designated as positive controls in which only antibody diluted in matrix is added. Appropriate spiked matrix standards (typically 2.5 to 2,500 ng/mL) containing an appropriate amount of female kidney homogenate are also analyzed in triplicate for calibration purposes. The remaining wells on the plate are used for analysis of samples, typically in triplicate.

Day 1: (1) coat each well of a flat-bottomed clear methacrylate 96-well microtiter plate (Clear Plate) with 200 µL of 1% BSA/PBS-Tween. Let plate stand overnight at about 4°C in a covered plastic container to prevent evaporation; (2) add 100 µL of 2000 ng/mL α_{2u}-globulin coating solution to

the appropriate wells of a black U-Bottom shaped pigmented styrene 96-well microtiter plate (Black Plate). Add 100 mL PBS to uncoated wells for use as negative controls. Let plate stand overnight at about 4°C in a covered plastic container to prevent evaporation.

Day 2: (3) thaw standards, primary antibody, matrix, and samples; (4) wash Clear Plate with PBS-Tween. The wash cycle includes three repeats of the following sequence: wells filled with about 300 μL of PBS-Tween, soaked for 10 seconds and emptied under vacuum; (5) add 60 μL of appropriate dilutions of samples, standards, or kidney matrix into the clear plate. Kidney matrix is added to both positive (antigen coated) and negative (PBS coated) control wells (in reference to sample positions on Black Plates). An α_{2u}-globulin calibration curve prepared at least at eight concentration levels between 2.5 to 2500 pg/mL will also be included; (6) on the Clear Plate, add 60 μL of an appropriate dilution of primary antibody (determined in Reagent Dilution Assay) prepared in PBS-Tween to each well; (7) incubate Clear Plate containing the antibody and antigen in solution for 90 minutes at 37 ± 2°C. The Clear Plate holds up to 96 different reactions involving binding of the antibodies to "free" antigen in solution; (8) transfer α_{2u}-globulin from wells in Black Plate to labeled plastic trough to be saved for coating the next day's plate(s). Ensure that the volume of α_{2u}-globulin is sufficient to coat subsequent plates. The α_{2u}-globulin solution may be used to coat up to 3 plates on subsequent days. Discard solution if this cannot be accomplished in that time frame. Wash Black Plate as in Step (4); (9) to the Black Plate, add 200 μL of PBS-Tween containing 1% BSA to each well and incubate for 90 minutes at 37 ± 2°C. The wells in the plate are now adequately blocked with 1 % BSA in an effort to reduce non-specific binding of primary and secondary antibodies; (10) following the 90 minute blocking incubation, repeat washing steps for Black Plate only described in Step (4); (11) transfer 100 μL from each well in Clear Plate to the corresponding location in the washed Black Plate and discard the Clear Plate. Incubate the Black Plate at 37 ± 2°C for 90 minutes. Excess antibody not bound to antigen free in solution during the incubation step in the Clear Plate can now react with the antigen bound to the

wells of the Black Plate; (12) Repeat washing steps described in Step (4); (13) the secondary antibody incubation step is performed by adding 100 μL of the secondary antibody preparation to each well and incubating the microtiter plate for 90 minutes at 37 ± 2°C. An enzyme, alkaline phosphatase is coupled to the secondary antibody that is directed toward mouse IgG. Therefore this incubation step enables secondary antibody to bind to the primary antibody; (14) Repeat washing steps described in Step (4) after 90 minute incubation in Step (13) is complete; (14) Wash wells manually two times with the MUP buffer, draining plate after each wash; (15) Add 100 μL of 0.1 mM MUP buffer solution to each well of Black Plate. MUP is converted to umbelliferyl phosphate (UP) by alkaline phosphatase. Read the initial fluorescence at excitation 360 nm and emission 460 nm using a microplate reader (such as the Cytofluor II Microplate Fluorescence Reader Biosearch Instruments, Millipore Corp., Bedford, MA), then hold the plate at room temperature and read fluorescence at about 15 minutes, about 30 minutes and 60 minutes.

Calculations: After sample analysis, the CytoFluor software can be set to automatically subtract fluorescence background and normalize the fluorescence values to 1000. Prepare a regression line using the standard curve data and a logarithmic curve fit. Determine mean, standard deviation, and standard error for each calibration concentration and sample (as applicable) using an electronic spreadsheet. The protein concentration in μg/mL is entered with the appropriate value for the dilution used for that sample. (a) ng α_{2u}-globulin /μg Soluble Protein = Concentration of α_{2u}-globulin in ng/mL/ protein concentration for dilution used in μg/mL. (b) nmol α_{2u}-globulin /g kidney = (ng α_{2u}-globulin / μg Soluble Protein) * ($1x10^6$ μg Soluble Protein/1 g Soluble Protein) * (1g α_{2u}-globulin / $1x10^9$ ng α_{2u}-globulin) * (g Soluble Protein / 100 mL kidney supernatant) * (1 mL kidney supernatant / 1 g kidney supernatant) * (2 g kidney supernatant / 1g kidney tissue) * (mol α_{2u}-globulin /18,730 g α_{2u}-globulin) * ($1x10^9$ nmol α_{2u}-globulin / 1 mol α_{2u}-globulin).

While the above description contains many specificities, these should not be construed as limitations on the scope of the invention, but rather as exemplification of preferred embodiments. Numerous other variations of the present invention are possible, and it is not intended herein to mention all of the possible equivalent forms or ramifications of this invention. Various changes may be made to the present invention without departing from the scope of the invention.

ASSAY FOR IDENTIFYING α_{2u}-GLOBULIN-MEDIATED NEPHROPATHY

CLAIMS

What is claimed:

Claim 1. An immunochemical assay for determining the amount of α2μ - globulin present in a test sample of rat tissue or rat fluid comprising:

A. Contacting said sample with a known quantity of a first antibody that is an anti- α2μ - globulin antibody in a first reservoir under conditions which allow binding of said first antibody to α2μ - globulin present in said sample to form an aqueous solution containing an α2μ -globulin/first antibody complex and free first antibody;

B. Immobilizing α2μ - globulin antigen on the surface of a second reservoir and contacting the immobilized antigen with a quantity of solution from Step (A);

C. Washing the surface of said second reservoir to remove α2μ - globulin/first antibody complex leaving the free anti- α2μ - globulin antibody bound to α2μ - globulin antigen immobilized on said second reservoir;

D. Adding a labeled secondary antibody which reacts with said first antibody to said second reservoir of Step(C); and

E. Adding a substrate that will react with the label on said labeled second antibody to create a measurable change and relating the amount of said measurable change in the test sample with the amount of such change found in standard samples and from a positive or negative control sample prepared in accordance with steps (A) - (D).

Claim 2. An immunochemical assay according to claim 1 wherein said tissue is kidney homogenate.

Claim 3. An immunochemical assay according to claim 1 wherein said fluid is urine, serum or plasma.

Claim 4. An immunochemical assay according to claim 3 wherein said fluid is urine.

Claim 5. The immunochemical assay of claim 1, wherein said first antibody is a monoclonal antibody.

Claim 6. The immunochemical assay of claim 1, wherein said first antibody is a polyclonal antibody.

Claim 7. The immunochemical assay of claim 5, wherein said first antibody is a mouse anti-α_{2u}-globulin monoclonal antibody.

Claim 8. The immunochemical assay of claim 1, wherein said labeled second antibody is alkaline-phosphatase-conjugated goat anti-mouse IgG heavy and light chain.

Claim 9. The immunochemical assay of claim 1, wherein said detectable change is fluorimetric.

Claim 10. The immunochemical assay of claim 1, wherein said detectable change is colorimetric.

Claim 11. The immunochemical assay of claim 8, wherein said chemical substrate is 4-methylumbelliferyl phosphate.

Claim 12. The immunochemical assay of claim 1, wherein said chemical substrate is 3-[2'-(spiroadamantane)-4-methyl-4-3"-phosphoryloxyphenyl-1-2-dioxetane, disodium salt].

Claim 13. The immunochemical assay of claim 1, wherein said chemical substrate is avidin-biotin.

Claim 14. The immunochemical assay of claim 1, wherein said first reservoir and said second reservoir are inert materials selected from the group consisting of glass, synthetic polymers, synthetic resins, and cellulose.

Claim 15. The immunochemical assay of claim 14, wherein said first reservoir is a 96-well flat bottom, clear, methacrylate plate.

Claim 16. The immunochemical assay of claim 14, wherein said second reservoir is a 96-well U-bottom, black pigmented, styrene plate.

Claim 17. A kit for detecting of α_{2u}-globulin in a test sample using the method of claim 1, comprising:

A. mouse anti-α_{2u}-globulin monoclonal antibody for use as a primary antibody for binding to α_{2u}-globulin;
B. labeled goat anti-mouse IgG for use as a second antibody for binding to said mouse anti-α_{2u}-globulin;
C. a chemical substrate for creating a detectable change when said chemical substrate contacts said labeled goat anti-mouse IgG;
D. a first reservoir for contacting said α_{2u}-globulin with said mouse anti-α_{2u}-globulin monoclonal antibody; and
E. a second reservoir for contacting said mouse anti-α_{2u}-globulin monoclonal antibody, said enzyme-conjugated goat anti-mouse IgG, and said substrate with one another.

Claim 18. A kit according to claim 17 further comprising purified α_{2u}-globulin for preparation of assay standards and controls.

Claim 19. A kit according to claim 17 further comprising kidney tissue from untreated female rats for use as a matrix in calibration standards and quality control samples.

Claim 20. A kit according to claim 17, further comprising kidney tissue from male rats for use as control samples in said assay, wherein said kidney tissue is derived from rats not exposed to a chemical known to induce α_{2u}-globulin nephropathy.

Claim 21. A kit according to claim 17, further comprising kidney tissue from male rats for use as control samples in said assay, wherein said kidney tissue is derived from rats exposed to a chemical known to induce α_{2u}-globulin nephropathy.

Claim 22. A kit according to claim 17, wherein the label on said second antibody is alkaline phosphatase.

Claim 23. A kit acceding to claim 22, wherein said chemical substrate is 4-methylumbelliferyl phosphate.

Claim 24. A kit according to claim 22, wherein said detectable change is fluorimetric.

Claim 25, A it according to claim 17, wherein said detectable change is colorimetric.

Claim 26. A kit according to claim 17, wherein said chemical substrate is 3-[2'-(spiroadamantane)-4-methyl-4-3"-phosphoryloxyphenyl-1-2-dioxetane, disodium salt].

Claim 27. A kit according to claim 17, wherein said chemical substrate is avidin-biotin.

Claim 28. A kit according to claim 17, wherein said first reservoir and said second reservoir are inert materials selected from the group consisting of glass, synthetic polymers, synthetic resins, and cellulose.

Claim 29. A kit according to claim 17, wherein said first reservoir is a 96-well flat bottom, clear, methacrylate plate.

Claim 30. A kit according to claim 17, wherein said second reservoir is a 96-well U-bottom, black pigmented, styrene plate.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/00 G01N33/50 G01N33/68 G01N33/53 G01N33/15

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 018, no. 399 (P-1776), 26 July 1994 (1994-07-26) & JP 06 118076 A (SUMITOMO CHEM CO LTD), 28 April 1994 (1994-04-28) cited in the application abstract ---	1-30
Y	US 5 534 431 A (MCDONALD THOMAS L) 9 July 1996 (1996-07-09) cited in the application abstract; claims ---	1-30
Y	US 5 139 932 A (BYGREN PER ET AL) 18 August 1992 (1992-08-18) cited in the application abstract; claims --- -/--	1-30

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 December 2001	18/12/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	GONCALVES M L F C

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/22906

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	US 5 654 158 A (MCDONALD THOMAS L) 5 August 1997 (1997-08-05) abstract; claims	1-30
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