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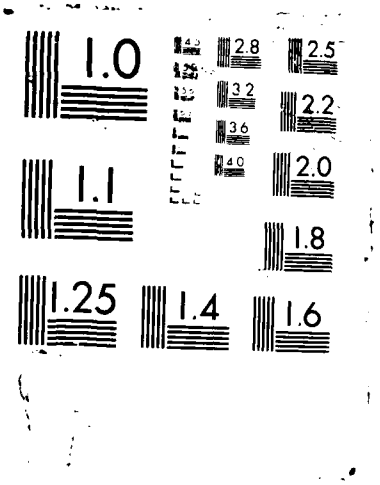
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Alpha-2U globulin is a low molecular weight urinary protein which may be associated with a hydrocarbon-induced proximal tubular cell degeneration in the male rat kidney. A new method was developed to obtain monospecific immunologic reagents for alpha-2U globulin using diafiltration, anion-exchange and hydroxylapatite chromatography. Isoelectric focusing techniques were developed to isolate the major isoelectric variants of the alpha-2U globulin molecule and to assess changes in alpha-2U globulin after experimental exposure to hydrocarbon compounds. Alpha-2U globulin was isolated from the urine of albino (Fischer 344 and Sprague-Dawley) and pigmented (Long-Evans and Fawn-Hooded) male rats to study strain susceptibility to the nephrotoxic process. An alpha-2U globulin isoelectric variant profile distinguishing albino from non-albino male rats was not apparent, however, strain differences were revealed. Fischer 344 male rats appear to have higher levels of the (Pi)=5.4 and 5.5 isoelectric variants than the other strains studied. These findings suggest that if a strain susceptibility to the hydrocarbon-induced nephrotoxic lesion exists, it may be associated with the alpha-2U globulin isoelectric variant profile.

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AFOSR 86-0313

FINAL REPORT

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Prepared by

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October, 1987

ABSTRACT

Alpha-2U globulin is a low molecular weight urinary protein which may be associated with a hydrocarbon-induced proximal tubular cell degeneration in the male rat kidney. A new method was developed to obtain monospecific immunologic reagents for alpha-2U globulin using diafiltration, anion-exchange and hydroxylapatite chromatography. Isoelectric focusing techniques were developed to isolate the major isoelectric variants of the alpha-2U globulin molecule and to assess changes in alpha-2U globulin after experimental exposure to hydrocarbon compounds. Alpha-2U globulin was isolated from the urine of albino (Fischer 344 and Sprague-Dawley) and pigmented (Long-Evans and Fawn-Hooded) male rats to study strain susceptibility to the nephrotoxic process. An alpha-2U globulin isoelectric variant profile distinguishing albino from non-albino male rats was not apparent, however, strain differences were revealed. Fischer 344 male rats appear to have higher levels of the (Pi)=5.4 and 5.5 isoelectric variants than the other strains studied. These findings suggest that if a strain susceptibility to the hydrocarbon-induced nephrotoxic lesion exists, it may be associated with the alpha-2U globulin isoelectric variant profile.

INTRODUCTION

Preliminary studies at AAMRL/THT, Wright-Patterson Air Force Base, suggested that alpha-2U globulin (A2U), a sexually dimorphic urinary protein might be involved in the hydrocarbon-induced nephrotoxic response of the adult male rat. The principal investigator, in collaboration with toxicologists at AAMRL/THT, designed this project to establish scientifically valid methods to evaluate certain potentially hazardous elements of petroleum-based Air Force fuels. The project was designed in two phases and has been supported by two AFOSR grants (# 84-0283, 9/1/84 to 8/31/86 and # 86-0313 9/1/86 to 8/31/87).

Phase I centered on the development of monospecific immunologic reagents to investigate the role of A2U in the nephrotoxic event. This involved the isolation and purification of A2U. A new technique was developed in the principal investigator's laboratory for removing contaminant urinary proteins from the final protein preparation. The purified A2U preparation was then used to develop monospecific antibodies for the detection of urinary, plasma and tissue-bound A2U. A rocket immunoelectrophoresis technique was developed in the principal investigator's laboratory to quantify plasma and urinary A2U.

Phase II was designed to investigate the mechanism of the nephrotoxic event from two perspectives: (1) to compare the association of A2U with the nephrotoxicity induced by pure hydrocarbon compounds and complex petroleum-based fuels; (2) to correlate alterations of the A2U with changes in the renal pathology. A decalin model which could induce a reproducible nephrotoxicity in male, Fischer 344 rats was developed in collaboration with toxicologists at AAMRL/THT. Although the remaining phase II goals of this project have been delayed by the principal investigator's move from Hahnemann University to the University of Illinois in August 1986, the genetic, histochemical, chromatographic and immunoelectrophoretic studies have been restarted and are progressing. This report is a final report for AFOSR project # 86-0313 which covers the project progress from September 1, 1986 to August 31, 1987. Although the present report will focus on the research supported by AFOSR grant # 86-0313, some aspects of AFOSR #84-0293 will be presented for the purpose of continuity.

RESEARCH OBJECTIVES

(I) To develop monospecific immunologic reagents against the isoelectric variants of urinary alpha-2U globulin from Fischer 344 male rats. These reagents will be used to evaluate alpha-2U globulin microheterogeneity with respect to the development of the hydrocarbon-induced nephrotoxic lesion.

(II) To determine what effect modification of the molecular profile of alpha-2U globulin has on the development of hydrocarbon-induced nephrotoxicity in male rats. One of the perplexing issues of hydrocarbon-induced nephrotoxicity is the strain selective nature of the lesion (e.g. cytoplasmic hyaline droplets and medullary casts). Albino male rats are the only experimental animals which appear to be susceptible to these pathologic effects.

MATERIALS AND METHODS

Laboratory Animals

A breeding colony of Fischer 344 rats was established by the principal investigator to provide a source of normal rat urine. The animals were housed in an AAALAC-approved facility and fed a standard rodent diet (Purina) and water by free choice.

Female, New Zealand white rabbits (3-5 kg) were used for the production of all immunologic reagents developed in this study. The animals were housed in an AAALAC-approved facility and fed a standard lagomorph diet (Purina) and water by free choice.

Isolation and Purification of A2U

Overnight urine specimens from young adult male Fischer 344 rats (100-200 days of age) were collected in a metabolic cage (Fisher Scientific). The urine was centrifuged and filtered through a 0.45 micrometer filter membrane in preparation for diafiltration. One volume of urine was diafiltered with ten volumes of 0.01 molar sodium phosphate buffer, pH 6.8 (PB) over a 5,000 dalton exclusion membrane (Amicon). This procedure replaced urinary salts with sodium phosphate salts and removed any urinary proteins with a molecular weight less than 5,000 daltons. Urine thus equilibrated with PB was added to a QMA anion exchange column (Waters) and the A2U peak zone recovered using gradient elution (0.01-0.50 molar PB). The A2U peak zone was equilibrated with PB by diafiltration over a 5,000 dalton exclusion membrane in preparation for hydroxylapatite chromatography. The equilibrated A2U peak zone was added to a hydroxylapatite matrix (Bio-Rad) and the purified A2U was recovered by gradient elution (Eurell, 1986).

Isoelectric Focusing of A2U

Isoelectric focusing was used to detect the molecular heterogeneity of the A2U protein and to prepare isoelectric variant antigens for antisera production. The A2U sample was isoelectrically focused in a polyacrylamide gel (analytical technique) or bead (preparative technique) matrix with a pH range of 3.0-8.0. Electrophoretic conditions were 2000 volts/25 milliamps/25 watts at 5oC for 2.5 hours.

The isoelectric pH gradient thus formed was determined: (1) using commercial standards (FMC), with unknown isoelectric point (pI) values being determined by inspection of the gel matrix, or (2) by direct measurement of the bead matrix.

Production of Immunologic Reagents

Antisera to the whole molecule and the isoelectic variants recovered through the A2U isolation and purification process were developed by an intramuscular adjuvant immunization procedure (Garvey, 1979). One ml of a Freund's complete adjuvant/antigen emulsion was divided into 5 different intramuscular injections given in the nuchal region and the thighs of each New Zealand White rabbit. The animals were rested for 4 weeks and a second set of intramuscular injections were given, with the antigen emulsion produced using Freund's incomplete adjuvant. The animals were rested for an additional 4 weeks and then bled to recover the resulting antiserum. The gamma globulin portion of the antiserum was purified by ammonium sulphate precipitation (Garvey, 1979). Residual ammonium sulphate salt was removed by dialysis and the final protein concentration of the purified antiserum made to 10 mg/ml.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight of the monomeric A2U molecule as well as any contaminant urinary proteins was determined using SDS-PAGE. The A2U sample was electrophoresed in a 12% gel for 18 hours using a constant voltage of 50 volts. Coomassie Brilliant Blue R-250 was used to stain the resulting protein bands. The electrophoresis was calibrated using standard proteins (Sigma) ranging from 66,000 to 14,200 daltons. All molecular weight and migration distance data were transformed to log values and evaluated using a linear regression analysis (Finney, 1971).

Exposure of Male Fischer 344 Rats to Decalin

Decalin was chosen as the initial nephrotoxic agent in this project because it is a pure hydrocarbon component of fuels, and has been used in previous nephrotoxicity studies (Alden, 1984; Bruner, 1984). Decalin was administered to male Fischer 344 rats (250-350 gm body weight), by gavage, at 1 ml/kg body weight (group A) and 2 ml/kg body weight (group B), daily for a period of two weeks. Control rats (group C) were given distilled water by gavage at a dose of 2 ml/kg body weight.

RESULTS

Research Objective (I)

Monospecific antisera have been produced in the principal investigator's laboratory against the whole Fischer 344 male rat alpha-2U globulin molecule. In addition, antisera produced against alpha-2U globulin isoelectric variants #2 and #3 (see Figure 1) are currently undergoing specificity evaluation. Development of specific antisera for alpha-2U globulin isoelectric variants #1, #4, and #5 is in progress.

Research Objective (II)

Studies regarding the mechanism of hydrocarbon-induced nephrotoxicity conducted in the principal investigator's laboratory have lead to strain comparisons of alpha-2U globulin (figures 2 and 3). Figure 2 reveals that the urine of albino as well as pigmented male rats contain the alpha-2U globulin molecule. However, a closer comparison provided by isoelectric focusing techniques (Figure 3) reveals a considerable strain variation in the alpha-2U globulin molecule. A relatively crude (anion-exchange only) preparation of urinary A2U from each of the strains was used in this comparison in order to minimize the effect of species variation on the A2U isolation. Fischer 344 alpha-2U globulin isoelectric variants #1-#5 are identified in the figure for a comparative reference.

Histopathologic evaluation of the animals exposed by gavage to decalin revealed consistent differences between the control and experimental groups (Table I). The nephrotoxic effect resulting from decalin exposure was similar to that most often reported in the literature for hydrocarbon-induced nephrotoxicity and included: (1) hyaline droplet formation in the cytoplasm of the proximal tubular epithelium of the kidney, (2) cortical tubular dilation, (3) proximal tubular epithelial necrosis, and (4) cortical-medullary cast formation. Another consistent finding in the present study was the occurrence of proximal tubular epithelial regeneration. Histopathology of the liver revealed no apparent difference between control and experimental animals. Two of the animals in the high dose treatment group (animals #04 and #05) developed clinical signs of marked weight loss and diarrhea.

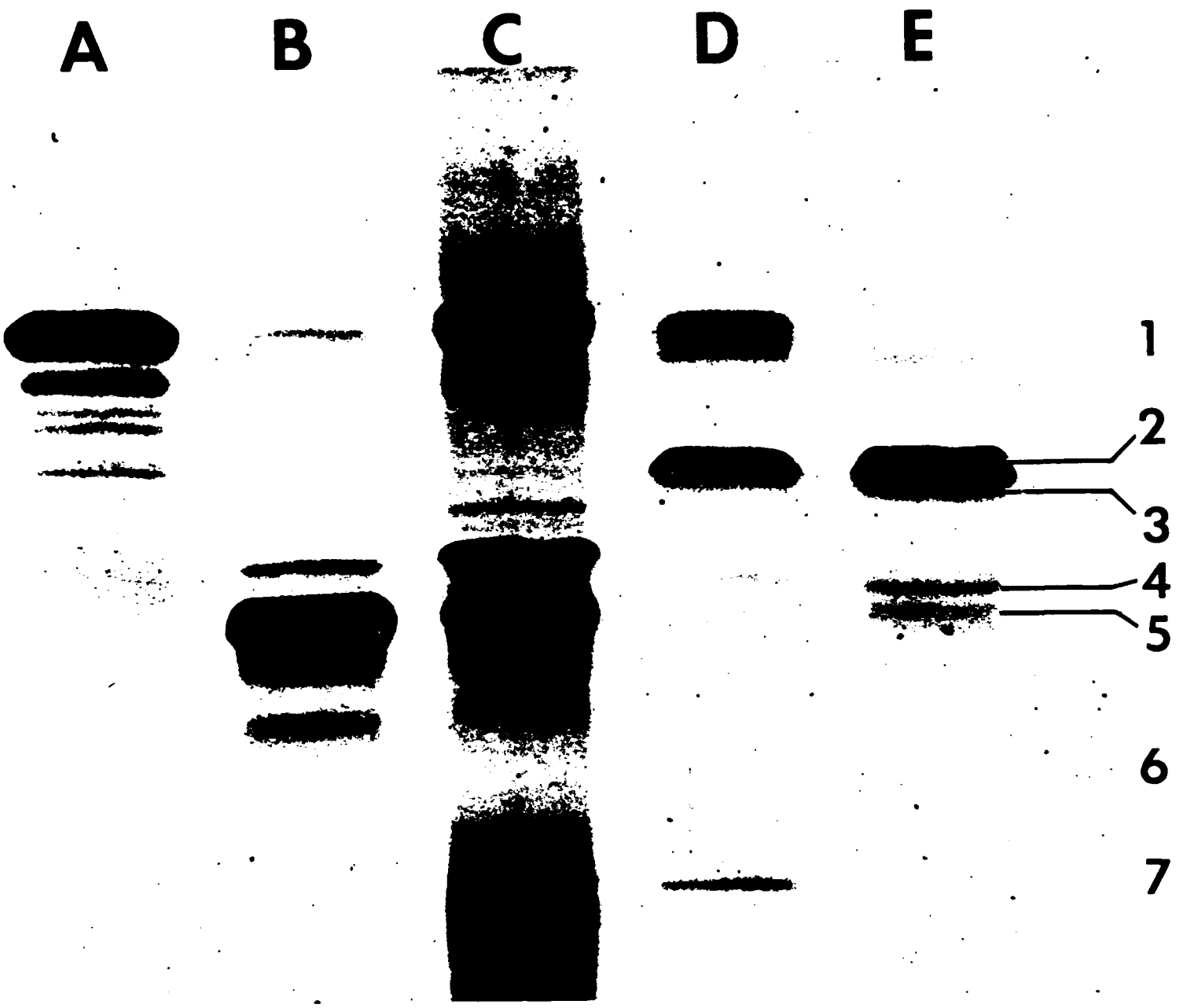

Figure 1. Isoelectric focusing pattern of A2U. Columns A, B, C, and D are protein standards and a crude extract of A2U used to calibrate the gel. The isoelectric variants of purified A2U are shown in column E and have the following pI values: (1)=6.0,(2)=5.5, (3)=5.4, (4)=5.3, and (5)=5.1.

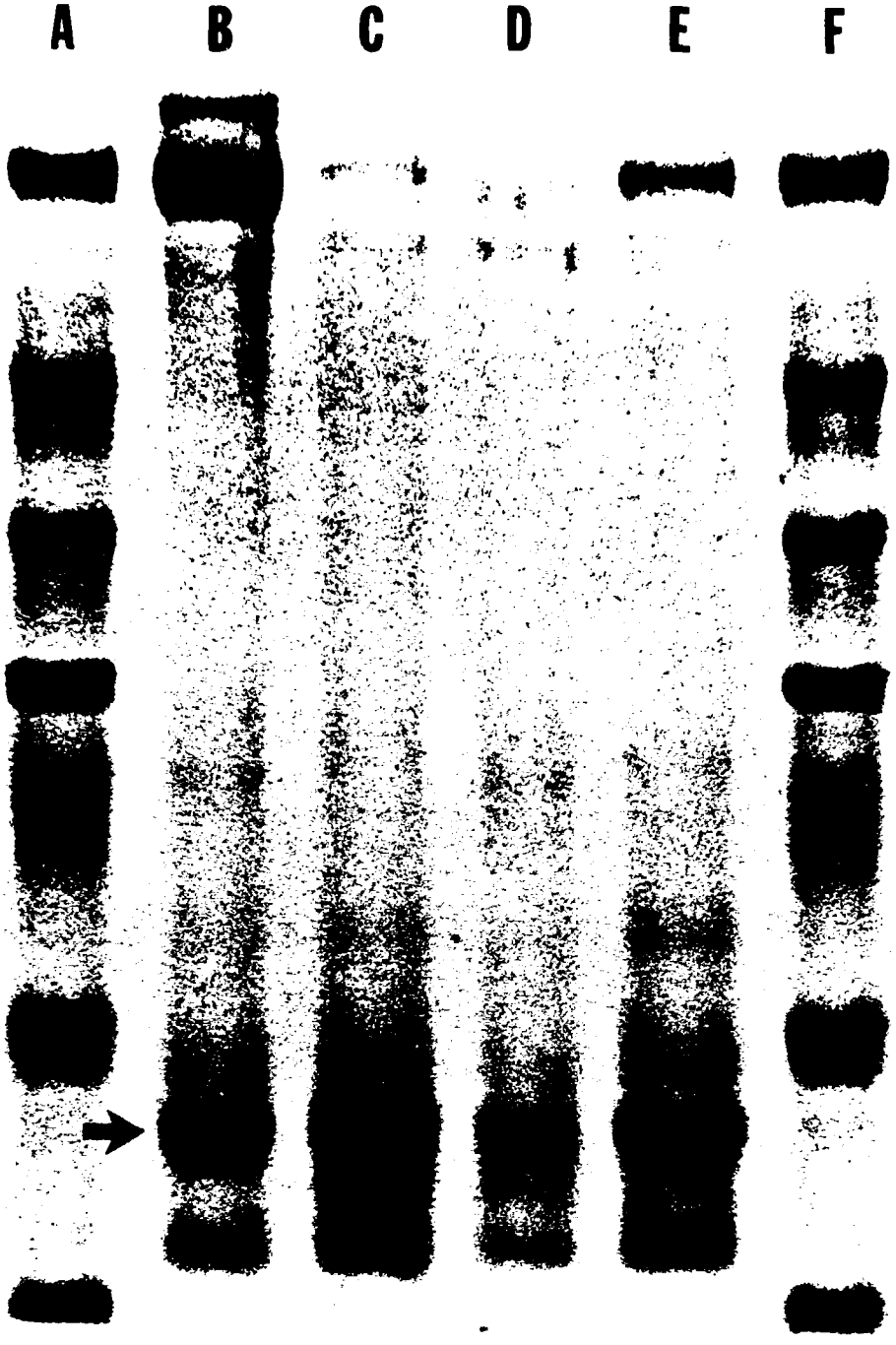

Figure 2. Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis of male urinary proteins from different rat strains. Lanes A and F represent standard protein molecular weight markers. Lane B=Fawn-Hooded strain, Lane C=Fischer 344 strain, Lane D=Long-Evans strain, and Lane E=Sprague-Dawley strain. Arrow indicates the alpha-2U globulin zone.

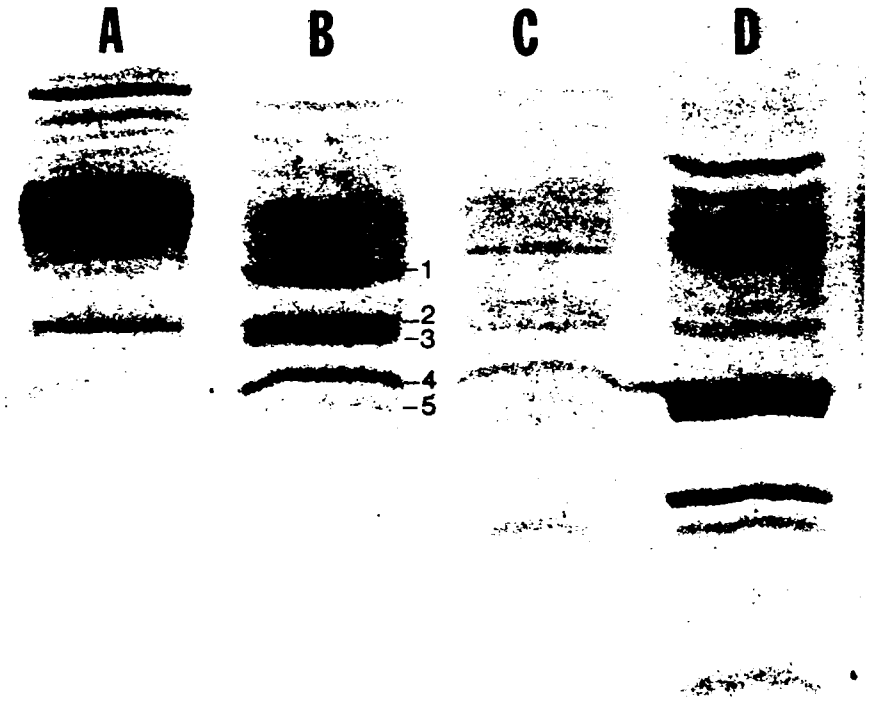

Figure 3. Isoelectric variant profile of male rat urinary alpha-2U globulin. Lane A=Fawn-Hooded strain, Lane B=Fischer 344 strain, Lane C=Long-Evans strain, and Lane D=Sprague-Dawley strain. pI values: (1)=6.0, (2)=5.5, (3)=5.4, (4)=5.3, and (5)=5.1. Note difference between isoelectric variants #2 and #3 in albino and pigmented rats.

Table I. Experimental Pathology Report Summary

TREATMENT	HYALIN DROPLETS IN PROX. EPI.	CORTICAL TUBULAR DILATION	PROXIMAL TUBULAR EPI. NECROSIS	CASTS	OTHER
H20 (2.0 ML/KG)					
86.4 (08)	+/-	+/-	-	-	-
86-12 (11)	+/-	1	-	-	-
86-1 (10)	+/-	+/-	-	-	-
86-6 (09)	+/-	1	-	-	-
DECALIN (1.0 ML/KG)					
86-3 (03)	2	1	1	OCC. (H)	#2
86-7 (00)	2	1	+/-	OCC. (H/C)	#2,#4
86-9 (02)	2	1	1	1 (H/C)	#2,#4
86-2 (01)	2	1	1	OCC. (H/C)	#2,#3
DECALIN (2.0 ML/KG)					
86-10 (04)	2-3	1	1-2	2 (H/C)	#2,#3 #4
86-8 (05)	2	2	1-2	1 (H/C)	#2,#3 #4
86-11 (06)	2	1	2	1 (H/C)	#2
86-5 (07)	2	1	2	2 (H)	#2,#4

NOTE: OCC.=OCCASIONAL; (H)=HYALIN; (H/C)=HYALIN/CELLULAR; #1=FOCAL HYPERPLASIA OF UROTHELIUM; #2=PROXIMAL TUBULAR EPI. REGENERATION; #3=FOCAL PERIVASCULAR LYMPHOID AGGREGATES; #4= CORTICAL TUBULAR NEPHROLITHS (BASOPHILIC OVOID BODIES)

SCORING- (+/-)=MINIMAL; (1)=MILD; (2)=MODERATE; (3)=SEVERE

DISCUSSION

Young adult male rat urine is a complex mixture of at least 20 urinary proteins, of which A2U is the major single protein element. A goal of this project was to develop antisera which would specifically detect A2U in urine without cross reacting with other urinary proteins. Cross reactive antibodies against contaminant urinary proteins would be present in an given antisera if the antigenic preparation used to induce that antisera contained urinary proteins other than A2U. Immunologic reagents containing antibodies against albumin would be particularly detrimental to accurate interpretation of test results as albumin is the second most prevalent protein in young adult male rat urine. The controversy regarding the association of A2U with hydrocarbon-induced nephrotoxicity may stem from the use of non-specific immunologic reagents which cross react with urinary proteins other than A2U.

In addition to the two major isoelectric variants of A2U (pI=5.5 and 5.4), the present study demonstrated three minor isoelectric variants (pI=5.1, 5.3, and 6.0; Figure 1, #5, #4, and #1,respectively). Prior studies have demonstrated that A2U is a complex protein which can exist in different molecular forms. A2U isolated from liver homogenate and blood serum of Sprague-Dawley male rats appears to be a single protein with a pI=5.2. The 5.2 pI protein is believed to be the parent form of the molecule (Lane, 1972)). Wistar male rat urinary A2U has been reported to consist of five isoelectric variants (pI=7.8, 6.1, 4.9, 4.1, and 3.7) (Roy, 1983), with the 6.1 and the 4.9 variants being the major components. Sprague-Dawley male rat urinary A2U has been reported to consist of four isoelectric variants (pI= 5.8, 5.4, 5.2, and 5.0 (Lane, 1972). Although the kidney is involved in urinary A2U isoelectric variant formation, the metabolic pathways or biological significance of this protein conversion are unknown.

The discrepancy between the referenced studies lead the principal investigator to compare rat strain variation in the A2U molecule. Alpha-2U globulin was isolated from the urine of albino (Fischer 344 and Sprague-Dawley) and pigmented (Long-Evans and Fawn-Hooded) male rats. An alpha-2U globulin isoelectric variant profile distinguishing albino from non-albino male rats was not apparent, however, strain differences were revealed. The isoelectric variant profile for Sprague-Dawley urinary A2U was similar to that previously reported (Lane, 1972). Isoelectric variant profiles for Fischer 344, Fawn-Hooded, or Long-Evans rats have not been previously reported. Fischer 344 male rats appear to have higher levels of the (pI)=5.4 and 5.5 isoelectric variants than the other strains studied.

Strain variation in A2U isoelectric variant profiles may be a central issue in hydrocarbon-induced nephrotoxicity as the Fischer 344 strain has been used most often in these studies, and may be particularly susceptible to the toxic effect. The findings in the present study suggest that if a strain susceptibility to the hydrocarbon-induced nephrotoxic lesion exists, it may be associated with the alpha-2U globulin isoelectric variant profile. This area is currently being actively pursued as the principal investigator in collaboration with Drs. M. Parnell and J. Cooper (AAMRL/THT and VS, WPAFB) have recently completed collecting data in a decalin exposure of Fischer 344, Long-Evans, and Fawn-Hooded male rats. Data analysis using the techniques and specific immunologic reagents developed by the principal investigator will provide the information necessary to determine if strain variation in A2U can be associated with hydrocarbon-induced nephrotoxicity in the male rat.

ABSTRACTS AND PUBLICATIONS

1. Eurell, T.E., Parnell, M.J., and Henningsen, G.M. Comparison of alpha-2U globulin isolated from the urine of albino and non-albino male rats. Submitted for 1988 annual meeting of the Society of Toxicology, Dallas, Tx, Feb., 1988.

2. Eurell, T.E., and Olson, C.T. A new technique for the isolation and purification of urinary alpha-2U globulin from Fischer 344 rats. (In preparation).

3. Eurell, T.E., Henningsen, G., and Olson, C.T. Comparison of strain differences between male rat urinary alpha-2U globulin. (In preparation).

PROFESSIONAL PERSONNEL ASSOCIATED WITH THE RESEARCH EFFORT

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INTERACTIONS

Consultation with AAMRL/THT toxicologists and pathologists at Wright-Patterson AFB:
(1) April 9-10, 1987
(2) June 24-25, 1987

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