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Using a lysosome specific, acid phosphatase stain developed by our research team, F344 and NBR male rats were found to respond to decalin, JP-4 and JP-8 exposure. Hydrocarbon-induced renal tubular lysosomal alterations were more closely related to the length of exposure rather than the strain of experimental animal. The NBR rats (extended exposure) had significantly enlarged lysosomes that would often be located in the basal aspect of the renal tubular epithelial cell in a manner similar to the characteristic F344 male rat response, whereas, the F344 rats (short exposure) showed groups of perinuclear lysosomal aggregates in a manner similar to the characteristic NBR male rat response. This effect could not be detected using H&E, LMBBF, and MH stains. This finding is important in regards to the controversy of alpha 2U-globulin's association with hyaline droplet nephropathy because: (1) the NBR rat demonstrates significant lysosomal alterations following extended hydrocarbon exposure in the presence of negligible concentrations of androgen-dependent alpha 2U-globulin and (2) the F344 rat demonstrates minimal lysosomal alteration following short hydrocarbon exposure in the presence of high concentrations of androgen-dependent alpha 2U-globulin. Immunohistochemical studies of renal tubular epithelial cells from NBR and F3444 male rats exposed to decalin, JP-4 and JP-8 revealed that the microtubules of the cytoskeleton form a characteristic aggregate pattern in the apical portion of the cell in association with hydrocarbon-induced lysosomal alterations. The nephrotoxic effect of decalin, JP-4 and JP-8 appeared to be equivalent as judged by renal tubular lysosomal and cytoskeletal alterations.

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The Effects of Three Hydrocarbons on the Histologic Structure of Male Rat Kidneys

AFOSR F49620-93-1-0432

Final Technical Report (7/1/93-6/30/94)

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SUMMARY

We used rat strain variation (Fisher 344 and NCI Black Reiter), different hydrocarbon compounds (JP-4, JP-8 and decalin) and length of exposure to study the mechanism of hydrocarbon-induced male rat nephrotoxicity. Using a lysosome specific, acid phosphatase stain developed by our research team, F344 and NBR male rats were found to respond to decalin, JP-4 and JP-8 exposure. Hydrocarbon-induced renal tubular lysosomal alterations were more closely related to the length of exposure rather than the strain of experimental animal. The NBR rats (extended exposure) had significantly enlarged lysosomes that would often be located in the basal aspect of the renal tubular epithelial cell in a manner similar to the characteristic F344 male rat response, whereas, the F344 rats (short exposure) showed groups of perinuclear lysosomal aggregates in a manner similar to the characteristic NBR male rat response. This effect could not be detected using H&E, LMBBF, and MH stains. This finding is important in regards to the controversy of alpha 2U-globulin's association with hyaline droplet nephropathy because: (1) the NBR rat demonstrates significant lysosomal alterations following extended hydrocarbon exposure in the presence of negligible concentrations of androgen-dependent alpha 2U-globulin and (2) the F344 rat demonstrates minimal lysosomal alteration following short hydrocarbon exposure in the presence of high concentrations of androgen-dependent alpha 2U-globulin. Immunohistochemical studies of renal tubular epithelial cells from NBR and F3444 male rats exposed to decalin, JP-4 and JP-8 revealed that the microtubules of the cytoskeleton form a characteristic aggregate pattern in the apical portion of the cell in association with hydrocarbon-induced lysosomal alterations. The nephrotoxic effect of decalin, JP-4 and JP-8 appeared to be equivalent as judged by renal tubular lysosomal and cytoskeletal alterations.

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INTRODUCTION

The principal investigator, in collaboration with toxicologists from the Armstrong Laboratory (AL/OET), Wright-Patterson AFB, is studying the mechanism of hydrocarbon-induced male rat nephrotoxicity. This investigation was designed to study the effects of decalin, JP-4, and JP-8 on the histologic architecture of renal tissue from Fischer 344 (F344) and NCI-Black Reiter (NBR) male rats. Comparison of rat strain renal response to hydrocarbon agents following gavage exposure was used to study the mechanism of hydrocarbon-induced nephrotoxicity.

The principal objectives of the study presented in this report were:

(1) Use quantitative histochemical and morphometric techniques to evaluate the lysosomal alterations induced by decalin, JP-4, and JP-8 following short exposures in F344 male rats and long exposures in NBR male rats.

(2) Use light microscopic and electron microscopic immunohistochemical methods to assess the effect of decalin, JP-4, and JP-8 exposure on the cytoskeleton of proximal tubular epithelial cells from F344 and NBR male rats.

This final technical report represents our efforts from July 1, 1993 through June 30, 1994.

USAF Relevancy

The hydrocarbon-induced nephrotoxicity which results from the exposure of laboratory animals to agents such as decalin, JP-4 and JP-8 currently serve as models of risk assessment for human exposure. Mechanistic studies of experimentally induced hydrocarbon nephropathy are necessary to evaluate the risks associated with hydrocarbon exposure to USAF personnel.

Technical Background-Objective 1

Excessive hyaline droplet formation in the cytoplasm of proximal tubular epithelial cells is a primary feature of hydrocarbon-induced male rat nephropathy. The accumulation of hyaline droplets following hydrocarbon exposure reflects an abnormality in renal protein catabolism which is believed to be associated with inefficient lysosomal degradation of low molecular weight proteins (principally alpha 2U globulin) (Swenberg, et al., 1989). The gene family that produces alpha 2U globulin (A2U) can occur in either an androgen-inducible or a constitutive form. The androgen-inducible A2U gene family occurs in the liver of male rats, whereas, the constitutive A2U gene family is expressed principally in the salivary and preputial glands (Chatterjee et al., 1989). The A2U found in the urine is thought to be primarily derived from the androgen-inducible A2U gene family. The contribution of the constitutive A2U gene products to systemic A2U concentrations are not known.

The NBR male rat is different from male rats of all other strains studied to date (albino and pigmented) because the androgen-inducible A2U gene family is almost undetectable, occurring in a concentration approximately the same as the normal female rat (Chatterjee et al., 1989). NBR male rats have been reported to neither form hyaline droplets nor accumulate any filtered protein in the renal cortex either spontaneously or after exposure to decalin. This finding was reported based on the electrophoretic analysis of kidney extracts and histologic analysis of kidney sections stained with the Mallory Heidenhain stain (Ridder, et al., 1990). A second study using Lee's Methylene Blue Basic Fuchsin stain reported spontaneous protein droplet formation in male NBR rats, however, no histologic

differences were observed between the kidney sections of treated and control rats (Dietrich and Swenberg, 1991). Both the Mallory Heidenhain and Lee's Methylene Blue Basic Fuchsin stains are non-specific protein stains which can make the differentiation of protein droplets and background cytoplasmic staining difficult. Using a specific stain for the acid phosphatase component of the lysosomal membrane, we found a significant alteration in the histologic appearance of renal tubular lysosomes following a 5 day hydrocarbon exposure in both F344 and NBR male rats (Eurell et al., 1992). However, the histologic appearance of the lysosomes in the NBR male rat suggested that it was less responsive to the hydrocarbon exposure than the F344 male rat.

If the major difference between NBR and F344 male rats is their degree of responsiveness to hydrocarbon exposure, it may be possible to minimize this difference by exposing NBR male rats to a relatively long hydrocarbon exposure while exposing F344 male rats to a relatively short hydrocarbon exposure.

Technical Background-Objective 2

A possible mechanism for the histologic alterations seen in renal sections of F344 and NBR male rats is a direct, hydrocarbon-induced alteration of cytoskeletal elements in the renal tubular epithelial cell. Several features of the cytoskeletal organization in epithelial cells and the lysosomal alterations in renal tubular cells following hydrocarbon exposure support this hypothesis: (1) the maturation and movement of endosomes, endolysosomes, and lysosomes occurs along components of the cytoskeleton (Bomsel et al., 1990; Kornfeld and Mellman, 1989), (2) the basal location of enlarged lysosomes in treated F344 rats is unusual because endolysosomes, lysosomes, and phagolysosomes are most often located at the median plane of the cell (Eurell et al., 1990) and (3) the NBR rat response to hydrocarbon exposure is unusual because of the lysosomal aggregates formed in the perinuclear cellular compartment (Eurell and Mattie, 1994).

Endosomes are important elements in the protein metabolism of renal tubular cells that contribute to cellular protein trafficking, recycling of cell surface components, and the biogenesis of lysosomes (Kawai and Hatae, 1991; Kornfeld and Mellman, 1989). Early endosomes occur in the apical cytoplasmic compartment and late endosomes (endolysosomes) occur in the perinuclear cytoplasmic compartment. Late endosomes can have several fates including fusion with lysosomes, fusion with Golgi-derived vesicles, and fusion with other late endosomes (Kornfeld and Mellman, 1989). The maturation and movement of endosomes from the apical to the perinuclear compartments and the fusion of late endosomes with lysosomes occurs along components of the cytoskeleton (Bomsel et al., 1990; Kawai and Hatae, 1991; Kornfeld and Mellman, 1991). A hydrocarbon-induced alteration in the cytoskeleton could induce cross- trafficking of the endolysosome/lysosome complex and result in the basal location of the enlarged lysosomes in the F344 rat, as well as, the perinuclear aggregate formation seen in NBR rats.

SUMMARY OF ACCOMPLISHMENTS OF RESEARCH PROJECT

Methods Development

1. Optimized acid phosphatase lysosomal staining procedure for NBR male rats.

2. Developed immunohistochemical methods for detection of cytoskeletal elements in renal tubular epithelial cells.

Application of Methods

All rat exposures were conducted at the AL/OEVM facility of Wright-Patterson AFB. All animals were housed, maintained, and used in accordance with DHHS (NIH) Publication 85-23 entitled "Guide for Care and Use of Laboratory Animals," and the Animal Welfare Act of 1966, as amended. All procedures involving the use of animals were approved by the AAMRL institutional animal care and use committee prior to any experimental procedures. Hydrocarbon exposures were conducted on F344 and NBR rats according to a standardized protocol and tissues were collected on 7/20/93, 7/21/93, 9/10/93, 10/15/93, 11/18/93, and 11/19/93. Tissues were recovered following gavage exposures to decalin (22 F344 + 27 NBR), JP-4 (22 F344 + 27 NBR), and JP-8 (22 F344 + 28 NBR). Thirty-eight control rats were also processed during the experiment. All tissues were processed by the principal investigator for immunohistochemical, histochemical and morphometric data.

RESULTS

Objective I-Histochemical and Morphometric Analysis

Control Animals There was no difference (P>.45) between the size of renal tubular lysosomes from control kidney specimens of F344 and NBR male rats (1.3 ± 0.5). The renal tubular lysosomes were distributed throughout the cytoplasm in kidney specimens from both rat strains.
This data is in agreement with previous reports from our research group.

F344 Male Rats Treated for Standard 1 Week Exposure The renal tubular lysosomes from kidney specimens of F344 rats exposed to decalin, JP-4 and JP-8 were significantly (P< 0.05) enlarged (3.7 ± 1.8) when compared to control lysosomes and primarily located at the basal surface of the cell. There was no significant difference (P>.20) between the size of renal tubular lysosomes from decalin, JP-4 and JP-8 treatment groups. This data is in agreement with previous reports from our research group.

NBR Male Rats Treated for Standard 1 Week Exposure The renal tubular lysosomes from kidney specimens of NBR rats exposed to decalin, JP-4 and JP-8 were not enlarged (P>.15) when compared to the size of lysosomes in kidney specimens from control animals. The lysosomes from decalin, JP-4 and JP-8 treated NBR male rats were primarily distributed along the median plane of the cell and occasionally formed into aggregates. There was approximately a 2-fold increase in the number of lysosomes in kidney sections from NBR male rats treated with decalin, JP-4 and JP-8 when compared to kidney sections from control animals. There was no significant difference (P>.50) between the size of renal tubular lysosomes from decalin, JP-4 and JP-8 treatment groups. This data is in agreement with previous reports from our research group.

F344 Male Rats Treated for 18 hours (Alternative Treatment)
Representative morphologic and histochemical features of the renal tubular response of F344 male rats to decalin, JP-4 and JP-8 are shown in Figure 1-Frames A, B and C. The renal tubular lysosomes were significantly (P< 0.05) reduced in size (0.6 ± 0.2) when compared to control F344 male rats. There was approximately a 10-15 fold increase in the number of lysosomes in kidney sections from F344 male rats treated with decalin, JP-4 and JP-8. Lysosomes were principally distributed between the median plane and basal aspect of the tubular epithelial cells. Distinct lysosomal aggregates occurred and were most commonly found in the perinuclear region of the cell. There was no significant difference (P>.32) between the size of renal tubular lysosomes from decalin, JP-4 and JP-8 treatment groups.

NBR Male Rats Treated for 2 Weeks (Alternative Treatment)
Representative morphologic and histochemical features of the renal tubular response of NBR male rats to the alternative treatment with decalin, JP-4 and JP-8 are shown in Figure 1-Frames D, E and F. The lysosomes were not significantly (P> 0.40) enlarged when compared to control NBR male rats. However, there was approximately a 5-fold increase in the number of lysosomes in kidney sections from NBR male rats treated with decalin, JP-4 and JP-8. The lysosomes were principally distributed along the median plane of the tubular epithelial cell. Distinct lysosomal aggregates occurred which were randomly distributed along the median plane of the tubular epithelial cell. There was no significant difference (P>.48) between the size of renal tubular lysosomes from decalin, JP-4 and JP-8 treatment groups.

Objective 2-Immunohistochemical Analysis

Light Microscopic Immunohistochemistry

Immunofluorescent detection of filamentous actin in rat renal tubular cells was performed using rhodamine-labelled phalloidin (Molecular Probes, Inc). A biotin-avidin linked, diaminobenzidine system was used in conjunction with monoclonal antibodies directed against beta-tubulin (microtubules) and pan-cytokeratin (intermediate filaments) to detect the presence of cytoskeletal elements in rat renal tubular cells. The biotin-avidin linked diaminobenzidine system used in conjunction with beta-tubulin antibodies produced the strongest and most specific reaction with the cytoskeleton in renal tissue from both F344 and NBR male rats. Representative kidney sections from F344 control animals and rats exposed to decalin (alternative dose of 18 hr) are shown in figures 2 and 3, respectively. The nephrotoxic effect of JP-4 and JP-8 on the cytoskeleton appeared to be equivalent to decalin in both the F344 and NBR male rats as judged by beta-tubulin immunoreactive sites in renal tubular epithelial cells. There was a strong and specific immunohistochemical reaction against beta-tubulin in renal tubular epithelial cells from control animals (both F344 and NBR), with an even distribution of immunoreactive sites throughout the epithelial cell (Figure 2). In F344 and NBR male rats treated with hydrocarbons (decalin or JP-4 or JP-8) there was: (1) a marked decrease in the concentration of beta-tubulin immunoreactive sites and (2) a distinct apical translocation of beta-tubulin immunoreactive sites (Figure 3).

Electron Microscopic Immunohistochemistry

A 2 step procedure was used to detect tubulin reactive sites at the ultrastructural level in renal tubular cells. First, kidney sections prepared for electron microscopy were incubated with monoclonal antibodies directed against beta-tubulin. Second, the kidney sections were incubated with a Protein A-gold (10 nm diameter) probe. We tried several different embedding and post-fixation procedures for this technique but were not able to obtain a specific reaction with the cytoskeleton. This may have been due to insufficient tissue fixation (osmium tetroxide was not used) or decreased antigenic reactivity of beta-tubulin associated with electron microscopy specimen preparation. The osmium tetroxide fixation step was omitted in processing the tissue for electron microscopic immunohistochemistry because the fixative interfered with the Protein-A gold reagent.

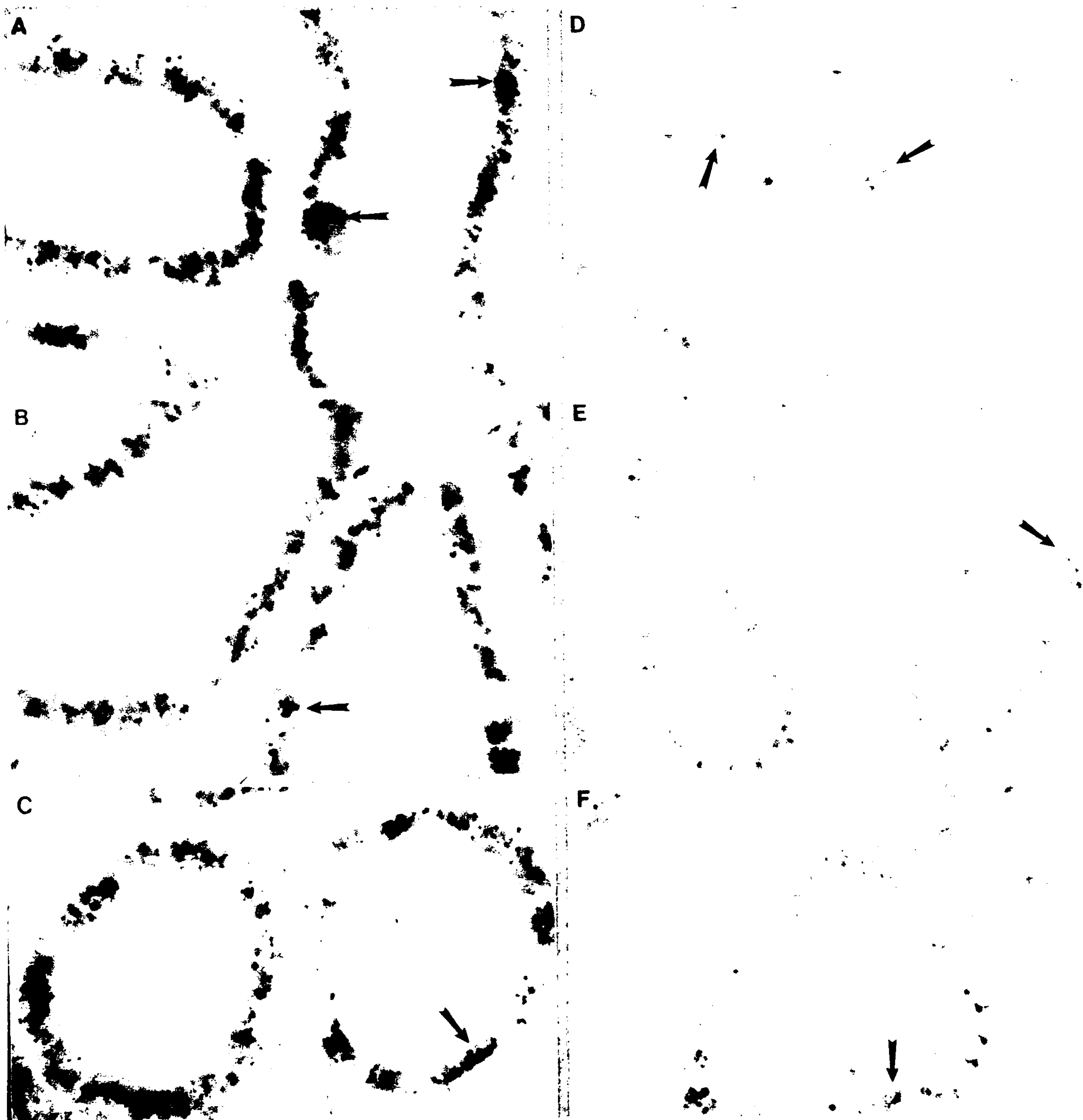

Figure1. Renal Tissue Sections from Exposed and Control Rats After Modified Dosing Period (Original Magnification=1000X). Frame A F344 male rat exposed to decalin for 18 hr. Frame B F344 male rat exposed to JP-4 for 18 hr. Frame C F344 male rat exposed to JP-8 for 18 hr. Frame D NBR male rat exposed to decalin for 2 wk. Frame E NBR male NBR rat exposed to JP-4 for 2 wk. Frame F NBR male rat exposed to JP-8 for 2 wk. Note lysosomal aggregates (arrows) distributed along the median plane of the tubular epithelial cells.

Figure 2. Immunohistochemical Localization of Beta-Tubulin Reactive Sites of the Cytoskeleton in Proximal Tubular Epithelial Cells of Control F344 Male Rats (Original Magnification=1000X). An avidin-biotin linked, diaminobenzidine system was used in conjunction with monoclonal antibodies against beta-tubulin. Immunoreactive sites were stained brown. Note even distribution of microtubular components of cytoskeleton throughout cell. Methyl green was used as a nuclear counterstain.

Figure 3. Immunohistochemical Localization of Beta-Tubulin Reactive Sites of the Cytoskeleton in Proximal Tubular Epithelial Cells of F344 Male Rats Exposed to Decalin (Original Magnification=1000X). An avidin-biotin linked, diaminobenzidine system was used in conjunction with monoclonal antibodies against beta-tubulin. Immunoreactive sites were stained brown. Note apical translocation of beta-tubulin immunoreactive

SIGNIFICANCE OF RESEARCH FINDINGS AND RECOMMENDATIONS

The results of this study confirm that NBR male rats demonstrate an intermediate form of hyaline droplet nephropathy, being greater than the negligible response of the female rat and less than the extensive response of the F344 male rat. This finding is important in regards to the controversy of A2U's association with hyaline droplet nephropathy since the NBR male rat demonstrates significant lysosomal alterations following hydrocarbon exposure in the presence of negligible concentrations of androgen-dependent A2U.

Decalin, JP-4 and JP-8 appeared equally effective in inducing lysosomal alterations in renal tubular epithelial cells following gavage exposure. Differences in the hydrocarbon-induced renal response of F344 and NBR male rats was minimized by altering the length of exposure. Alteration in the histologic appearance of the renal tubular lysosomes occurred rapidly in the F344 male rat and slowly in the NBR male rat. Our research group, as well as others, have noted that the lysosomes of F344 male rats appear enlarged and are located in the basal aspect of the renal tubular epithelial cell after 3 days of hydrocarbon exposure. Therefore, the transition of F344 renal tubular lysosomes from small, aggregated bodies in the median plane of the cell to large, singular structures in the basal aspect of the cell occurs between 18 and 72 hours following hydrocarbon exposure.

Immunohistochemistry clearly demonstrated that hydrocarbon exposure reduced the concentration of cytoskeleton immunoreactive sites and induced an apical translocation of the microtubular elements of the cytoskeleton. The hydrocarbon-induced alteration of the renal tubular cytoskeleton in F344 and NBR male rats (Figure 3) occurred in conjunction with the lysosomal aggregation seen using the acid phosphatase stain (Figure 1). We propose that the unifying concept for the histologic evidence presented above and the mechanism of renal tubular cell exfoliation is a direct, hydrocarbon-induced alteration of cytoskeletal elements in the renal tubular epithelial cell. Two general features of the cytoskeletal organization in epithelial cells support this hypothesis: (1) the maturation and movement of endosomes, endolysosomes, and lysosomes occurs along components of the cytoskeleton and (2) anchoring junctions between epithelial cells and the basal lamina (extracellular matrix) are joined to the cytoskeleton. A hydrocarbon-induced alteration in the cytoskeleton would induce cross-trafficking of the endolysosome/lysosome complex and result in the aggregate formation seen in F344 and NBR rats (Figure 1). The anchoring junctions of the renal tubular epithelial cell (between adjacent epithelial cells and to the extracellular matrix) are joined to the cytoskeleton through adherens junctions, desmosomes, and hemidesmosomes. Alterations in the cytoskeleton can affect the adherens junctions causing detachment of the cell from the extracellular matrix. This is the mechanism of action for drugs such as cytochalasin which are used to disrupt cell-substrate attachments in tissue culture systems. Such a mechanism could explain the early exfoliation of proximal tubular epithelial cells seen following hydrocarbon exposure of male rats (Figure 1).

The electron microscopic immunohistochemistry didn't provide a specific reaction with the renal tubular cytoskeleton. We believe that confocal microscopy might provide a better method for the demonstration of the cytoskeletal network than electron microscopic immunohistochemistry. We are currently working with Dr. Steve Channel (AL/OETA) at Wright Patterson AFB to confirm the efficacy of this procedure with the cytoskeleton of male rat renal tubular cells.

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PUBLICATIONS FROM RESEARCH PROJECT

Eurell, TE, Mattie, DR, Dunovant, VS, Marit GB, and Flemming CD. Hematology and blood chemistry values for F344 and NBR male rats after exposure to decalin and JP-8. The Toxicologist 13(1): 205, 1993.

Eurell, T.E. and Mattie, D.R. The comparison of lysosomal alterations in fischer 344 and nci-black reiter rats following short and prolonged exposures to decalin, JP-4 and JP-8. The Toxicologist 14(1):66, 1994.

Eurell, TE, and Mattie, DR. The comparison of lysosomal alterations in Fisher 344 and NCI-Black Reiter male rats following gavage exposure to decalin, JP-4 and JP-8. Submitted to Toxicologic Pathology -pending authors response to reviewer's comments.

Manuscripts In Preparation

Eurell, TE, Mattie, DR, Dunovant, VS, Marit GB, and Flemming CD. Hematology and blood chemistry values for F344 and NBR male rats after exposure to decalin and JP-8.

Davis, MA, and Eurell, TE. Evaluation of the association between alpha 2U globulin and lysosomes in male rats exposed to decalin.

Eurell, TE, and Mattie, DR. Alteration of lysosomal morphology and endosomal marker distribution in renal cells of male rats exposed to decalin.

INTERACTIONS (COUPLING ACTIVITIES)

Dr. Eurell conferred with Dr. David Mattie (AL/OETA) at Wright-Patterson AFB on 7/20/93, 7/21/93, 9/10/93, 10/15/93, 11/18/93, and 11/19/93.. During the visits Dr. Eurell presented an update on this project and was involved in tissue collection.