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EFFECT OF CARBON TETRACHLORIDE ON HAMSTER TRACHEAL EPITHELIAL CELLS

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EFFECT OF CARBON TETRACHLORIDE ON HAMSTER TRACHEAL EPITHELIAL CELLS

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This investigation was designed to assess cytotoxicity of carbon tetrachloride (CCl_4) in hamster trachea. Adult, male, Syrian golden hamsters were treated with 2.5 ml/kg CCl_4, and controls received the vehicle only. Animals were sacrificed at various intervals following treatment. Tracheas were fixed and embedded for light and electron microscopy. Cell damage was evaluated by light and electron microscopy. Glycoprotein-containing cells were evaluated by light microscopy using Alcian blue/Periodic acid Schiff (AB-PAS). In control hamsters secretory cells were columnar, and contained abundant smooth endoplasmic reticulum. Cytochemically, most secretory cells were PAS positive and contained neutral glycoproteins. CCl_4 produced injury in secretory cells and ciliated cells. Injury was characterized by loss of staining, dilatation of the nucleus and other intracellular organelles. However, the extent of injury varied in different regions and levels of tracheal epithelium. This chemical also caused alterations in glycoprotein-containing cells. For example CCl_4 produced transient shift from neutral to acidic glycoproteins. Cells containing homogenous, PAS positive material were absent in control,

but were obvious after CCl_4. In other experiments, hamsters were fed ad lib or fasted for 24 hrs or were pretreated with 0.6 ml/kg diethylmaleate (DEM) for 30 min prior to administration of CCl_4. All hamsters received 1.0 ml/kg CCl_4 or vehicle. Fasted animals were further starved after administration of CCl_4 or vehicle. Animals were killed 24 hrs later. Tissues were again studied with light and electron microscopy. Fasting alone produced injury in secretory cells and ciliated cells. Fasting potentiated CCl_4-induced injury. DEM produced injury in both secretory cells and ciliated cells, however, the extent of injury varied in different regions and levels of the epithelium.

Since CCl_4-induced cell injury is dependent on metabolism of this agent by intracellular NADPH dependent cytochrome P-450 monoxygenases, this study suggests that secretory cells and ciliated cells of hamster trachea have the potential to bioactivate CCl_4. Depletion of glutathione and/or induction of the microsomal enzyme system by fasting may have potentiated CCl_4-induced injury. The observation that DEM produced injury in hamster trachea rendering its effect on CCl_4 toxicity difficult to ascertain from these studies.

DEDICATION

In memory of my Father, Seyed Mohamed Tagi Ahmadizadeh and my advisor Dr. Robert Echt, who would have delighted in sharing the joy of this achievement.

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TABLE OF CONTENTS

Page

CHAPTER I

GENERAL INTRODUCTION
- Conducting Airways of the Lungs. 1
- Microsomal Mixed-Function Oxidases 6
- Components of the Mixed Function Oxidase (MFO) System. 7
- Pulmonary Drug Metabolism. 9
- Cellular Localization of Cytochrome P-450 Enzyme System in Airway Epithelium 10
- Role of Metabolism and Covalent Binding in Lung Injury 11
- Role of Glutathione. 11
- The Airway Epithelial Cells as Potential Targets for Chemicals Requiring Metabolic Activation
  - 4-Ipomeanol 14
  - Carbon Tetrachloride. 16
  - Benzo(a)pyrene. 18
- Effect of Chemical Irritants on Glycoprotein Cells of the Airways of Lung 20

PURPOSE 21

REFERENCES. 23

CHAPTER II

EFFECT OF CARBON TETRACHLORIDE ON HAMSTER TRACHEAL EPITHELIAL CELLS

ABSTRACT. 31

INTRODUCTION. 32

MATERIALS AND METHODS 33

RESULTS 35

DISCUSSION. 46

REFERENCES. 52

TABLE OF CONTENTS Continued:

Page

CHAPTER III

EFFECT OF CARBON TETRACHLORIDE ON GLYCOPROTEIN CONTAINING CELLS OF HAMSTER TRACHEA

ABSTRACT. 55

INTRODUCTION. 56

MATERIALS AND METHODS 58

RESULTS 60

DISCUSSION. 76

REFERENCES. 80

CHAPTER IV

EFFECT OF FASTING OR DIETHYLMALEATE ON CARBON TETRACHLORIDE INDUCED INJURY IN HAMSTER TRACHEAL EPITHELIAL CELLS

ABSTRACT. 83

INTRODUCTION. 84

MATERIALS AND METHODS 85

RESULTS 87

DISCUSSION. 115

REFERENCES. 119

CHAPTER V

GENERAL CONCLUSION. 122

LIST OF TABLES

Page

CHAPTER II:

Table

1 Number of damaged cells in various levels and regions of tracheal epithelia of hamsters treated with CCl_4. 45

CHAPTER III:

1 Number of glycoprotein-containing cells (GP) of various types in levels of dorsal regions of trachea of hamsters treated with CCl_4 for various times. 73

2 Number of glycoprotein-containing cells (GP) of various types in levels of lateral regions of trachea of hamsters treated with CCl_4 for various times. 74

3 Number of glycoprotein-containing cells (GP) of various types in levels of ventral regions of trachea of hamster treated with CCl_4 for various times. 75

CHAPTER IV:

1 Effect of diethylmaleate (DEM) or fasting on CCl_4-induced injury in tracheal epithelial cells. 111

2 Effect of diethylmaleate (DEM) or fasting on CCl_4-caused alterations in glycoprotein-containing cells (GP) of various levels of dorsal regions of tracheal epithelia 112

3 Effect of diethylmaleate (DEM) or fasting on CCl_4-caused alterations in glycoprotein-containing cells (GP) of various levels of lateral regions of tracheal epithelia. 113

4 Effect of diethylmaleate (DEM) or fasting on CCl_4-caused alterations in glycoprotein-containing cells (GP) of various levels of ventral regions of tracheal epithelia. 114

LIST OF FIGURES

Page

CHAPTER I:

Figure

1 A simplified scheme showing the major components of xenobiotic-metabolizing enzyme system. 8

2 Schematic representation of relationships between toxification and detoxification pathways for xenobiotic agents 13

CHAPTER II:

1 Light micrograph of ventral region of upper trachea of hamster treated with vehicle only (control) 38

2 Light micrograph of laterial region of lower trachea of 1 hr CCl_4 treated hamster 39

3 Transmission electron micrograph of ventral region of control hamster trachea. 40

4 Transmission electron micrograph of laterial region of tracheal epithelial cells of hamster treated 1 hr previously with CCl_4. 41

5 Transmission electron micrograph of laterial regions of tracheal epithelial cells of hamster treated 4 hrs previously with CCl_4 42

6 Transmission electron micrograph of laterial region of tracheal epithelial cells of hamster treated 12 hrs previously with CCl_4. 43

7 Transmission electron micrograph of ventral region of tracheal epithelial cells of hamster treated 24 hrs previously with CCl_4. 44

LIST OF FIGURES (Continued):

Page

CHAPTER III:

Figure

1 Light micrograph of lateral region of upper trachea of hamster treated 12 hrs previously with CCl_4. 63

2 Light micrograph of ventral region of upper trachea of hamster treated 4 hrs previously with CCl_4. 64

3 Light micrograph of dorsal region of upper trachea of hamster treated 12 hrs previously with CCl_4. 65

4 Light micrograph of dorsal region of lower trachea of hamster treated 4 hrs previously with CCl_4. 66

5 Light micrograph of lateral region of lower trachea of hamster treated 24 hrs previously with CCl_4. 67

6 Transmission electron micrograph of lateral region of tracheal epithelial cells of control hamster. 68

7 Transmission electron micrograph of lateral region of tracheal epithelium of hamster treated 1 hr previously with CCl_4. 69

8 Transmission electron micrograph of lateral region of tracheal epithelium of hamster treated 4 hrs previously with CCl_4 70

9 Transmission electron micrograph of ventral region of tracheal epithelium of hamster treated 12 hrs previously with CCl_4. 71

10 Transmission electron micrograph of lateral region of tracheal epithelial cells of hamster treated 24 hrs previously with CCl_4. 72

LIST OF FIGURES (Continued):

Page

CHAPTER IV:

Figure

1 Light micrograph of ventral region of upper trachea of peanut oil treated hamster fed ad lib 93

2 Light micrograph of lateral region of upper trachea of peanut oil treated fasted hamster. 94

3 Light micrograph of ventral region of upper trachea of peanut oil treated hamster pretreated with DEM. 95

4 Light micrograph of ventral region of upper trachea of CCl_4 treated hamster pretreated with DEM 96

5 Light micrograph of dorsal region of upper trachea of CCl_4 treated hamster fed ad lib 97

6 Light micrograph of lateral region of lower trachea of CCl_4 treated fasted hamster 98

7 Light micrograph of dorsal region of lower trachea of CCl_4 treated hamster pretreated with DEM 99

8 Transmission electron micrograph of ventral region of trachea of peanut oil treated hamster fed ad lib 100

9 Transmission electron micrograph of lateral region of CCl_4 treated hamster fed ad lib. 101

10 Transmission electron micrograph of lateral region of peanut oil treated hamster 102

11 Transmission electron micrograph of lateral region of trachea of CCl_4 treated fasted hamster. 103

12 Transmission electron micrograph of lateral region of trachea of peanut oil treated hamster treated with DEM 104

13 Transmission electron micrograph of lateral region of trachea of peanut oil treated hamster treated with DEM 105

LIST OF FIGURES (Continued):

Page

CHAPTER IV Continued:

Figure

14 Transmission electron micrograph of lateral region of trachea of CCl_4 treated hamster treated with DEM 106

15 Light micrograph of lateral region of upper trachea of peanut oil treated, fasted hamster. 107

16 Light micrograph of lateral region of upper trachea of a peanut oil treated hamster treated with DEM 108

17 Light micrograph of dorsal region of lower trachea of a peanut oil treated fasted hamster. 109

18 Light micrograph of lateral region of upper trachea of CCl_4 treated hamster treated with DEM 110

CHAPTER I

GENERAL INTRODUCTION

Conducting Airways of the Lungs

The conducting airways are a system of branching tubes of regular, cylindrical, or somewhat irregular cross sections that extend from the trachea to the respiratory bronchioles. The conducting airway complexity has only been recognized recently. It comprises an epithelial lining, a mechanical and an immunological defense organ, and an exocrine and endocrine gland. The tracheobronchial epithelial lining is tall-columnar in which at least eight cell types have been identified by electron microscopy. These cell types are: basal, intermediate, brush, goblet, serous, Clara, neurosecretory and ciliated cells. However, based on species variation one or more cell types may be absent in the respiratory airway. Furthermore, the population of each cell type varies among different species. A description of each of these cell types follows:

1) Basal cells. The basal cells rest on the epithelial basement membrane, but they do not reach the airway lumen. These cells are found in the airway epithelium as distally as bronchioles, but they are more numerous in the trachea. They characteristically contain large nuclei that fill most of the cell. The cytoplasm is

electron-dense and contains many tonofilaments, numerous ribosomes, small Golgi zones, and a few mitochondria. Basal cells are generally regarded as the progenitors of the other cell types (Blenkinsopp, 1967; Breeze et al., 1976; Breeze and Truck, 1984).

2) Intermediate cells. The intermediate cells seem to represent stages between basal cells and fully differentiated cells. These cells are found in proximal and distal airways. Intermediate cells are roughly spindle shaped and usually, but not always, reach the airway lumen. The abundant cytoplasm contains mitochondria and profiles of rough endoplasmic reticulum. They have large oval nuclei. As the intermediate cell differentiates, it becomes more electron dense as it starts to accumulate mucin granules. Conversely, it become more electron lucent with onset of ciliogenesis (Breeze et al., 1976; Breeze and Wheeldon, 1977).

3) Brush cells. The brush cells are columnar, rest on a basement membrane and reach the airway lumen. These cells are found in the epithelia of trachea, bronchi, and bronchioles. The cytoplasm of a brush cell contains many free ribosomes and a few profiles of rough endoplasmic reticulum. The nucleus is not lobulated and sometimes contains a nucleolus. The presence of a pronounced luminal brush border of microvilli is the distinguishing characteristic of these cells. The function of brush cells is unknown (Breeze and Wheeldon, 1977).

4) Goblet cells. The goblet cells have chalice-like shapes. These cells are abundant in the trachea and bronchi of man, horse, guinea pig, cat and dog, but they are relatively sparse in the rat, mouse, hamster and rabbit. The base of the goblet cell contains an elongated nucleus which has moderately condensed euchromatin. The surrounding cytoplasm is packed with rough endoplasmic reticulum, and a few mitochondria are present. A well developed Golgi apparatus is usually found above the nucleus. Many mucous granules are present in the apical cell cytoplasm. The protein moiety of mucus is synthesized in the rough endoplasmic reticulum and passes to the Golgi apparatus where it combines with carbohydrate and is sulfated before release from the cell.

Within the goblet cells, neutral or acidic glycoprotein granules can be identified by the Alcian blue/Periodic acid schiff (AB-PAS) technique at pH 2.6. The PAS positive granules stain red and contain neutral glycoproteins, while AB positive granules stain blue and contain acidic glycoproteins (Jones et al., 1973). Goblet, serous and Clara cells are thought to produce the mucous coating of the tracheobronchial tree (Breeze and Wheeldon, 1977; Reid and Jones, 1980). Goblet cells also contribute lining material in proximal airways (trachea and bronchi).

5) Serous cells. Epithelial serous cells were described in the trachea and extrapulmonary bronchi of rat (Jeffery and Reid, 1975). These cells contain nuclei with irregular outlines and abundant rough endoplasmic reticulum in the perinuclear cytoplasm. The membrane bound, electron dense vesicles in the apical cytoplasm and

basal nuclei are characteristic features of these cells. Epithelial serous cell granules contain neutral glycoproteins and resemble those of the serous cells of the submucosal glands. The function of the serous epithelial cells is not known, although it has been suggested that they may contribute to the periciliary fluid layer beneath the mucus. Serous cells produce a secretion of lower viscosity than that of mucous cells (Meyrick and Reid, 1975). Serous cells are believed to secrete neutral glycoproteins, lysozyme, and the epithelial transfer component of IgA (Breeze and Truk, 1984).

6) Clara cells. Nonciliated bronchiolar epithelial cells were first described by Kolliker (1881). Later, Clara (1937) described their structure in more detail; therefore, these cells are now termed Clara cells. They are numerous in bronchioles (Azzopardi and Thurlbeck 1969; Cutz and Conen, 1971; Smith et al., 1979; Plopper et al., 1980 a,b,c), but are found also in bronchi and tracheas of rats, mice, hamsters, and rabbits (Hansell and Moretti, 1969; Pack et al., 1980, 1981; Plopper et al., 1983). Clara cells contain membrane-bound, electron dense secretory granules in the apical cytoplasm. The most characteristic features of these cells is the abundant smooth endoplasmic reticulum (SER) which fills the majority of the cytoplasm in the apical area (Plopper et al., 1980 a,b,c). This abundant SER, which is present in many species is believed to be the site of cytochrome P-450 dependent mixed function oxidase (MFO).

There is general agreement that the cytoplasmic granules reflect a secretory function of Clara cells, although the secretory function and its mechanism are not completely understood. The Clara cells granules have been shown to be PAS-positive (Azzopardi and Thurlbeck, 1969; Cutz and Conen, 1971; Pack et al., 1980). An apocrine secretory mechanism was originally suggested by Clara (1937). This suggestion was later supported by Etherton et al. (1973) and by Smith et al. (1974); however, the work of other investigators supports a merocrine secretory mechanism (Kuhn et al., 1974; Yoneda, 1977; Yoneda and Birk, 1981). Morphological evidence, using transmission and scanning electron microscopy indicates that the secretory activity of tracheal nonciliated Clara cells in mice may be either apocrine or merocrine (Pack et al., 1980).

7) Neurosecretory cells. Neurosecretory cells are commonly demonstrated in the airways by three methods: 1) electron microscopic identification of the specific granules. 2) the localization of intracytoplasmic, fluorogenic monoamines using Falck technique and 3) their argyrophilic properties after Grimelus silver nitrate staining. These cells are found at all levels of the tracheobronchial and bronchiolar epithelium. The characteristic features of neurosecretory cells include the existence of small, basal granules with a electron dense core. The cytoplasm contains abundant Golgi apparatus, smooth endoplasmic reticulum and free ribosomes. Neurosecretory cells have a capacity for amine precursor uptake, decarboxylation (APUD) and storage. The cells have a triangular shape and a round or oval nucleus. Pulmonary

endocrine cells may be single or occur in groups called neuroepithelial bodies. The functions of neurosecretory cells are not known. Suggested roles include a stretch receptor or a CO_2 chemoreceptor, with possible involvment in regulation of the pulmonary circulation in the neonatal period or under hypoxic conditions (Breeze and Turk, 1984).

8) Ciliated cells. The luminal surface of ciliated cells is characterized by numerous erect and free standing cilia that are visible with light microscopy. Approximately 250 cilia are found on the luminal surface of each cell. The ciliated cells are present throughout the airway epithelium. These cells are columnar, and they have a relatively electron lucent cytoplasm containing scattered ribosomes, rough endoplasmic reticulum and many apical mitochondria. The cilia beat about 1000 times per minute (Breeze and Truk, 1984). The effective beat in the upper or lower resiratory tract is always toward the pharynx. Functionally, cilia beat in waves over the surface of the epithelium to move material across the epithelium on a layer of fluid. Cilia are particulary important in removing inhaled particulate matter (Breeze and Wheeldon, 1977; Breeze and truk, 1984).

Microsomal Mixed-Function Oxidases

The cytochrome P-450 dependent, microsomal mixed-function oxidase (MFO) system occurs in smooth endoplasmic reticulum (SER) of the liver and other organs and metabolizes steroids, hormones, vitamins, and fatty acids as well as a multitude of organic compounds. These include

drugs, pesticides, food additives and environmental chemicals such as polycyclic hydrocarbons and industrial by-products. Metabolism by MFO invariably results in products that are more polar than the parent compound. In most cases, the metabolites are also less toxic. However, microsomal xenobiotic metabolism does not necessarily result in substrate detoxification. Many cases are known where the metabolite is more toxic than the parent compound.

For many years, major emphasis has focused on the xenobiotic metabolizing enzymes of mammalian liver, because the liver of most species contains a large concentration of these enzymes. It is widely recognized, however, that several extrahepatic tissues (e.g. lung, kidney, skin) also possess microsomal enzyme systems. The hepatic microsomal enzyme system is quantitatively more important for the biotransformation of most orally administered drugs than are extrahepatic enzymes. However, xenobiotic metabolizing enzymes of tissues in direct contact with the environment (e.g. lung or skin) appear to play an important role in determining local fate and toxicity of certain chemicals.

Components Of The Mixed Function Oxidase (MFO) System

The MFO enzyme systems located in SER membranes consist of cytochrome P-450, NADPH cytochrome P-450 reductase and lipids and requires NADPH and molecular oxygen for activity (Fig. 1).

a) Cytochrome P-450: The terminal oxidase of the MFO system is a hemoprotein (or group of proteins) called cytochrome P-450. The name cytochrome P-450 is derived from the fact that the cytochrome (or pigment) exhibits an absorbance maximum at 450 nm when reduced

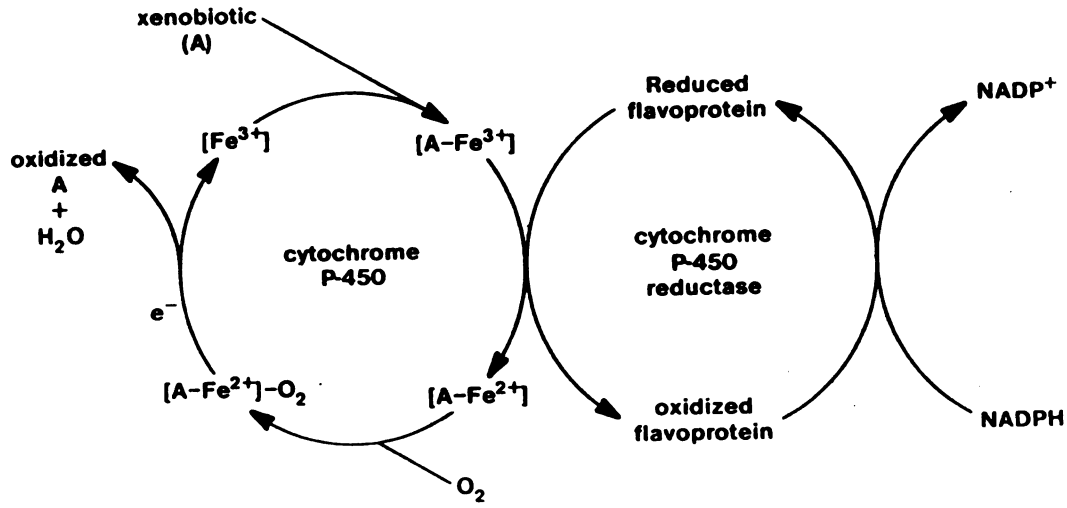

Figure 1: A simplified scheme showing the major components of the xenobiotic-metabolizing enzyme system. A foreign compound (A) binds with the oxidized form of cytochrome P-450. The resulting xenobiotic-cytochrome complex then combines with molecular oxygen. Cytochrome P-450 reductase transfers an electron from NADPH to the heme iron, reducing the iron from Fe^{+++} to Fe^{++}. The complex then binds molecular oxygen, and a second electron reduces the O_2, forming a radical which is now able to oxidize compound A bound to cytochrome P-450.

and complexed with carbon monoxide.

b) Cytochrome P-450 reductase: Cytochrome P-450 reductase is a flavin enzyme which carries electrons from NADPH to the heme iron of cytochrome P-450. The flavoprotein is sometimes referred to as NADPH-cytochrome C reductase.

c) Lipids: Phospholipids are essential for activity of the reconstituted system. It has been suggested that lipids may be required for substrate binding, facilitating electron transfer or providing a "template" for the interaction of cytochrome P-450 and NADPH-cytochrome P-450 reductase.

Pulmonary Drug Metabolism

The lung, like the liver, possesses a system containing cytochrome P-450 that metabolizes exogenous substrates. This system is similar in many respects to that in the liver. As with liver, the xenobiotic metabolizing activity is concentrated in the microsomal fraction of the lung (Hook et al., 1972), and cytochrome P-450 is apparently required for the oxidation of a number of xenobiotic agents (Bend et al., 1972). Oxidative xenobiotic metabolism in the lung is thought to involve the same electron transport system as the liver (Matsubara and Tochino, 1971). In addition, the lung also contains multiple forms of cytochrome P-450 (Liem et al., 1980). Thus, as far as major constituents are concerned, the system closely resembles that of the liver.

A number of drugs and carcinogens induce the synthesis of microsomal enzymes in rodent liver. However, several studies have shown induction of these enzymes in the lung (Grover et al.,1974;

Grover, 1974; Matsubara et al.,1974; Burk and Prough, 1976). Pretreatment of the rat with 3-methylcholanthrene (3-MC) or phenobarbital (PB) caused induction of hepatic microsomal activities. 3-MC induced the pulmonary microsomal enzyme system; however, PB had no effect on the lung enzymes (Matsubara et al. (1974). Like 3-MC, pretreatment of mouse and rabbit with 2,3,7,8- tetrachlorodibenzo-p-dioxin (TCDD) caused induction of both pulmonary and hepatic MFO activity (Poland et al., 1974; Liem et al., 1980). Agents such as cytochrome c, SKF 525A, and others inhibit microsomal mixed-function oxidase activities in lung as well as in liver in vitro (Bend et al., 1972; Litterst et al., 1977). These have provided useful tools with which to study P-450-dependent xenobiotic metabolism.

Cellular Localization of Cytochrome P-450 Enzyme System in Airway Epithelium

The cytochrome P-450 dependent monooxygenase enzyme system occurs in smooth endoplasmic reticulum and provides a major pathway for metabolism of xenobiotic agents. The most characteristic feature of Clara cells is the abundant smooth endoplasmic reticulum. There is much evidence to suggest that these cells are a primary site of cytochrome P-450 dependent monooxogenases reactions. Cytochrome P-450 was identified in pulmonary Clara cells of rats, guinea pigs and rabbits by using immunoflorescence techniques (Dees et al., 1980; Serabjit-Singh et al., 1980; Devereux et al., 1981). However, microsomal enzyme systems in other epithelial cells of airway have not been reported.

Role of Metabolism and Covalent Binding in Lung Injury

During the past several years it has become evident that the carcinogenicity, mutagenicity and cytotoxicity produced by certain, chemically inert, foreign compounds are due to their reactive metabolites (Gillette et al., 1974; Gillette, 1974). The term "reactive metabolite" suggests that the molecule in question is chemically unstable and reacts quickly with suitable, nearby molecules to form a more stable configuration. These alkylation and arylation reactions generally involve electron sharing and covalent bond formation. The reactive metabolites are thought usually to be formed in close proximity to the molecular alkylation sites. Thus, the presence of covalently bound metabolites within an organ suggests that the activation occured directly in the target tissue.

The relationship between the reactive metabolite formation and acute tissue injury has been studied in the rodent liver, where positive correlations between the covalent binding of reactive metabolite to hepatic macromolecules and hepatotoxicity have been demonstrated (Ilett et al., 1973). More recently, correlations between metabolite binding to lung and chemically induced lung injury have been reported (Boyd, 1977; Boyd et al., 1978; Devereux et al., 1982). The presence of covalently bound metabolites within cells with high SER content in the lung suggests that the activation occurs within the lung tissue.

Role of Glutathione

Glutathione (GSH), a sulfhydryl tripeptide, is found in high concentration in the liver and may play a key role in protecting the liver from the effect of toxic metabolites of foreign chemicals (Fig.

2). It has been suggested that one of the physiological functions of GSH is to protect cellular components from attack by electrophilic chemicals or their metabolites that may cause tissue damage by reaction with macromolecules (Boyland and Chasseaud, 1969; Jakoby, 1978; Meister and Anderson, 1983). Various drugs and environmental chemicals have been shown to deplete the liver of GSH. This appears to occur by a reaction of GSH at an electrophilic position in the compound or one of its metabolites. This reaction can be either nonenzymatic or catalyzed by a group of GSH-dependent enzymes known as the gluthione S-transferases (Jakoby, 1978; Meister and Anderson, 1983). The product of the reaction is either secreted into bile or further metabolized and excreted into the urine as the N-acetylcysteine derivative, more commonly known as mercapturic acid. Consequently, it is believed that one of the physiological functions of GSH is to scavenge electrophilic compounds which can potentially cause tissue damage by reacting covalently with macromolecules.

Previous studies have demonstrated that depletion of GSH in lungs of rats by diethylmaleate (Richardson and Murphy, 1975) enhances covalent binding and toxicity of 4-ipomeanol in target tissue (Boyd and Burka, 1978). Thus, it seems likely that GSH protects the lung against certain electrophilic, toxic metabolites.

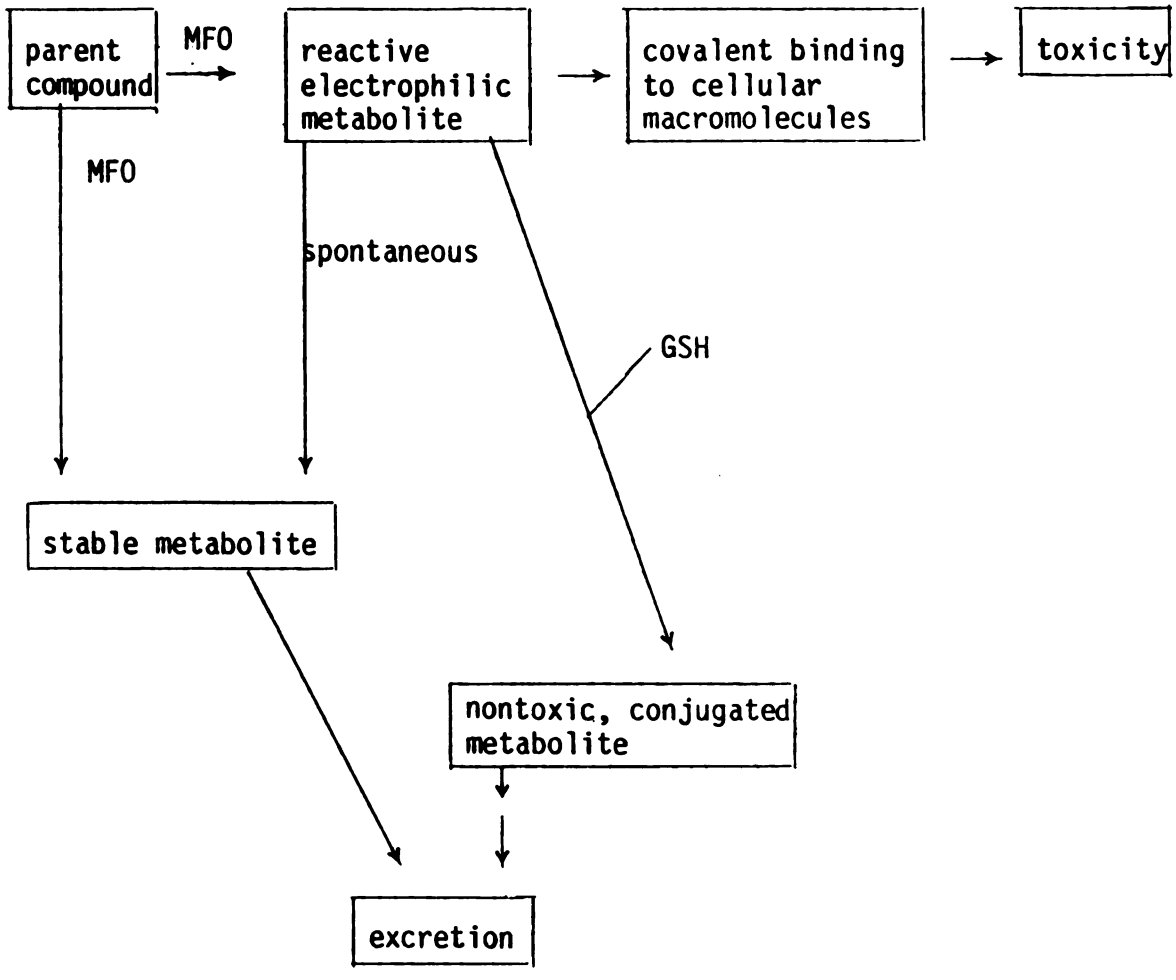

Figure 2: Schematic representation of relationships between toxification and detoxification pathways for xenobiotic agents.

The Airway Epithelial Cells as Potential Targets for Chemicals Requiring Metabolic Activation

4-Ipomeanol

A derivative from moldy sweet potatoes, 4-ipomeanol, is highly toxic to the lungs of experimental animals (Boyd et al., 1973). Boyd et al. (1978) demonstrated that rat pulmonary microsomes mediated the covalent binding in vitro of this pulmonary toxin when radiolabeled 4-ipomeanol (^{14}C-4-ipomeanol) was incubated in the presence of NADPH and oxygen with various subcellular fractions of rat lung. No binding occured in the absence of NADPH or oxygen. Boyd et al. (1978) found that 4-ipomeanol is not sufficiently reactive to alkylate tissue components without prior metabolism.

The effects of various inhibitors and inducers of MFO on the metabolism, covalent binding and toxicity of 4-ipomeanol were investigated. Pretreatment with agents that reduce MFO activity, such as pyrazole, piperonyl butoxide and cobaltous chloride, markedly reduced covalent binding and pulmonary injury from 4-ipomeanol (Boyd, 1977; Boyd and Burka, 1978; Boyd et al., 1978). Interestingly, SKF 525A prevented the covalent binding of 4-ipomeanol in vitro, but had no effect on the covalent binding or pulmonary toxicity of 4-ipomeanol in rats in vivo (Boyd and Burka, 1978). One possible explanation for this observation is that the concentration of SKF 525A in vivo was too low to inhibit the metabolic activation of this toxin.

Effects of the MFO inducers, 3-methylcholanthrene (3MC) and phenobarbital (PB), on covalent binding and pulmonary toxicity of 4-ipomeanol were investigated. Pretreatment of rats with 3MC decreased

covalent binding of 4-ipomeanol *in vivo* in the lung, but actually increased it in the liver. This was associated with centrilobular necrosis, but pulmonary damage was relatively minimal (Boyd, 1975; Boyd and Burka, 1978). These findings demonstrated a positive correlation between 4-ipomeanol toxicity and the level of covalent binding to target tissue.

Pretreatment of rats with PB increased covalent binding of 4-ipomeanol to liver microsomes *in vitro*, but no change occured in binding to lung microsomes *in vitro*. Boyd et al. (1978) concluded that 4-ipomeanol can be metabolized by forms of cytochrome P-450 induced by 3MC or PB. Interestingly, covalent binding of 4-ipomeanol was reduced *in vivo* in lung and liver of rats pretreated with PB. This was paralleled by decreased toxicity of 4-ipomeanol in the lung and liver of rats. Thus, it appears that PB induced detoxifying pathways *in vivo* rather than ones that result in activation (Boyd, 1976). Previous studies demonstrated that the reactive metabolite formed by 4-ipomeanol metabolism is highly electrophilic (Boyd and Burka, 1978; Boyd et al., 1978; 1979; Buckpitt and Boyd, 1980). Addition of glutathione (GSH) markedly reduced covalent binding of 4-ipomeanol *in vitro*. GSH did not prevent reactive metabolite formation, but it did inhibit its covalent binding by acting as alternate nucleophile (Boyd et al., 1978; Buckpitt and Boyd, 1980). Pretreatment of rats with diethylmaleate (DEM), which depleted pulmonary GSH (Richardson and Morphy, 1975), markedly enhanced 4-ipomeanol covalent binding *in vivo* (Boyd and Burka, 1978). Thus, it appears that GSH may play an important role in protecting the lung from the toxic, reactive 4-ipomeanol metabolite(s).

The pulmonary cellular specifity for the metabolic activation of 4-ipomeanol *in vivo* has been studied by autoradiography (Boyd, 1977).

4-ipomeanol was covalently bound preferentially to the intrapulmonary Clara cells of rat, mouse, and hamster. 4-ipomeanol also caused necrosis in these cells. To determine whether metabolism of 4-ipomeanol was required to develop radioactive binding to Clara cells, animals were pretreated with piperonyl butoxide prior to administering 4-ipomeanol. Pretreatment of animals with this MFO inhibitor markedly decreased covalent binding of 4-ipomeanol to Clara cells. Histological examination of the lungs from these animals revealed an absence of necrosis. The metabolic activation of 4-ipomeanol was also studied in several cell types isolated from rabbit lung (Devereux et al. 1981). The highest rates of cytochrome P-450 mediated meabolism of 4-ipomeanol was observed in isolated Clara cells. A considerable amount of activity, but much less than that in Clara cells, was also seen in isolated alveolar type II cells.

In summary, biochemical and morphological studies have demonstrated that 4-ipomeanol is metabolized *in situ* by pulmonary microsomal enzymes to a highly reactive metabolite which covalently binds to cellular macromolecules. 4-ipomeanol produced injury preferentially to pulmonary Clara cells. It has been suggested that Clara cells are susceptible to 4-ipomeanol-induced injury due to their capacity to activate this chemical metabolically.

Carbon Tetrachloride

Carbon tetrachloride (CCl_4) induced injury has been extensively studied in the liver (Recknagel, 1967: Recknagel and Glende, 1973). However, a few studies have focused on the pulmonary toxicity of this chemical (Valdivia and Sonnad, 1966; Chen et al., 1977; Willis and

Recknagel, 1979; Boyd et al., 1980; Ahmadizadeh and Echt, 1985; Ahmadizadeh et al., 1987). It is generally agreed that CCl_4 is metabolized by the cytochrome P-450 locus in smooth endoplasmic reticulum of liver to the trichloromethyl free radical ($CCl_3^{\bullet}$), which is capable of producing injury either by stimulating lipid peroxidation or by binding covalently to tissue macromolecules (Reynolds, 1967; Recknagel and Glende, 1973; Comporti, 1985).

Chen et al. (1977) found pulmonary cytochrome P-450 to be markedly decreased in rat lung 4 hrs after oral administration of 2.5 ml/kg CCl_4. These authors also found that the concentration of cytochrome P-450 was decreased in rat lung microsomes in vitro 30 min after the addition of CCl_4 in the presence of NADPH. Similarly, Boyd et al. (1980) reported that the oral administration of 2.5 ml/kg of CCl_4 to rats caused a marked reduction in rat pulmonary cytochrome P-450. Boyd et al. (1980) also noted that the metabolism of 4-ipomeanol in vitro was impaired in lung preprations from mice or rats treated in vivo with 2.5 ml/kg CCl_4. These results led to the conclusion that pulmonary cytochrome P-450 dependent monooxygenase activities participate in the bioactivation of CCl_4 in lung.

However, the mechanism by which CCl_4 causes lung injury is a subject of controversy. For example, Willis and Recknagel (1977) reported that CCl_4 stimulated the production of thiobarbituric acid (TBA) reactive material in rat lung microsome preparations, suggesting lipid peroxidation. In contrast, Villarruel et al. (1977) found no alteration in the ultraviolet absorbance of lipid from lungs of CCl_4-treated rats, but they found significant amounts of ^{14}C irreversibility bound to lung lipids 3 hours after oral administration of 1 ml/kg of

$^{14}CCl_4$ to rats. These authors suggested that the irreversible binding to cellular components rather than lipid peroxidation is the major mechanism in CCl_4-induced lung injury. It appears that CCl_4 is metabolized by pulmonary a cytochrome P-450 to electrophilic, toxic metabolite(s) capable of causing injury by stimulating lipid peroxidation and/or binding covalently to tissue macromolecules.

Studies have shown that CCl_4 produced injury to type II pneumocytes of guinea pigs and rats (Valdivia and Sonnad, 1966; Gould and Smuckler, 1971; Chen et al., 1977). Boyd et al. (1980) reported that oral administration of 2.5 ml/kg of CCl_4 to mice or rats caused injury in pulmonary Clara cells. Boyd et al. (1980) found that CCl_4 induced injury that was strikingly selective to Clara cells, whereas adjacent ciliated airway epithelial cells appeared to be normal. These authors suggested that pulmonary Clara cells are susceptible to CCl_4-induced injury due to their capacity to activate this chemical metabolically. However, the finding of CCl_4-induced injury in type II pneumocytes raises the possibility that these cells also contain a microsomal, P-450 MFO system. This is further supported by the studies of Devereux and Fouts (1981) and of Jones et al. (1983) suggesting the presence of cytochrome P-450 system in alveolar type II cells of rabbit and rat.

Benzo(a)pyrene

Polycyclic aromatic hydrocarbons are ubiquitous pollutants of air, soil and water. One of the most abundant of this class of compounds is benzo(a)pyrene (BP). This chemical is known to be metabolized to epoxides, phenols, and quinones by cytochrome P-450 dependent monooxygenases and by epoxide hydrases. The resultant metabolites are then conjugated to form more hydrophilic metabolites by various

conjugating enzyme systems. Most of the metabolites are excreted. However, the MFO enzyme system will also convert BP to more reactive forms that can bind covalently to cellular macromolecules (Gelboin, 1980). Many studies have indicated that the carcinogenic, mutagenic and toxic effects of BP depend on its enzymatic conversion to active forms.

Arylhydrocarbon hydroxylase (AHH), also called benzpyrene hydroxylase, is an example of a mixed function of oxygenase enzyme system that metabolizes BP. AHH has a central role in detoxification as well as inactivation of polycyclic hydrocarbons. AHH activity has been demonstrated in many tissues and many species (Wiebel et al., 1971, 1973; Nebert and Gelboin, 1969; Nebert and Gielen, 1972; Ahmadizadeh et al., 1984). The activity has been found in both Clara cells and type II cells of rabbit lung (Devereux et al., 1981).

Stowers and Anderson (1984) showed that treatment of mice or rabbits with BP resulted in the binding of BP metabolites to DNA in lungs of mice and rabbits. In another study, binding of BP metabolites was determined in specific cell populations from lungs of rabbits treated with BP (Horton et al., 1985). Binding of radioactivity was found in Clara cells and type II cells. Kaufman et al. (1973) reported covalent binding of BP-3H in tracheal epithelial cells of hamster after tracheas were isolated from untreated hamsters and incubated in vitro as short-term organ cultures containing radiolabeled BP. These authors found that incubation of the trachea in a medium containing 7,8-benzoflavone, an inhibitor of AHH activity (Wiebel, 1971), prevented the covalent binding of BP-3H. They suggested that the hamster tracheal epithelium can metabolize BP and that the metabolism is

necessary for the binding of BP to tissue. Reznik-Schuller and Mohr (1974 a,b; 1975) demonstrated injury in airway epithelial cells of hamsters treated with BP in vivo. It appears that the lesions were associated with the binding of BP metabolites to bronchiolar and tracheal epithelial cells.

Effect of Chemical Irritants on Glycoprotein Cells of the Airways of Lung

As noted above, several different epithelial cell types contribute to glycoprotein secretion in the respiratory tract. These cells are Clara, goblet and serous cells (Azzopardi and Thurlbeck, 1969; Lamb and Reid, 1969; Jeffery and Reid, 1975, 1977; Becci et al., 1978a; Spicer et al., 1980; Al-Ugaily et al., 1980; Pack et al., 1980, 1981). Many studies have demonstrated that acute and chronic inhalation of certain chemical irritants increased the secretion of glycoproteins in the airway. Exposure of animals to chemical irritants such as sulfur dioxide, ammonia vapor and tobacco smoke caused structural and functional changes in the secretory elements of the airways. Lamb and Reid (1968) reported that exposure of rats to sulfur dioxide (SO_2) increased the number of glycoprotein-containing cells in the trachea. Seltzer et al. (1984) found hypersecretion of mucus in dogs after exposure to SO_2. Freeman and Haydon (1964) reported that exposure of rats to nitrogen dioxide (NO_2) increased the population of glycoprotein-containing cells in the respiratory airway. Exposure of rats to tobacco smoke increased mitotic activity and increased the number of goblet cells in tracheas of rats (Jones et al., 1973; Jones and Reid, 1978).

Becci et al. (1978b) observed hypertrophy of glycoprotein cells in hamster tracheobronchial epithelium 24 hours after intratracheal instillation of mixture of benzo(a)pyrene and ferric oxide (BP-Fe_2O_3). These authors found that glycoprotein became more acidic in BP-Fe_2O_3 treated animals when compared to that of unexposed animals. These effects were attributed to the BP and not to the Fe_2O_3 particles. Since BP requires metabolic activation by the microsomal enzyme system, it seemed likely that glycoprotein cells of trachea can be altered by chemicals that are bioactivated by the mixed function oxidases.

After exposure to irritants, the origin of new goblet cells in trachea and bronchi of rats was investigated. Under conditions of exposure to irritants such as tobacco smoke and sulphur dioxide, serous cells transformed to goblet cells (Jeffery et al., 1977). In the distal airways, the Clara cells transformed to goblet cells (Meyrick, 1977).

In summary, exposure of animals to chemical irritants caused alterations in glycoprotein cells of airways. Modification of glycoprotein-containing cells of airways after exposure to a chemical that is bioactivated by microsomal enzyme system was also reported. Thus, it appears that glycoprotein-containing cells of airways can be altered by chemicals that are bioactivated by MFOs.

PURPOSE

Increasing evidence suggests that epithelial cells of airways have potential for metabolizing xenobiotic compounds. Carbon tetrachloride induced cell injury is dependend on metabolism of this agent by intracellular NADPH-dependent cytochrome P-450 monooxygenases. CCl_4

produced injury in intrapulmonary airway epithelial cells. However, the effect of CCl_4 on tracheal epithelial cells has not been investigated. It seemed possible that this agent might produce injury in tracheal epithelial cells. To test this hypothesis, the effect of CCl_4 on hamster tracheal epithelial cells was examined, with special emphasis on glycoprotein containing cells. In addition, the effect of diethylmaleate or fasting on CCl_4 induced injury in hamster tracheal epithelial cells was studied.

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CHAPTER II

THE EFFECT OF CARBON TETRACHLORIDE ON HAMSTER TRACHEAL EPITHELIAL CELLS

ABSTRACT

This study was designed to assess cytotoxicity of carbon tetrachloride (CCl_4) in hamster tracheal epithelium. Adult, male, Syrian golden hamsters were treated with 2.5 ml/kg CCl_4 and controls received the vehicle (peanut oil) only. Animals were sacrificed after 1, 4, 12, and 24 hours. Tissue samples from upper and lower trachea were fixed and embedded in glycol methacrylate for light microscopy. One or two tracheal rings were fixed in formaldehyde/glutaraldehyde cacodylate buffer for transmission electron microscopy. For histopathologic evaluation of the tracheal epithelial cells, each tracheal level was cut transversely at 3 um and stained with toluidine blue. CCl_4 produced ciliated and nonciliated cell injury in the various levels and regions of hamster trachea. The severity of CCl_4-induced injury differed in various levels and regions of tracheal epithelium. For example, in trachea of hamsters sacrificed 1 hour after CCl_4 treatment, the number of damaged cells was considerably higher in the dorsal region of lower trachea when compared to those of ventral region of upper trachea. The number of damaged cells markedly increased after 4 and 12 hours in the ventral region of lower trachea. By 24 hours, the number of injured cells had decreased so that no

significant difference from control was evident. The ultrastructural alterations of epithelial cells were obvious as early as 1 hour post CCl_4 treatment. Intracellular organelles, including smooth and rough endoplasmic reticulum, mitochondria and Golgi apparatuses, were damaged by this chemical. Since CCl_4 induced cell injury is dependent on metabolism of this agent by intracellular NADPH-dependent cytochrome P-450 monooxygenases, this study suggests that hamster tracheal epithelial cells have the potential to bioactivate CCl_4.

INTRODUCTION

The mixed function oxidases (MFOs) occur in smooth endoplasmic reticulum (SER) and provide a major pathway for metabolism of xenobiotic agents. Generally, the MFO system produces metabolites that are less toxic than the parent compounds. However, in many cases, MFO metabolism leads to the formation of metabolites more toxic than their parent chemicals. For example, carbon tetrachloride (CCl_4) is metabolized by MFO components to a free radical(s) that is highly cytotoxic.

Cellular localization of cytochrome P-450-dependent monooxygenases in the respiratory system has generated considerable interest due to the fact that many environmental chemicals gain access to the body through the respiratory system. In this respect, detoxification or toxification of a chemical by the pulmonary MFO system may in part determine its site and mechanism of action.

Clara cells have been suggested as a primary site of MFO enzyme activity in the lung (Boyd et al., 1977; Serabjit-Singh et al., 1980).

The presence of Clara-like cells have been identified in tracheal epithelium of several species, including hamster, mouse, and rabbit (Hansell and Moretti, 1969; Pack et al., 1980; Al-Ugaily et al., 1980; Plopper et al., 1983). CCl_4 produced injury in intrapulmonary Clara cells of mice and rats (Boyd et al., 1980). It has been suggested that these cells are susceptible to CCl_4 injury because of their capacity to metabolize this chemical to a toxic intramediate(s). Since tracheal Clara cells contain abundant amounts of smooth endoplasmic reticulum similar to intrapulmonary Clara cells, it seemed likely that a xenobiotic agent such as CCl_4 might produce injury in tracheal epithelial cells. To test this hypothesis, I examined the effect of CCl_4 on various regions and levels of tracheal airway epithelium.

MATERIALS AND METHODS

Adult, male, Syrian golden hamsters (100-140g) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.) and housed in groups of three in clear polypropylene cages in a light cycle (12 hr light and 12 hr dark) and temperature controlled room. Animals were allowed food (Wayne Lab Blox, Chicago, IL) and tap water ad libitum. Carbon tetrachloride (CCl_4) was obtained from Sigma Chemical Company (St. Louis, MO), dissolved in peanut oil and administered i.p. at dose of 2.5 ml/kg body weight. Control animals received vehicle only. 1, 4, 12, or 24 hrs after treatment, animals were sacrificed with 100-300 mg/kg of sodium pentobarbital (i.p.). Three hamsters were used for each group.

Light Microscopy:

Tracheal tissues were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2). Each specimen was divided into upper and lower levels and embedded in glycol methacrylate (GMA, Polyscience, Inc.). Tracheas were transversely sectioned at 3 um from proximal to distal ends with glass knives on a JB-4 microtome. Three histological sections, each at least 15 um apart, were taken from each tissue block and stained with 1% toluidine blue.

The luminal epithelium was subdivided into dorsal, right and left lateral, and ventral regions. The total number of injured cells/mm/region was counted at 400X magnification and compared statistically. Only those cells with distinct nuclei were counted. The criteria for cell injury included: nuclear dilation, loss of staining capacity and obvious cellular swelling.

Data were expressed as mean $\pm$ standard error. The results were analyzed by analysis of variance, completely randomized design, and treatment differences were identified by the method of Newman-Keuls (Steel and Torrie, 1960). $P < 0.05$ was used as the criterion for significance. Since the data were nonsymmetrical and the sample size at each time period was small (n=3 animals) a standard $\log_{10}$ transformation was used to compensate for the nonsymmetry, while preserving the relative pattern among groups. The transformed data in each morphological level and region of trachea were then subjected to a one-way analysis of variance across time.

Transmission Electron Microscopy:

One or two rings of trachea from each animal were cut into pieces approximately 1 mm^2, and 1-2 blocks from each region (dorsal, lateral or ventral) were prepared for electron microscopy. Tissues were fixed in formaldehyde-glutaraldehyde cacodylate buffer (Karnovsky, 1965). The tissues were washed in 0.2 M cacodylate buffer, post-fixed in 2% osmium tetroxide, dehydrated with ethanol and embedded in epon-araldite (Mollenhauer, 1964). Sections 1 um thick were cut from selected areas and stained with toluidine blue for examination by light microscopy. Thin sections (approximately 90 nm) were cut from selected areas. The sections were stained with uranyl acetate and lead citrate and examined with a Philips 201 transmission electron microscope.

RESULTS

Administration of peanut oil vehicle alone did not produce detectable injury to hamster tracheal epithelial cells (Fig. 1). However, cell injury was observed in the various morphological levels and regions of the trachea following CCl_4 treatment. Light microscopy revealed that both ciliated and nonciliated tracheal epithelial cells were swollen, had loss of staining capacity, and nuclei appeared to be dilated. CCl_4 produced injury in tracheal epithelial cells as early as 1 hour following CCl_4 treatment (Fig. 2). However, the degree of injury varied in different levels and regions of tracheal epithelium.

Quantitative Analysis by Light Microscopy:

1) Dorsal region

a) Upper trachea: In this region, CCl_4 did not produce a significant effect by 1 hour post-treatment. However, the number of damaged epithelial cells was markedly increased at 4 and 12 hours. By 24 hours, the number of injured cells had decreased so that no significant difference from control was evident.

b) Lower trachea: CCl_4 produced a significant effect by 1 hour post treatment. However, the number of damaged cells was considerably decreased at 4, 12, and 24 hours so that no significant differences from control were evident.

2) Lateral region

a) Upper trachea: In this region, tracheal epithelium remained essentially normal in appearance at 1 hour after CCl_4 treatment. However, the number of damaged epithelial cells was increased at 4, 12, and 24 hours when compared to those of control animals.

b) Lower trachea: Numbers of damaged epithelial cells were increased at 1 and 12 hours CCl_4 post-treatment when compared with control values. However, number of damaged cells decreased at 4 and 24 hours so that no significant differences from control were evident.

3) Ventral region

a) Upper trachea: In this region, CCl_4 did not produce a significant effect at 1, 12, and 24 hours. However, the number of damaged epithelial cells was elevated 4 hours post CCl_4 treatment.

b) Lower trachea: In lower trachea, the population of damaged epithelial cells was larger at 1, 4, and 12, but not 24 hours after CCl_4 when compared with control values.

Transmission Electron Microscopy:

In control hamster tracheal epithelium, tall columnar, nonciliated cells were observed that contained abundant smooth endoplasmic reticulum, numerous mitochondria, well-developed rough endoplasmic reticulum (RER), and prominent Golgi complexes. In addition, the apical cytoplasm contained membrane bound, electron lucent, secretory granules (Fig. 3). Ciliated cells were columnar, and numerous mitochondria, RER, and Golgi complexes were seen in electron lucent cytoplasm (Fig. 3). Ultrastructural alterations in nonciliated and ciliated cells were noted as early as 1 hour following CCl_4 treatment (Fig. 4). Damage in intracellular organelles of nonciliated cells was characterized by dilation of SER, ribosomal disaggregation, swelling of mitochondria and numerous cytoplasmic vacuoles. The ultrastructural features of ciliated cells injured by CCl_4 were dilation of RER, swelling of mitochondria, disaggregation of ribosomes and numerous cytoplasmic vacuoles (Figs. 4-7). However, the degree of intracellular injury varied in different regions of hamster trachea in a manner similiar to the light microscopic observations.

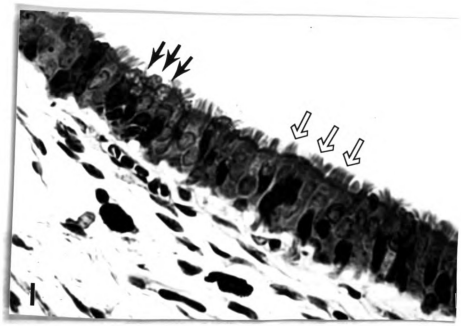

Figure 1: Light micrograph of ventral region of upper trachea of hamster treated with vehicle only (control). The ciliated cells (open arrow) and nonciliated cells (solid arrows) are intact. Note pseudostratified appearance of tracheal epithelium. X 400.

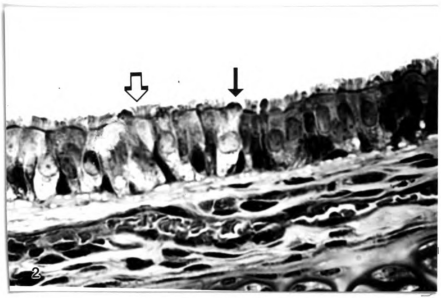

Figure 2: Light micrograph of laterial region of lower trachea 1 hr CCl_4 treated hamster, showing injury (loss of staining capacility and dilatation of cytoplasm and nucleus) to ciliated cells (open arrow) and nonciliated cells (solid arrow). X 400.

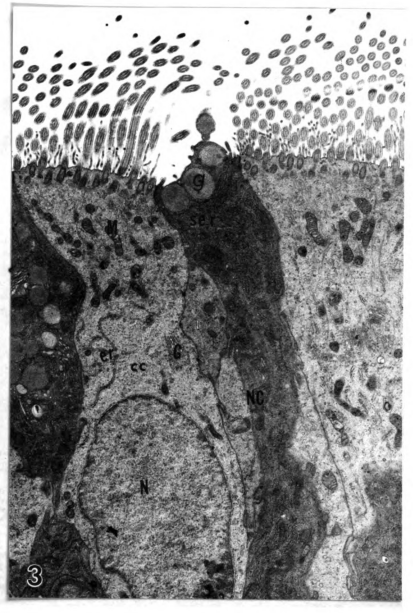

Figure 3: Transmission electron micrograph of ventral region of control hamster trachea, showing ciliated (CC) and nonciliated (NC) cells. Note electron lucent, membrane bound secretory granules (g). Mitochondria (M), rough endoplasmic reticulum (er), smooth endoplasmic reticulum (ser), Golgi complex (G) and nucleus (N). X 6272.

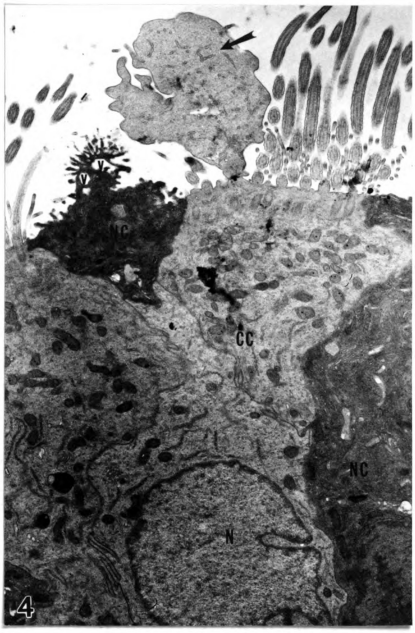

Figure 4: Transmission electron micrograph of laterial region of tracheal epithelial cells of hamster treated 1 hr previously with CCl_4. Vacuoles (V) are evident in nonciliated (NC) cells. Note ciliated cells (CC) with cytoplasmic bleb (arrow). X 15000.

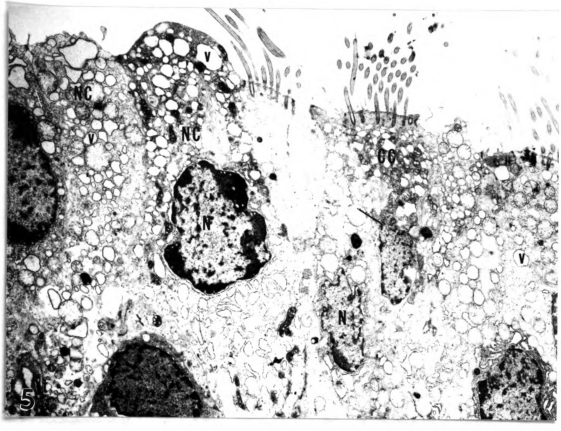

Figure 5: Transmission electron micrograph of laterial region of tracheal epithelial cells of hamster treated 4 hrs previously with CCl_4. Severe damage (dilatation of intracellular organelles including mitochondria, rough endoplasmic reticulum and numerous vacuoles) is shown in both ciliated (CC) and nonciliated (NC) cells. Nucleus (N). vacuole (V). X 5732.

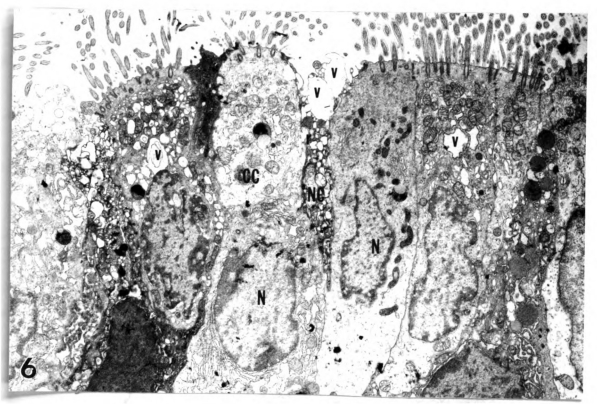

Figure 6: Transmission electron micrograph of laterial region of tracheal epithelial cells of hamster treated 12 hrs previously with CCl_4. Note injury (dilatation of intracullular organelles including mitochondria, rough endoplasmic reticulum and numerous vacuoles) in both ciliated (CC) and nonciliated (NC) cells. Nucleus (N). X 5732.

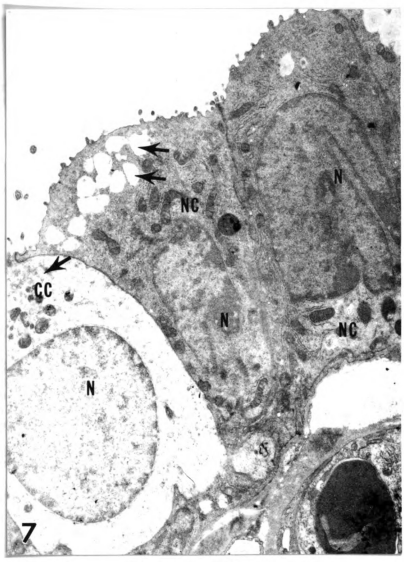

Figure 7: Transmission electron micrograph of ventral region of tracheal epithelial cells of hamster treated 24 hrs previously with CCl_4. Vacuoles (arrows) are evident in ciliated (CC) and nonciliated (NC) cells; however cells look less injured than at earlier times. Nucleus (N). X 8624.

Table 1: Numbers of damaged cells in various levels and regions of tracheal epithelia of hamsters treated with CCl_4.*

		CCl_4 Treatment			
	Control	1 hr	4 hr	12 hr	24 hr
Dorsal					
UT	0±0	2.27±1.31^{cd}	18.50±4.72^{ab}	13.32±1.78^{ab}	2.22±0.63^{cd}
LT	0±0	18.12±8.30^{a}	1.31±0.37	4.26±0.92	0.74±0.13^{b}
Lateral					
UT	0±0	1.30±0.54^{d}	14.96±5.24^{ab}	12.81±0.90^{ab}	3.24±0.55^{abcd}
LT	0±0	11.69±4.38^{a}	3.18±1.09^{d}	18.49±5.31^{ac}	2.98±0.40^{d}
Ventral					
UT	0±0	0.94±0.46^{cd}	7.74±1.96^{ab}	6.96±3.98^{b}	2.00±0.54
LT	0±0	9.68±4.27^{a}	8.92±1.62^{a}	17.77±2.74^{a}	1.56±0.66^{bcd}

Adult, male, Syrian golden hamsters were treated with CCl_4 (2.5 ml/kg). Control animals were given vehicle only. Animals were killed 1, 4, 12, or 24 hrs later. Tracheal tissues were removed and subdivided into upper (UT) and lower (LT) levels, and prepared for histological determination of number of damaged cells as described in Methods. Three hamsters were used for each group.

*Number of damaged cells per 1 mm length of tracheal epithelium. Control values are given for comparison.

a=Significantly different from that of the control group ($p<0.05$).

b=Significantly different from that of the group killed 1 hr after CCl_4 ($p<0.05$).

c=Significantly different from that of the group killed 4 hrs after CCl_4 ($p<0.05$).

d=Significantly different from that of the group killed 12 hrs after CCl_4 ($p<0.05$).

DISCUSSION

The use of 2-3 um glycol methacrylate histologic sections allows for an accurate identification of cell types in tracheal epithelium and, therefore, permits reliable quantitative analyses of cellular structural responses after exposure to a toxicant such as CCl_4. This study demonstrated that intraperitionial administration of CCl_4 produces differential injury. I observed that the extent of CCl_4-induced injury varied in different levels and regions of hamster trachea. Since the cytotoxicity produced by CCl_4 requires metabolic activation, this finding suggests that MFO metabolic activity of hamster airway tracheal epithelial cells varied in different morphological regions. Gabridge et al. (1977) used a nitroblue tetrazolium assay for overall metabolic activity of airway tracheal epithelial cells in hamster and reported that the metabolic activity of tracheal epithelial cells was different in various rings of trachea. These regional differences point out the importance of specifying the location of a cellular response to toxic chemicals both in vivo and in vitro.

It is widely accepted that CCl_4 is metabolized to a toxic species by a cytochrome P-450 dependent monooxygenase enzyme system. Boyd et al. (1980) reported that CCl_4 produced injury in intrapulmonary Clara cells of mice and rats. This suggested that these cells were susceptible to injury by CCl_4 due to their capacity to activate the chemical metabolically.

I observed an abundance of smooth endoplasmic reticulum (SER) in nonciliated hamster tracheal epithelial cells. This raised the possibility that these cells may have the potential for bioactivation of CCl_4. Recently, Plopper et al. (1983) reported that hamster tracheal Clara cells were structurally similiar to intrapulmonary Clara cells and contained large amounts of SER, with electron dense granules in the apical cytoplasm. In the present study nonciliated cells containing electron lucent granules in the apical portions of cytoplasm were observed. These findings confirmed those of others (Reznik-Schuller and Mohr, 1974; Becci et al., 1978; Kennedy et al., 1978), who have reported the presence of electron lucent granules.

Many studies have demonstrated that chemical carcinogens produced injury in hamster tracheal epithelial cells. These chemicals are known to be metabolized by a microsomal enzyme system, resulting in the generation of alkylating intermediates that are responsible for the mutagenic, toxic and carcinogenic effects of the parent compound (Health, 1962; Malling, 1971; Magour and Nievel, 1971; Kaufman, et al., 1973; Czygan et al., 1973; Bartsch et al., 1975; Reznik-Schuller and Tomaszewski, 1980; Reznik-Schuller and Hague, 1981). For example, studies conducted by Reznik-Schuller and Mohr (1974) demonstrated that benzo(a)pyrene is carcinogenic for the tracheal epithelium of Syrian golden hamster. In this respect, the observation that CCl_4-induced cell injury suggests the presence of a NADPH-dependent monooxygenase enzyme system in epithelial cells of hamster trachea.

The finding that the extent of CCl_4-induced cell injury was different among various levels and regions of hamster tracheal epithelium raised the possibility that some tracheal epithelial cells

may be more capable than others of bioactivating CCl_4. Alternatively, cells of other regions and levels of trachea also might be capable of activating CCl_4, but the cells which appeared to be less sensitive may more effectively detoxify the reactive CCl_4 metabolite and thereby prevent toxicity.

The mechanism(s) by which CCl_4 causes cell injury is not completely resolved. However, a large body of evidence suggests that CCl_4 is bioactivated by a NADPH-dependent cytochrome P-450 locus in SER of hepatocytes to the trichloromethyl free radial (CCl_3), which then interacts with intracellular organelles leading to an alteration of cellular integrity. CCl_4 may cause injury to essential macromolecules by stimulating lipid peroxidation and/or binding covalently to them (Recknagel, 1967; Recknagel and Glende, 1973; Comporti, 1985).

Biochemical mechanisms exist which protect cells from reactive metabolites. For example, substances such as glutathione (GSH) can react with free radicals and prevent cell necrosis (Orrenius and Jones, 1978; Meister and Anderson, 1983). Aldehyde dehydrogenases (ALDH) are capable of detoxifying a variety of aldehydes (Weiner, 1980); several investigators suggested that products of toxic aldehydes generated during CCl_4-induced lipid peroxidation inhibit ALDH activity in livers. Inhibition of cytosolic and mitochondrial ALDH may be important in the hepatotoxic effects of CCl_4 (Hjelle et al., 1981; Hjelle and Petersen, 1981; Hjelle et al., 1983). ALDH is present in hamster tracheal epithelium (Ahmadizadeh and Echt, unpublished results). Thus, the extent of CCl_4-induced epithelial cell injury may also be related to the intracellular concentration of GSH and/or ALDH activity.

In addition to CCl_4-induced damage to nonciliated cells, I observed injury to ciliated tracheal epithelial cells in various

regions and levels of hamster trachea as early as 1 hour after CCl_4 treatment. This finding raises several possibilities. First, the ciliated cells may possess microsomal enzyme systems and may be capable of bioactivating this chemical. Gabridge et al. (1977), using the nitroblue tetrazolium assay, suggested that hamster ciliated airway epithelial cells have a high metabolic activity. Harris et al. (1973) reported radioactive binding of benzo(a)pyrene (BP) in ciliated cells of hamster trachea after tracheas were isolated from untreated hamsters and incubated in vitro as short-term organ cultures containing radiolabeled BP. These authors found that binding was markedly reduced by the addition of 7,8-benzoflavone, an inhibitor of arylhydrocarbon hydroxylase (AHH) activity. Thus ciliated cells appear to have the capacity to metabolize foreign chemicals in the hamster.

Interestingly, Castro et al. (1983) reported that highly purified rat liver mitochondria were capable of metabolizing CCl_4. These authors suggested that cytochrome P-450 of mitochondria participates in bioactivation of reactive metabolites. This finding raised the possibility that not only SER, but intracellular organelles, such as mitochondria, may be associated with an MFO enzyme system. Ciliated cells of trachea of hamsters contain numerous mitochondria. However, whether this organelle can participate in activation of CCl_4 to a toxic, reactive metabolite remains to be determined.

A second possible explanation for CCl_4-induced injury in ciliated tracheal epithelial cells is that bioactivation of CCl_4 may occur in nonciliated tracheal epithelial cells, and toxic metabolites translocate to ciliated cells via cell-cell communication. Alterations of the intercellular junction have been associated with pathological

changes in various tissues. James et al. (1986) observed that CCl_4 increased gap junction size in rat hepatocytes. The presence of gap junctions in guinea pig tracheal airway epithelial cells was reported by Inoue and Hogg (1974). In this way, translocation of toxic metabolite(s) from nonciliated to ciliated cells might be responsible in part for ciliated cell injury.

It is generally accepted that toxic aldehydes generated during CCl_4-induced lipid peroxidation have relatively long life spans and, therefore, can have an effect on subcellular targets distant from the production of a toxic metabolite (Comporti, 1985). A third possible mechanism for CCl_4-induced ciliated cell injury is that the generation of a toxic metabolite may occur in the liver, and subsequent translocation of the toxic intermediate(s) to the lung may account at least in part for tracheal epithelial cell injury. However, further studies will be needed to clarify these hypotheses.

With light microscopy, the number of injured cells decreased in all regions and levels of hamster trachea 24 hours after CCl_4 treatment. This finding suggests that CCl_4-induced tracheal epithelial cell injury is reversible. This observation is consistent with the results of Boyd et al. (1980), who reported that CCl_4 produced reversible injury in intrapulmonary Clara cells in mice and rats. Thus, it appears that CCl_4 induced intra- and extrapulmonary epithelial cell lesions are to some extent reversible.

In summary, this study indicates clearly that CCl_4 produced injury in tracheal epithelial cells of hamster. Inasmuch as CCl_4 requires bioactivation for toxicity, both nonciliated and ciliated cells may have the capacity to metabolize CCl_4 in the hamster. I observed that CCl_4-induced cell injury varied in different levels and regions of

trachea. This finding raised the possibility that some tracheal epithelial cells may possess a greater capacity for bioactivating CCl_4. Alternatively, the cells which appeared to be less sensitive may detoxify the metabolites of CCl_4 more effectively and thereby avoid cell injury.

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CHAPTER III

EFFECT OF CARBON TETRACHLORIDE ON GLYCOPROTEIN-CONTAINING CELLS IN HAMSTER TRACHEAL EPITHELIUM

ABSTRACT

This study was designed to examine the distribution of glycoprotein containing cells in hamster trachea and their histochemical response to carbon tetrachloride (CCl_4). Adult, male, Syrian golden hamsters were treated with 2.5 ml/kg CCl_4 and controls received a vehicle (peanut oil) only. Animals were killed after 1, 4, 12 or 24 hrs. Tracheal tissues from upper (UT) and lower (LT) tracheal levels were fixed and embedded in glycol methacrylate for light microscopy. One or two tracheal rings were fixed in formaldehyde-glutaraldehyde cacodylate buffer and processed for transmission electron microscopy. For cytochemical evaluation of glycoprotein-containing cells, each tracheal level was cut transversely at 3 um and stained with Alcian blue/Periodic acid-Schiff (AB-PAS) at pH 2.6. Granules of acid glycoproteins were AB positive, and those of neutral glycoproteins were PAS positive.

In control hamster most of the glycoprotein cells were PAS-positive. A few contained AB-PAS-positive granules. However, the frequency distribution of the glycoprotein-containing cells varied in different regions and levels. The secretory cells were columnar and

contained electron lucent, membrane bound secretory granules. Intracellular organelles, including abundant smooth and rough endoplasmic reticulum, mitochondria and Golgi complexes, were observed in these cells.

CCl_4 produced numerous changes in glycoprotein-containing cell numbers and cytochemistry depending upon the time elapsing since treatment. For example, cells appeared that contained PAS-positive glycoprotein that was distributed homogenously in the cell rather than discrete granules. Numbers of these cells markedly increased after 12 and 24 hrs post CCl_4 treatment. However, numbers of cells containing acidic glycoproteins (AB-PAS-positive) increased 1 and 4 hrs after CCl_4 treatment.

Ultrastructural alterations of the secretory cells were obvious as early as 1 hr after CCl_4 treatment. Intracellular organelles, including smooth and rough endoplasmic reticulum, mitochondria, Golgi complexes, were damaged by CCl_4. This chemical also produced injury in electron lucent secretory granules. Since CCl_4-induced cell injury depends on metabolism of this agent by intracellular, NADPH-dependent cytochrome P-450 monooxygenases, this study suggests that glycoprotein containing cells in hamster trachea have the potential to bioactivate this chemical.

INTRODUCTION

One of the most important defense barriers in the tracheobronchial tree is mucus, consisting primarily of secreted glycoproteins. The goblet, serous and Clara cells contribute to glycoprotein secretion in the respiratory tract (Spicer et al., 1971; 1974; Jeffery and Reid,

1977; Spicer et al., 1980; Pack et al.,1980; 1981). Numerous studies have demonstrated that acute or chronic inhalation of chemical irritants increases secretion of glycoproteins in the airway. Exposure of rats to chemical irritants such as sulfur dioxide (Ried, 1963; Spicer et al, 1974; Freeman and Haydon, 1964 Lamb and Reid, 1968) and tobacco smoke (Lamb and Reid, 1969; Jones et al., 1973; Jeffery and Reid, 1977; Jones and Reid, 1978; Coles et al., 1979) causes hypertrophy and hyperplasia of glycoprotein cells in airways.

Modification of glycoprotein-containing cells in the trachea after exposure to a chemical carcinogen was also reported. Becci et al. (1978a) found that treatment of hamsters with a mixture of benzo(a)pyrene and ferric oxide (BP-Fe_2O_3) caused hypertrophy and hyperplasia of glycoprotein cells in hamster trachea. These authors reported that the glycoprotein became more acidic in treated animals when compared to that of unexposed hamsters. These effects were attributed to BP and not to the Fe_2O_3. Benzo(a)pyrene is metabolized by mixed function oxidases (MFO) to an electrophile that is highly cytotoxic; thus, it appears that glycoprotein cells can be altered by chemicals that are bioactivated by MFO.

Carbon tetrachloride (CCl_4) induced cell injury is dependent on metabolism of this agent by intracellular NADPH-dependent cytochrome P-450 monooxygenases. Since BP requires metabolic activation by MFO and since it altered glycoprotein-containing cells, it seems likely that a xenobiotic agent such as CCl_4 might also alter glycoprotein-containing cells of respiratory airways. To test the hypothesis, I examined the effect of CCl_4 administration <u>in vivo</u> on glycoprotein-containing cells of various regions and levels of hamster trachea.

MATERIALS AND METHODS

Adult, male, Syrian golden hamsters (100-140g) were obtained from Harlan Sprague Dawley (Indianapolis, Ind.) and housed in groups of three in clear polypropylene cages in a light cycle (12 hr light and 12 hr dark) and temperature controlled room. Animals were allowed food (Wayne Lab Blox, Chicago, IL) and tap water ad libitum. Carbon tetrachloride (CCl_4) was obtained from Sigma Chemical Company (St. Louis, MO), dissolved in peanut oil and administered i.p. at a dose of 2.5 ml/kg body weight. Control animals received the vehicle only. At 1, 4, 12, or 24 hrs after treatment, animals were sacrificed with 100-300 mg/kg of sodium pentobarbital (i.p.). Three hamsters were used for each group.

Light Microscopy:

Tracheal tissues were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2). Each specimen was divided into upper and lower levels and embedded in glycol methacrylate (GMA, Polyscience, Inc.). Tracheas were sectioned transversely at 3 um from proximal to distal ends with glass knives on a JB-4 microtome. Three histological sections at least 15 um apart were taken from each tissue block and stained with Alcian blue/ Periodic acid-Schiff (AB-PAS) stain at pH 2.6.

The luminal epithelium was subdivided into dorsal, right and left lateral, and ventral regions. The total number of glycoprotein

containing cells/mm/region was counted at 400X magnification and compared statistically. Data were expressed as mean $\pm$ standard error. The results were analyzed by analysis of variance and treatment differences were identified by the method of Newman-Keuls (Steel and Torrie, 1960). $P<0.05$ was used as the criterion for significance. Since the data were nonsymmetrical and the sample size at each time period was small (n=3 animals) a standard $\log_{10}$ transformation was used to compensate for the nonsymmetry while preserving the relative pattern among comparison groups. The transformed data in each morphological level and region of the trachea were subjected to a one-way analysis of variance.

Transmission Electron Microscopy:

One or two rings of trachea from each animal were cut into pieces approximately 1 mm^2, and 1-2 blocks from each region (dorsal, lateral or ventral) were prepared for electron microscopy. Tissues were fixed in formaldehyde-glutaraldehyde cacodylate buffer (Karnovsky, 1965). The tissues were washed in 0.2 M cacodylate buffer, post-fixed in 2% osmium tetroxide, dehydrated with ethanol and embedded in epon-araldite (Mollenhauer, 1964). Sections 1 um thick were cut from selected areas and stained with toluidine blue for examination by light microscopy. Thin sections (approximately 90 nm) were cut from selected areas. The sections were stained with uranyl acetate and lead citrate and examined with a Philips 201 transmission electron microscope.

RESULTS

Within the glycoprotein containing cells, the presence of granules of acid and neutral glycoproteins were identified by the Alcian blue/Periodic acid Schiff (AB-PAS) stain at pH 2.6. Granules of acid glycoproteins are AB positive, and those of neutral glycoproteins are PAS positive (Jones et al., 1973). Based on the amount of intracellular granules, the glycoprotein containing cells were described as small (S) or large (L). A small glycoprotein-containing cell was one containing granules at its apex, and a large cell was one containing granules throughout its cytoplasm (Figs. 1-4). Glycoprotein cells containing PAS-positive, material that was distributed homogenously rather than in discrete granules was observed in some groups. These cells were described as small (PAS-H-S) or large (PAS-H-L). A small cell was one containing homogenous PAS-positive material only at its apex; and a large was one containing homogenous, PAS-positive material throughout its cytoplasm (Fig. 5).

Glycoprotein Containing Cells In Control Animals:

In tracheas from control animals, most glycoprotein-containing cells were PAS-positive (Tables 1-3). The small PAS-positive (PAS-G-S) cells were more predominant in the lateral region of the upper trachea (UT) and ventral region of the lower trachea (LT). However, the large PAS-positive (PAS-G-L) cells were more numerous in the

lateral region of UT. The glycoprotein cells containing both AB and PAS (AB-PAS) positive granules were found infrequently. No cells were apparent with only AB positive granules. Similarly, glycoprotein cells containing homogenous, PAS-positive material in the cytoplasm were absent.

Transmission electron microscopy of trachea from peanut oil treated hamsters revealed that the secretory cells were columnar, and the cytoplasm was electron-dense and contained electron lucent, membrane bound, secretory granules in the apical region or throughout the cytoplasm (Fig. 6). Intracellular organelles, including abundant smooth and rough endoplasmic reticulum, mitochondria and Golgi complex, were observed in these cells.

Changes In Glycoprotein Cells After CCl_4 Treatment:

The effect of CCl_4 on intracellular glycoprotein is shown in tables 1-3. Unlike controls, in CCl_4- treated hamster trachea, glycoprotein cells containing homogenous, PAS-positive material were observed. In the dorsal region, the number of these cells markedly increased in upper trachea (UT) and lower trachea (LT) 12 or 24 hrs after CCl_4 treatment. In lateral regions, glycoprotein cells containing homogenous, PAS-positive material increased in UT at 1 hr, LT at 4, 12, and 24 hrs after CCl_4-treatment. In ventral regions, the cells containing PAS-positive, homogenous material increased in UT at 1, 4, 12, 24, and in LT at 24 hrs post CCl_4 treatment.

In contrast, the glycoprotein cells containing PAS-positive granules decreased in trachea of hamsters treated with CCl_4. In dorsal regions, these cells decreased in UT and LT at 1 , 4 and 24 hrs after CCl_4 treatment. In lateral regions, the glycoprotein cells containing

PAS-positive granules decreased in UT at 1, 24, and in LT at 1, 4, and 24 hrs after CCl_4 treatment. In ventral regions, the PAS-positive granule-containing cells decreased in LT at 1, 4, and UT at 12 hrs and UT and LT at 24 hrs after CCl_4 treatment.

Glycoprotein-containing cells with AB-PAS-positive granules were rarely seen in control animals. However, these cells were predominant in all regions of UT and LT at 1 and 4 hrs after CCl_4 treatment. Cells containing AB-positive granules were absent in control hamster, but were observed infrequently in CCl_4 treated hamsters.

Transmission electron microscopy revealed that CCl_4 produced injury in secretory cells as early as 1 hr CCl_4 post-treatment. Damage in intracellular organelles was characterized by rupture of membrane bound secretory granules, dilation of Golgi apparatuses, smooth and rough endoplasmic reticulum, swelling of mitochondria and numerous cytoplasmic vacuoles (Figs. 7-10).

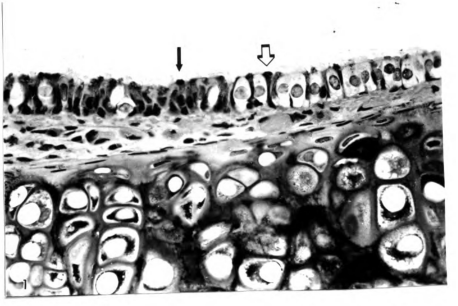

Figure 1: Light micrograph of lateral region of upper trachea of hamster treated 12 hrs previously with CCl_4. Note cells containing PAS-positive granules in the apical cytoplasm (solid arrow) or throughout the cytoplasm (open arrow). X 400.

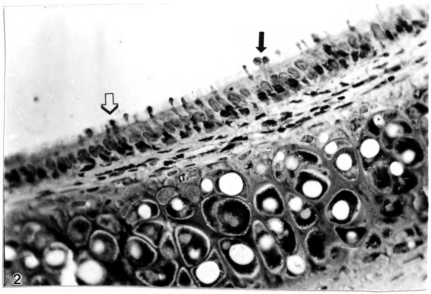

Figure 2: Light micrograph of ventral region of upper trachea of hamster treated 4 hrs previously with CCl_4. Note cells containing AB-PAS positive granules in the apical cytoplasm (solid arrow) or throughout the cytoplasm (open arrow). X 400.

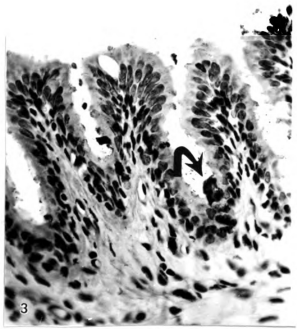

Figure 3: Light micrograph of dorsal region of upper trachea of hamster treated 12 hrs previously with CCl_4. Note cell containing AB-positive granules in the apical cytoplasm (arrow). X 400.

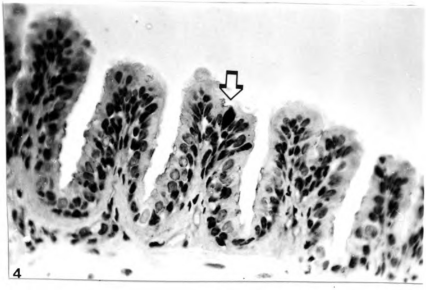

Figure 4: Light micrograph of dorsal region of lower trachea of hamster treated 4 hrs previously with CCl_4. Note cell containing AB-positive granules throughout cytoplasm (arrow). X 400.

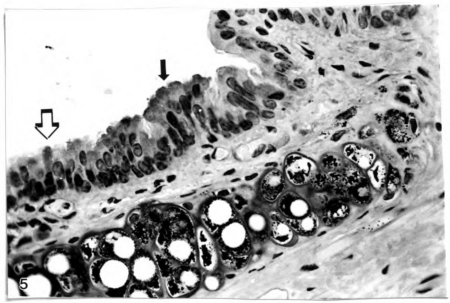

Figure 5: Light micrograph of lateral region of lower trachea of hamster treated 24 hrs previously with CCl_4. Note cells containing homogenous, PAS-positive material in the apical cytoplasm (solid arrow) or throughout the cytoplasm (open arrow). X 400.

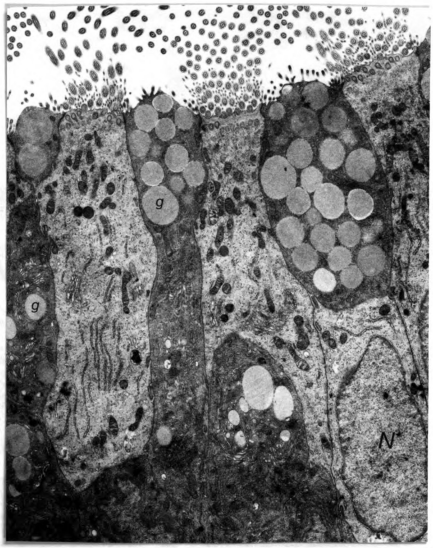

Figure 6: Transmission electron micrograph of lateral region of tracheal epithelial cells of control hamster. Note electron lucent, membrane bound, secretory granules (g) in apical cytoplasm (center) or throughout the cytoplasm (left) of nonciliated cells. X 6272.

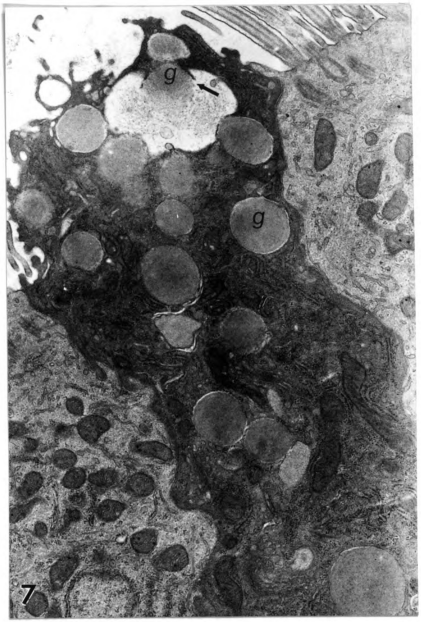

Figure 7: Transmission electron micrograph of lateral region of tracheal epithelium of hamster treated 1 hr previously with CCl_4. Note rupture of membrane bound secretory granules (arrow). Secretory granules (g). X 7165.

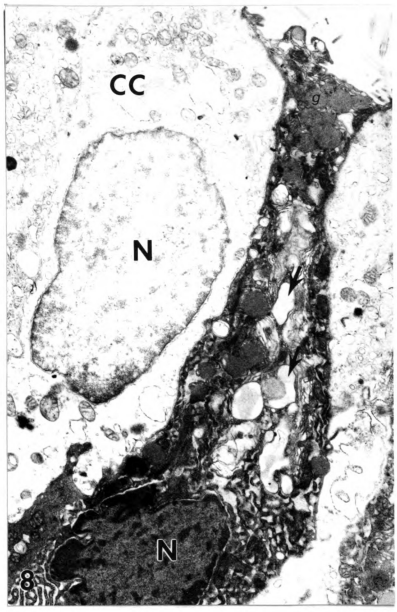

Figure 8: Transmission electron micrograph of lateral region of tracheal epithelium of hamster treated 4 hr previously with CCl_4. Note injury (dilatation of intracellular organelles including mitochondria, rough endoplasmic reticulum, Golgi apparatuses and many vacuoles) in both ciliated and nonciliated cells. Vacuole (arrow), Secretory granule (g), nucleus (N). X 8624.

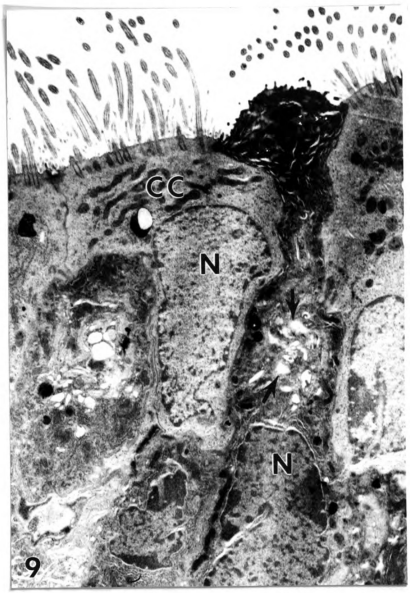

Figure 9: Transmission electron micrograph of ventral region of tracheal epithelium cells of hamster treated 12 hrs previously with CCl_4. Note many vacuoles (arrows) in the cytoplasm of the nonciliated cell. Nucleus (N), ciliated cells (CC). X 8624.

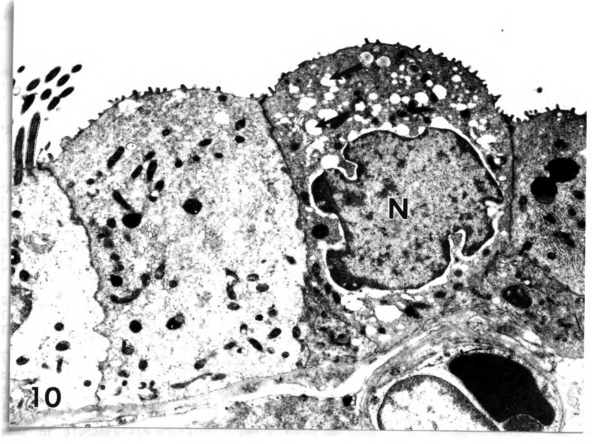

Figure 10: Transmission electron micrograph of lateral region of tracheal epithelial cells of hamster treated 24 hrs previously with CCl_4. Note many vacuoles (arrows) in the cytoplasm of nonciliated cell. Nucleus (N). X 8820.

Table 1: Number of glycoprotein-containing cells (GP) of various types in levels of dorsal regions of trachea of hamsters treated with CCl_4 for various times.*

GP	Control	CCl_4 treatment			
		1 hr	4 hr	12 hr	24 hr
PAS-G-S					
UT	9.11±1.60	0.56±0.25[ad]	1.75±1.01[ad]	12.82±5.26[bc]	2.53±0.79
LT	4.00±0.71	0.32±0.06[ad]	1.77±0.79	5.91±1.77[b]	0.83±0.60[ad]
PAS-G-L					
UL	7.56±2.30	0.52±0.18[acd]	3.39±0.51[b]	8.00±2.75[b]	1.38±0.35[abd]
LT	2.48±0.32	0.39±0.05[ad]	0.23±0.0[ad]	3.05±0.69[bc]	0.45±0.22[ad]
PAS-H-S					
UT	0	1.27±0.99	3.74±1.15	3.72±2.86	14.32±4.45[ab]
LT	0	2.66±0.24	3.09±0.83	8.08±2.82[a]	17.26±7.73[abc]
PAS-H-L					
UT	0	2.52±1.00	4.54±1.89	5.53±1.26[a]	32.93±3.86[abcd]
LT	0	4.14±1.40	2.71±0.87	6.68±2.12[a]	24.55±5.33[abcd]
AB-PAS-G-S					
UT	0.76±0.23	11.27±3.86[a]	6.24±2.62	7.70±2.87[a]	0.21±0.21[b]
LT	0.09±0.09	7.94±1.62[a]	7.96±3.35[a]	1.7±1.08	0[bc]
AB-PAS-G-L					
UT	0.12±0.12	4.41±1.93[a]	4.60±1.27[a]	0[bc]	0.83±0.83
LT	0.09±0.09	3.95±1.14	2.60±0.14	0.93±0.81	0
AB-G-S					
UT	0	0.11±0.10	0	0.69±0.68	0
LT	0	0	0.12±0.06[abd]	0	0[c]
AB-G-L					
UT	0	0.19±0.19	0	0	0
LT	0	0.29±0.14	0.25±0.12	0	0

Adult, male, Syrian golden hamsters were treated with CCl_4 (2.5 ml/kg). Control animals were given vehicle only. Animals were killed 1, 4, 12, or 24 hrs later. Tracheal tissues were removed and subdivided into upper (UT) and lower (LT) levels and prepared for cytochemical determination of glycoprotein-containing cells as described in Methods. Three animals were used in each group.

*Number of glycoprotein-containing cells in 1 mm length of tracheal epithelium. Values are mean ± standard error.

a=Significantly different from that of the control group ($p<0.05$).

b=Significantly different from that of the group killed 1 hr after CCl_4 ($p<0.05$).

c=Significantly different from that of the group killed 4 hrs after CCl_4 ($p<0.05$).

d=Significantly different from that of the group killed 12 hrs after CCl_4 ($p<0.05$).

Table 2: Number of glycoprotein-containing cells (GP) of various types in levels of lateral regions of trachea of hamsters treated with CCl_4 for various times.*

		CCl_4 treatment			
GP	Control	1 hr	4 hr	12 hr	24 hr
PAS-G-S					
UT	18.81±4.00	1.02±0.35^{acd}	7.46±0.54^{b}	21.20±1.99^{b}	2.25±0.69^{abcd}
LT	7.15±0.81	1.15±0.88^{a}	1.22±0.43^{ad}	7.79±1.86^{bc}	1.86±0.58^{ad}
PAS-G-L					
UL	10.71±4.22	1.85±0.59^{acd}	9.15±2.03^{b}	21.31±2.42^{b}	0.78±0.35^{acd}
LT	4.06±1.25	1.73±0.88	1.25±0.03	4.87±1.76	0.30±0.11^{abcd}
PAS-H-S					
UT	0	4.06±1.68^{c}	0.61±0.30^{bd}	4.90±1.32^{c}	2.26±0.68^{c}
LT	0	3.32±1.25	6.39±2.74^{a}	12.96±4.08^{a}	17.69±4.64^{ab}
PAS-H-L					
UT	0	7.04±2.38^{ac}	1.47±0.59^{b}	2.33±0.53	0.85±0.29^{b}
LT	0	7.49±4.66	5.15±2.89	11.48±4.49^{a}	26.81±8.05^{a}
AB-PAS-G-S					
UT	0.56±0.45	11.66±2.14^{ad}	13.98±1.26^{ad}	1.91±0.52^{ac}	0.12±0.12^{bc}
LT	0.04±0.03	18.19±3.06^{ad}	16.77±0.35^{ad}	1.28±0.09^{bc}	0^{bc}
AB-PAS-G-L					
UT	0.28±0.14	1.65±1.05	6.64±2.14	1.46±0.69	0.09±0.09
LT	0.03±0.03	2.77±0.28^{a}	3.72±1.14^{a}	0.53±0.35	0^{bc}
AB-G-S					
UT	0	0.07±0.03	0.36±0.08	0.05±0.52	0
LT	0	0	0.25±0.15	0.12±0.12	0
AB-G-L					
UT	0	0	0.17±0.11	0	0
LT	0	0	0.04±0.02	0	0

Adult, male, Syrian golden hamsters were treated with CCl_4 (2.5 ml/kg). Control animals were given vehicle only. Animals were killed 1, 4, 12, or 24 hrs later. Tracheal tissues were removed and subdivided into upper (UT) and lower (LT) levels and prepared for cytochemical determination of glycoprotein-containing cells as described in Methods. Three animals were used in each group.
*Number of glycoprotein-containing cells in 1 mm length of tracheal epithelium. Values are mean ± standard error.
a=Significantly different from that of the control group ($p<0.05$).
b=Significantly different from that of the group killed 1 hrs after CCl_4 ($p<0.05$).
c=Significantly different from that of the group killed 4 hrs after CCl_4 ($p<0.05$).
d=Significantly different from that of the group killed 12 hrs after CCl_4 ($p<0.05$).

Table 3: Number of glycoprotein-containing cells (GP) of various types in levels of ventral regions of trachea of hamsters treated with CCl_4 for various times.*

		CCl_4 treatment			
GP	Control	1 hr	4 hr	12 hr	24 hr
PAS-G-S					
UT	7.56±2.88	2.10±1.11[d]	7.66±1.45	20.17±3.30[b]	3.54±1.01
LT	19.46±4.50	0.43±0.17[acd]	2.22±0.35[abd]	10.60±2.77[bc]	0.72±0.37[acd]
PAS-G-L					
UL	5.96±1.95	3.33±1.44	6.26±1.67[d]	15.40±2.45[ac]	1.04±0.20[acd]
LT	7.95±0.75	2.00±0.28[a]	1.33±0.16[a]	3.52±1.93	0.62±0.13[abd]
PAS-H-S					
UT	0	3.07±1.77	0.25±0.02[abd]	3.56±1.62[c]	27.07±1.49[abcd]
LT	0	2.28±0.57	1.80±1.40	7.51±2.79	12.94±6.92[a]
PAS-H-L					
UT	0	5.16±2.82[ac]	0	4.57±2.14[ac]	6.32±1.44[ac]
LT	0	2.74±0.84	2.99±2.78	2.58±0.59	17.34±11.67
AB-PAS-G-S					
UT	0.06±0.06	12.22±6.65[ad]	19.62±2.07[ad]	1.35±0.32[bc]	1.06±0.29[bc]
LT	0.01±0.1	14.76±2.18[ad]	25.03±2.33[ad]	1.66±1.45[c]	0[bc]
AB-PAS-G-L					
UT	0	3.56±2.04	5.74±1.36	0	0
LT	0	2.81±1.00	5.24±1.80[ad]	1.17±1.16	0[c]
AB-G-S					
UT	0	0.08±0.48	0.46±0.45	0.38±0.38	0
LT	0	0	0.19±0.19	0.53±0.52	0
AB-G-L					
UT	0	0	0.06±0.06	0	0
LT	0	0	0	0.12±0.11	0

Adult, male, Syrian golden hamsters were treated with CCl_4 (2.5 ml/kg). Control animals were given vehicle only. Animals were killed 1, 4, 12, or 24 hrs later. Tracheal tissues were removed and subdivided into upper (UT) and lower (LT) levels and prepared for cytochemical determination of glycoprotein-containing cells as described in Methods. Three animals were used in each group.

*Number of glycoprotein-containing cells in 1 mm length of tracheal epithelium. Values are mean + standard error.

a=Significantly different from that of the control group (p 0.05).

b=Significantly different from that of the group killed 1 hrs after CCl_4 (p 0.05).

c=Significantly different from that of the group killed 4 hrs after CCl_4 (p 0.05).

d=Significantly different from that of the group killed 12 hrs after CCl_4 (p 0.05).

DISCUSSION

The cytochemical results presented demonstrate that most cells in control hamster contained neutral glycoproteins (PAS-positive). A few contained acidic glycoproteins (AB-PAS). This indicates that hamster tracheal epithelial cells have the potential to synthesize both acidic and neutral glycoproteins. This was consistent with observations of Becci et al. (1978b), Goldman and Baseman (1980) and Kim et al. (1985).

The frequency distribution of glycoprotein cells in various regions and levels of hamster trachea has not been described previously. This cytochemical study revealed that the number of glycoprotein-containing cells differed in various regions and levels of the trachea. For example, small PAS-positive cells were more predominant in lateral region than in ventral or dorsal region of upper trachea. However, these cells were more numerous in ventral region of lower trachea. Jones and Reid (1978) reported that the population of glycoprotein-containing cells in rat trachea varied in different levels of trachea. They found that PAS-positive cells were more predominant in lower than uppper trachea. Robinson et al. (1986) observed that the majority of glycoprotein-containing cells in ferret trachea contained both neutral and acidic glycoproteins (AB-PAS). These authors reported that the population of the glycoprotein cells were fairly evenly distributed around the anterior and lateral regions, but there were fewer in the posterior region. The biological role of glycoprotein-containing cells in airway epithelium is not fully known. However, the

the frequency distribution of these cells in different regions or levels of trachea might be related to the important function of these cells, such as defense barriers against microorganisms, protection aganist dehydration, as well as trapping airborne particles.

Abundant smooth endoplasmic reticulum (SER) were observed in nonciliated secretory cells. This finding raised the possibility that secretory cells of hamster trachea may have the potential to bioactivate CCl_4. Recently, Plopper et al. (1983) reported that hamster tracheal epithelium contained Clara cells. These cells were structurally similar to Clara cells in intrapulmonary airways and contained large amounts of SER, with electron dense secretory granules in the apical cytoplasm. In the present study nonciliated cells containing electron lucent secretory granules were observed in the apical portion of cytoplasm. These findings confirmed those of others (Becci et al., 1978b; Kennedy et al., 1978) who have reported the presence of electron lucent secretory granules.

CCl_4 caused alterations in glycoprotein-containing cells of various regions and levels of trachea. Glycoprotein cells containing PAS-positive, homogenous material were observed in CCl_4 treated hamsters, but not in controls. This suggests that CCl_4 produced injury to membrane-bound secretory granules, resulting in their disruption and subsequent homogeneous staining rather than staining as discrete granules. This hypothesis is supported by the finding that the number of glycoprotein-containing cells with PAS positive granules decreased. By contrast, the population of glycoprotein cells with PAS-positive, homogenous material, increased 24 hrs after CCl_4 treatment. Alternatively, the homogenous material may have arisen as a result of

the production of abnormal glycoprotein or from abnormal proportions of normal glycoprotein that failed to form secretory granules.

The cytochemical study demonstrated that the number of cells containing both neutral and acidic glycoproteins were rarely seen in trachea of control hamster trachea. However, the number of these cells markedly increased 1 or 4 hrs CCl_4 posttreatment. These findings suggest that the infrequent distribution of acidic glycoprotein containing cells in controls may reveal a balance between the rate of biosynthesis and discharge of the secretory product. The increased number of these cells after CCl_4 may be due to an altered balance between the rates of synthesis and discharge of glycoproteins.

Becci et al. (1978a) observed hypertrophy of glycoprotein-containing cells in trachea of hamster 24 hrs after administration of a mixture of benzo(a)pyrene-ferric oxide (BP-Fe_2O_3). These authors reported that the glycoproteins became more acidic in treated hamsters when compared to that of unexposed animals. These effects were attributed to the benzo(a)pyrene and not to the Fe_2O_3 particles. Since BP requires metabolic activation by the microsomal system, it seemed likely that glycoprotein containing cells of trachea can be altered by chemicals that are bioactivated by the mixed function oxidase system. My findings support this hypothesis.

Many studies have shown that acute or chronic inhalation of chemical irritants caused hypertrophy and hyperplasia of glycoprotein-containing cells in the airways. However, the mechanism by which chemical irritants act to alter the secretion of glycoprotein in the airway has not been investigated. It is well documented that the toxicity of CCl_4 is related to its metabolism by a microsomal enzyme system (Recknagel, 1967; Recknagel and Glende, 1973). A large body of

evidence in liver cells suggests that CCl_4 caused destruction of microsomal enzyme systems, depression of protein synthesis, impaired ATP biosynthesis and alteration of intracellular calcium (Smuckler et al., 1964; Recknagel, 1967; Recknagel and Glende, 1973; Lowery et al., 1981; Brattin, 1985; Comporti, 1985).

CCl_4-produced injury in nonciliated secretory cells of hamster trachea. Intracellular organelles including smooth and rough endoplasmic reticulum, mitochondria and Golgi apparatuses were damaged by this chemical. Thus, alterations of glycoprotein-containing cells in hamster trachea after CCl_4 might be the consequence of the effect of this toxic agent on subcellular organelles that are involved in the biosynthesis of glycoproteins.

In summary, secretory cells of hamster trachea are columnar and contain electron-lucent secretory granules. Intracellular organelles included abundant smooth endoplasmic reticulum in these cells. Cytochemically, I observed both neutral and acidic glycoprotein granules in nonciliated secretory cells. CCl_4 caused alterations in glycoprotein-containing cells. This chemical also produced injury in secretory cells. Since CCl_4 induced injury is dependent on metabolism of this agent by intracellular NADPH-dependent cytochrome P-450 monooxygenases, this study suggests that glycoprotein containing cells in hamster trachea have the potential to bioactivate CCl_4.

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CHAPTER IV

EFFECT OF FASTING OR DIETHYLMALEATE ON CCl_4-INDUCED INJURY IN TRACHEAL EPITHELIAL CELLS

ABSTRACT

Fasting or diethylmaleate (DEM) was found by others to potentiate toxicity of carbon tetrachloride (CCl_4) in rat liver. This study was designed to examine the effect of fasting or DEM on CCl_4-induced injury in trachea. Adult, male, Syrian golden hamsters were treated with 0.6 ml/kg DEM for 30 minutes or were fasted for 24 hrs prior to the administration of 1.0 ml/kg CCl_4 or peanut oil (vehicle) only. For comparison, another series of hamsters had access to food and were given CCl_4 or vehicle. All animals were killed 24 hrs after administration of CCl_4 or peanut oil. Tracheal tissues were removed, fixed and embedded for light and electron microscopy.

CCl_4 produced injury in both secretory cells and ciliated cells. In peanut oil treated fasted hamsters, injured secretory cells and ciliated cells were observed infrequently. Fasting caused ultrastructural alterations in nonciliated secretory cells and ciliated cells. Fasting potentiated cytotoxicity of CCl_4 in hamster trachea. DEM produced injury in both nonciliated secretory cells and ciliated cells. No potentiation of CCl_4 injury in DEM treated animals was

noted. This study suggests that nonciliated secretory cells, as well as ciliated cells, have the capacity to bioactivate CCl_4. Depletion of glutathione and/or induction of the microsomal enzyme by fasting may have potentiated CCl_4-induced injury in trachea. DEM itself produced marked injury to hamster trachea, rendering its effects on CCl_4 toxicity difficult to ascertain from these studies.

INTRODUCTION

It is generally agreed that carbon tetrachloride (CCl_4) is metabolized by the cytochrome P-450 locus in smooth endoplasmic reticulum of liver cells to the trichloromethyl free radical ($\dot{C}Cl_3$) which then reacts with tissue macromolecules, leading to cell injury (Recknagel and Glende, 1973). Substances such as glutathione markedly inhibit the covalent binding of CCl_4 to liver microsomes (Corsini et al., 1972), and protect liver from toxic effect of CCl_4 (Gravela and Dianzani, 1970). Thus, it appears that reduced glutathione (GSH) plays an important role in cellular defense against toxic electrophile(s) generated by the bioactivation of CCl_4. Depletion of GSH by diethylmaleate, DEM (Boyland and Chasseaud, 1970, Lindstrom et. al., 1978) or fasting (Maruyama et al., 1968; Harris and Anders, 1980) potentiated hepatotoxicity of CCl_4 (Krishnan and Stenger, 1966; Diaz Gomez et al., 1975, Harris and Anders, 1980).

Previous studies demonstrated that depletion of GSH in lungs of rat by DEM (Richardson and Murphy, 1975) enhanced covalent binding and toxicity of 4-ipomeanol in the target tissue (Boyd and Burka, 1978). Since pneumotoxicity of 4-ipomeanol is produced by its electrophilic

metabolite(s), it seemed likely that GSH protects the lung against electrophilic toxic metabolite(s) of 4-ipomeanol.

Recently we observed CCl_4-induced injury in tracheal epithelial cells of hamsters. Therefore, we suggested that hamster tracheal cells possess microsomal enzyme systems capable of bioactivaing CCl_4 (Ahmadizadeh and Echt, 1985; Ahmadizadeh et al., 1987). It seemed likely that fasting or pretreatment with DEM might also potentiate CCl_4-induced injury in trachea. To test this hypothesis, I examined the effect of DEM or fasting on injury in hamster trachea caused by CCl_4.

MATERIALS AND METHODS

Adult, male Syrian golden hamsters (100-140g) were obtained from Harlan (Indianapolis, Ind.) and housed in groups of three in clear polypropylene cages in a light cycle (12 hr light and 12 hr dark) and temperature controlled room. Diethylmaleate (DEM) and carbon tertachloride (CCl_4) were obtained from Sigma Chemical Company (St.Louis, MO). In one series of experiments, hamsters were fed ad libitum with regular rodent diet (Wayne Lab Blox, Chicago, IL) and received 0.6 ml/kg undiluted DEM, ip, 30 minutes prior to administration of CCl_4 (Harris and Anders, 1980). In another set of experiments, hamsters were fasted by withholding food for 24 hrs; water was available ad libitum. Following pretreatments, hamters were given 1.0 ml/kg of CCl_4, ip (in peanut oil). Control animals received peanut oil alone (each group had a separate control). The fasted hamsters starved further after administration of CCl_4 or vehicle. All animals were killed 24 hrs later. Hamsters were sacrificed with 100-300 mg/kg of sodium pentobarbital (ip). Three hamsters were used for each

treatment.

Light microscopy. Tracheal tissues were fixed in 4% paraformaldehyde in phosphate buffer (pH 7.2). Each specimen was divided into upper and lower levels and embedded in glycol methacrylate (GMA, Polyscience, Inc.). Tracheas were sectioned transversely at 3 um from proximal to distal ends with glass knives on a JB-4 microtome. Three histological sections, each at least 15 um apart, were taken from each tissue block and stained with toluidine blue. Adjacent sections were used for localization of intracellular glycoprotein and stained with Alcian blue/Periodic acid-Schiff (AB-PAS) at pH 2.6 (Jones et al.,1973).

The luminal epithelium was subdivided into dorsal, right and left lateral, and ventral regions. The total number of damaged or glycoprotein-containing cells/mm/region was counted at 400X magnification and compared statistically. The criteria for a damaged cell included obvious cellular swelling, loss of staining capacity and nuclear dilation. Only those cells with distinct nuclei were counted. Date were expressed as mean $\pm$ standard error. The results were analyzed by analysis of variance, completely randomized design, and treatment differences were identified by the method of Newman-Keuls (Steel and Torrie, 1960). $P < 0.05$ was used as the criterion for significance. Since the data were nonsymmetrical and the sample size at each time period was small (n=3 animals) a standard $\log_{10}$ transformation was used to compensate for the nonsymmetry while preserving the relative pattern among comparison groups. The transformed data in each morphological level and region of trachea were subjected to a one-way analysis of variance.

Transmission electron microscopy. One or two rings of trachea from each animal were cut into pieces approximately 1 mm^2, and 1-2 blocks from each region (dorsal, lateral or ventral) were prepared for electron microscopy. Tissues were fixed in formaldehyde-glutaraldehyde cacodylate buffer (Karnovsky, 1965). The tissues were washed in 0.2 M cacodylate buffer, post-fixed in 2% osmium tetroxide, dehydrated with ethanol and embedded in epon-araldite (Mollenhauer, 1964). Sections 1 um thick were cut from selected areas and stained with toluidine blue for examination by light microscopy. Thin sections (approximately 90 nm) were cut from selected areas. The sections were stained with uranyl acetate and lead citrate and examined with a Philips 201 transmission electron microscope.

RESULTS

A. Histopathology evaluation. Hamster tracheal epithelia were pseudostratified. This was true whether animals were fed or fasted for 24 hrs (Figs. 1-2). However, diethylmaleate (DEM) caused an alteration in morphology of tracheal epithelium. An occasional transition from pseudostratified to cuboidal- appearing epithelium was noted, especially in the lateral or ventral regions of upper and lower trachea (Fig. 3). Pretreatment with DEM and subsequent administration of CCl_4 resulted in effects on hamster tracheal epithelium that were indistinguishable from those in DEM treated groups (Fig. 4).

Damaged cells in peanut oil treated animals. There were no damaged cells in peanut oil treated animals fed ad lib (Fig. 1). However,

injured nonciliated and ciliated cells were observed infrequently in peanut oil treated, fasted hamsters (Fig. 2). In peanut oil treated hamsters pretreated with DEM, injury to nonciliated cells and ciliated cells was observed frequently (Fig. 3).

Damaged cells in CCl_4-treated animals. CCl_4 effects in animals fed ad lib: In animals fed ad lib, CCl_4 produced injury in both nonciliated cells and ciliated cells (Fig. 5). However, the number of damaged cells was significantly increased in the dorsal region of upper trachea (UT) compared to that in peanut oil treated animals fed ad lib (Po-fed) animals. Numbers of damaged cells in other levels and regions of trachea remained minimal; no significant differences from Po-fed animals were evident (Table 1).

CCl_4 effects in fasted animals: In this group, injury to nonciliated cells and ciliated cells was observed (Fig. 6). However, in CCl_4-treated, fasted hamsters, the number of damaged cells was markedly increased in dorsal and lateral regions of lower trachea (LT) and ventral regions of UT and LT, so that significant differences from CCl_4-treated hamsters fed ad lib were evident (Table 1).

CCl_4 effects in DEM treated animals: In this group, CCl_4 produced injury to nonciliated cells and ciliated cells (Fig. 7). However, CCl_4 did not worsen the injury in DEM pretreated animals (Table 1).

Transmission electron microscopy. In peanut oil treated hamsters fed ad lib, tall columnar nonciliated cells were observed that contained abundant smooth endoplasmic reticulum, numerous mitochondria, well developed rough endoplasmic reticulum (RER) and prominent Golgi complexes. In addition, membrane bound, electron lucent secretory

granules were observed in apical cytoplasm or throughout the cytoplasm (Fig. 8). Ciliated cells were columnar, and numerous mitochondria, RER, and Golgi complexes were seen in electron lucent cytoplasm.

Ultrastrucural alterations in nonciliated secretory cells and ciliated cells were noted in CCl_4-treated hamsters fed ad lib (Fig. 9). Damage to intracelluar organelles of nonciliated cells was characterized by dilation of SER, ribosomal disaggregation, swelling of mitochondria and numerous cytoplasmic vacuoles. The ultrastructural features of ciliated cells injured by CCl_4 were dilated RER, swollen mitochondria, disaggregated ribosomes and numerous cytoplasmic vacuoles.

Fasting produced ultrastructural alterations in hamster trachea (Fig. 10). Both secretory cells and ciliated cells were damaged by fasting. The mitochondria were considerably enlarged, RER was disorganized and numerous vacuoles were observed in cytoplasms of both secretory cells and ciliated cells. These changes were also noted in CCl_4-treated fasted hamsters (Fig. 11).

DEM produced injury in secretory cells and ciliated cells (Figs. 12-13). Intracellular organelles including smooth and rough endoplasmic reticulum, mitochondria, electron lucent secretory granules were damaged by this chemical. Similar ultrastructural alterations were noted in CCl_4 treated hamsters pretreated with DEM (Fig. 14). However, similar to the light microscopy, ultrastructural alterations varied in different regions of trachea.

B. Evaluation of intracellular glycoproteins. Within the glycoprotein-containing cells, the presence of granules of acid and

neutral glycoproteins were identified by the Alcian blue/ Periodic acid Schiff (AB-PAS) stain at pH 2.6. Granules of acidic glycoproteins are AB positive, and those of neutral glycoproteins are PAS positive (Jones et al., 1973). Based on the amount of intracellular granules, the glycoprotein-containing cells were described as small (G-S) or large (G-L). A small, glycoprotein-containing cell was one containing granules at its apex, and a large cell was one containing granules throughout the cytoplasm (Figs.15-17). The glycoprotein cells containing homogenous, PAS-positive material (PAS-H) were described as small or large. A small cell (PAS-H) was one containing homogenous PAS-positive material only its apex, and a large cell (PAS-H-L) was one containing homogenous, PAS-positive material throughout its cytoplasm (Fig. 18).

Glycoprotein cells in peanut oil treated animals:

a) Peanut oil-treated, animals fed ad lib. In this group, most glycoprotein-containing cells had PAS-positive granules (Tables 2-4). The small, PAS-positive cells (PAS-G-S) were most predominant in lateral region of upper trachea (UT) and ventral region of lower trachea (LT). The large, PAS-positive cells (PAS-G-L) were more numerous in the lateral region of UT. The glycoprotein cells that contained granules positive for both PAS and AB (AB-PAS) were found infrequently. No cells were apparent with only AB positive granules. Glycoprotein-containing cells with homogenous, PAS-positive material in the cytoplasms were not observed.

b) Peanut oil treated, fasted animals: Fasting had little effect on the distribution of cell types in peanut oil-treated hamsters. However, glycoprotein-containing cells with homogenous, PAS-positive material were observed in these animals. These cells were most pronounced in lateral regions of UT so that a significant difference from Po-fed animals was evident.

c) Peanut oil treated animals pretreated with DEM: In this group, the number of glycoprotein-containing cells with small PAS-positive granules (PAS-G-S) decreased in ventral region of LT, and the number of large, PAS-positive granules (PAS-G-L) decreased in the dorsal region of UT. Unlike controls, cells with glycoprotein distributed homogeneously in the cytoplasm were evident. Large, homogenous, PAS-positive cells (PAS-H-L) increased in number the lateral region of upper trachea when compared to those in Po-fed hamsters.

Glycoprotein cells in CCl_4 treated animals:

a) CCl_4 treated, animals fed ad lib: Number of glycoprotein cells containing PAS-positive granules markedly decreased in most regions of both levels of trachea. Glycoprotein-containing cells with homogenous, PAS-positive material were observed after CCl_4-treatment; and numbers of these cells increased markedly in most regions of upper and lower trachea (Tables 2-4).

b) CCl_4 treated, animals fed ad lib: Number of glycoprotein cells significant alterations of glycoprotein cells noted when compared to those in CCl4-treated animals fed ad lib (Tables 2-4).

c) CCl_4 treated animals pretreated with DEM: In this group, glycoprotein-containing cells with PAS-positive granules increased in the dorsal region of upper trachea, so that a significant difference from CCl_4 treated hamsters fed ad lib was evident (Table 2). However, there were no significant alterations of other types of glycoprotein-containing cells when compared to those in CCl_4-treated hamster fed ad lib (Tables 2-4).

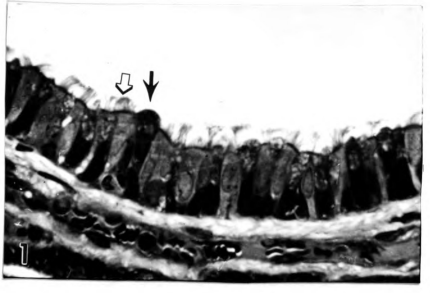

Fig. 1: Light micrograph of ventral region of upper trachea of peanut oil treated hamster fed ad lib. Nonciliated cells (solid arrow) and ciliated cells (open arrow) are intact. Note pseudostratified appearance of tracheal epithelium. X 400.

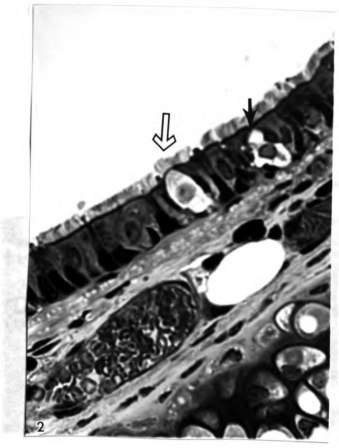

Fig. 2: Light micrograph of lateral region of upper trachea of peanut oil treated, fasted hamster. Note pseudostratified appearance of tracheal epithelium and injury (loss of staining capacity and dilatation of cytoplasm and nucleus) to nonciliated cell (solid arrow) and ciliated cell (open arrow). X 400

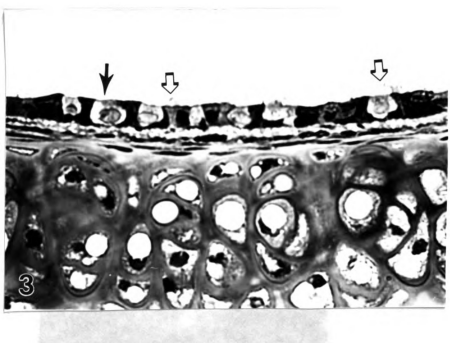

Fig. 3: Light micrograph of ventral region of upper trachea of peanut oil treated hamster pretreated with DEM. Transition of pseduostratified tracheal epithelium to cuboidal type with loss of cilia is apparent. Note injury (loss of staining capacity and dilatation of cytoplasm and nucleus) to nonciliated cells (solid arrow) and ciliated cells (open arrow). X 400.

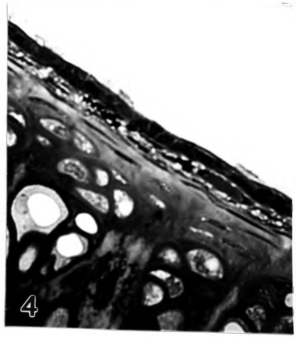

Fig. 4: Light micrograph of ventral region of upper trachea of CCl_4 treated hamster pretreated with DEM. Transition of pseudostratified tracheal epithelium to cuboidal type with loss of cilia is apparent. X 400.

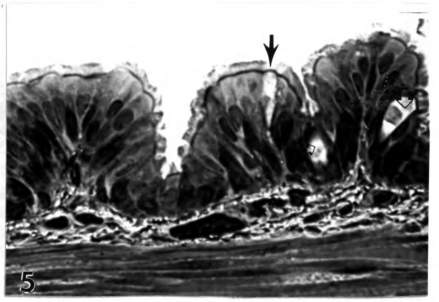

Fig. 5: Light micrograph of dorsal region of upper trachea of CCl_4 treated hamster fed ad lib. Note injury (loss of staining capacity and dilatation of cytoplasm and nucleus) to nonciliated (solid arrow) and ciliated cells (open arrow). X 400.

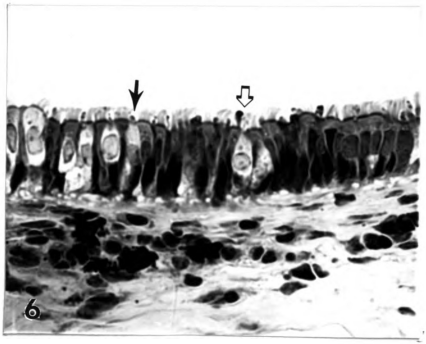

Fig. 6: Light micrograph of lateral region of lower trachea of CCl_4 treated fasted hamster. Note injury (loss of staining capacity and dilatation of cytoplasm and nucleus) to nonciliated cells (solid arrow) and ciliated cells (open arrow). X 400.

Fig. 7: Light micrograph of dorsal region of lower trachea of CCl_4 treated hamster pretreated with DEM. Note injury (loss of staining capacity and dilatation of cytoplasm and nucleus) to nonciliated cells (solid arrow) and ciliated cells (open arrow). X 400.

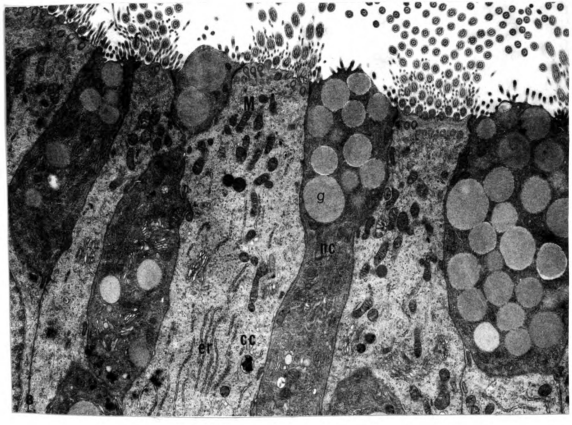

Fig. 8: Transmission electron micrograph of ventral region of trachea of peanut oil treated hamster fed ad lib. Note electron lucent, membrane bound secretory granules (g) in apical cytoplasm or throughout the cytoplasm of nonciliated cells (nc). Ciliated cells (CC) contained numerous mitochondria (M) and rough endoplasmic reticulum (er). X 7840.

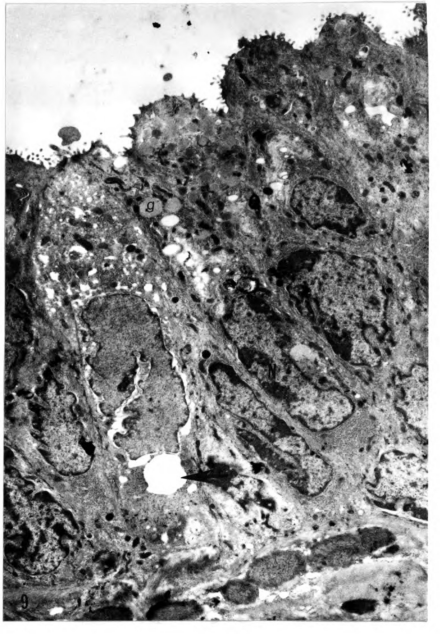

Fig. 9: Transmission electron micrograph of lateral region of CCl_4 treated hamster fed ad lib. Note numerous vacuoles (arrows) in nonciliated cells. Nucleus (N), secretory granules (g). X 5589.

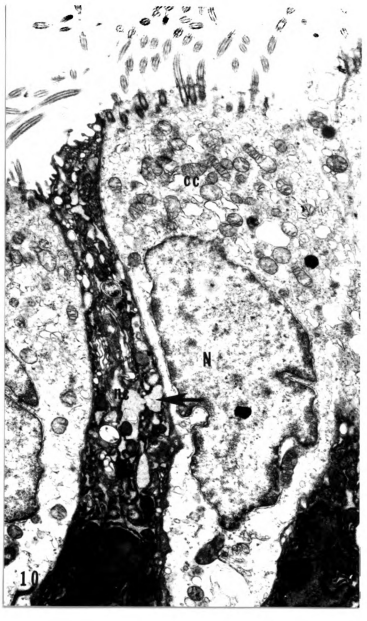

Fig. 10: Transmission electron micrograph of lateral region of peanut oil treated, fasted hamster. Injury (dilatation of intracellular organelles including mitochondria, rough endoplasmic reticulum, and Golgi apparatuses) to both nonciliated cell (nc) and ciliated cells (cc) is apparent. Note numerous vacuoles (arrows) in these cells. Nucleus (N). X 8624.

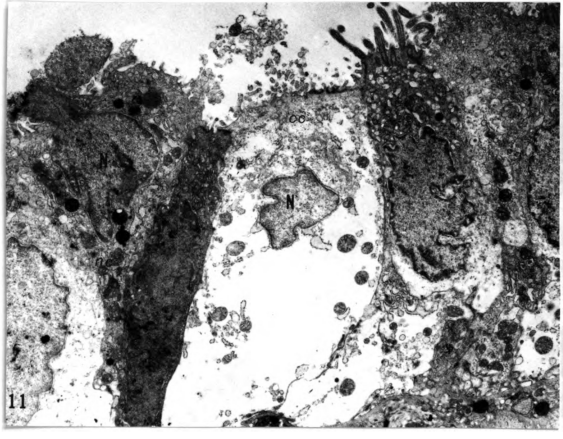

Fig. 11: Transmission electron micrograph of lateral region of trachea of CCl_4 treated fasted hamster. Note injury (dilatation of intracellular organelles including mitochondria and rough endoplasmic reticulum) to nonciliated cells (nc) and ciliated (cc) cells. Nucleus (N). X 6664.

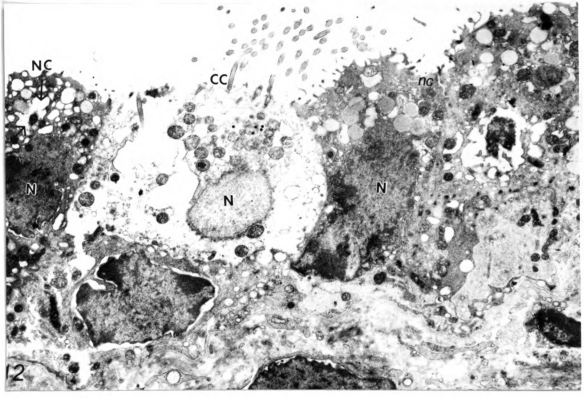

Fig. 12: Transmission electron micrograph of lateral region of trachea of peanut oil treated hamster treated with DEM. Note injury (dilatation of intracellular organelles including mitochondria, and rough endoplasmic reticulum) to nonciliated cells (nc) and ciliated cells (cc). Cells are also less columnar than normal. Nucleus (N). X 6468.

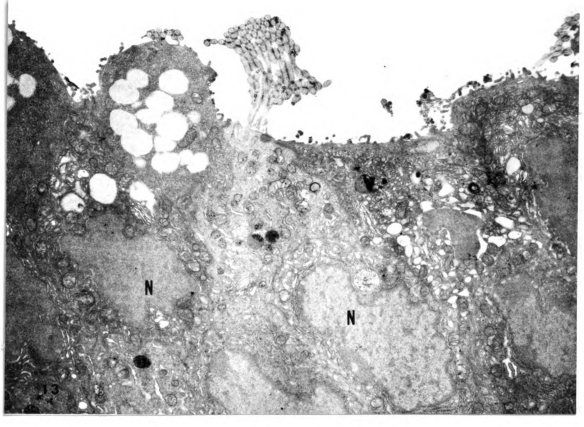

Fig. 13: Transmission electron micrograph of lateral region of trachea of peanut oil treated hamster treated with DEM. Note injury (dilatation of intracellular organelles including mitochondria, rough endoplasmic reticulum, Golgi apparatuses and loss of cilia) to ciliated cell. Nucleus (N). X 8232.

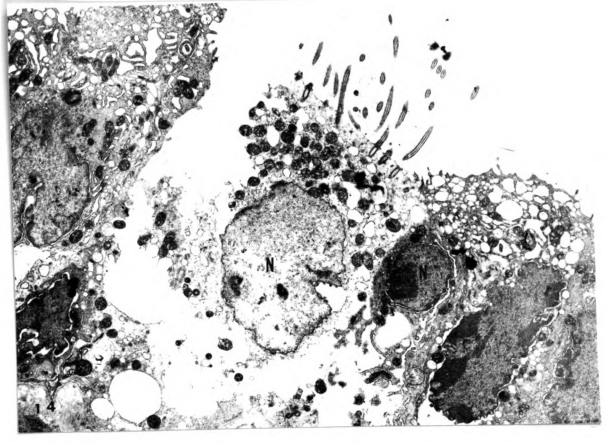

Fig. 14: Transmission electron micrograph of lateral region of trachea of CCl_4 treated hamster treated with DEM. Note injury (dilatation of intracellular organelles including mitochondria, rough endoplasmic reticulum and numerous vacuoles) to both ciliated and nonciliated cells. Nucleus (N). X 6468.

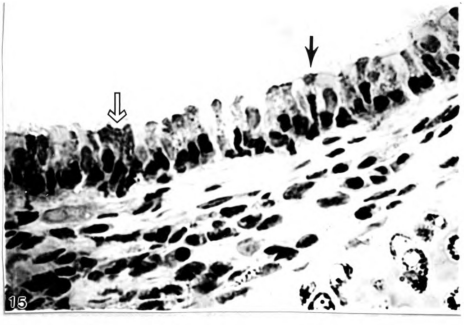

Fig. 15: Light micrograph of lateral region of upper trachea of peanut oil-treated, fasted hamster. Note cells containing PAS positive granules in the apical (solid arrow) cytoplasm or throughout the cytoplasm (open arrow). X 400.

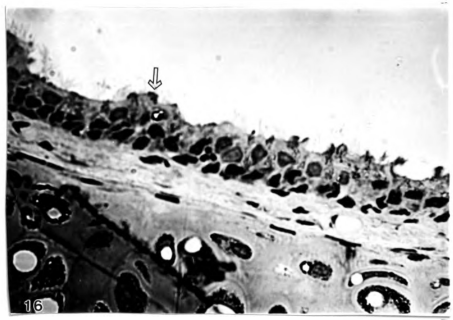

Fig. 16: Light micrograph of lateral region of upper trachea of a peanut oil treated hamster treated with DEM. Note cells containing AB-PAS positive granules in the apical portion of cytoplasm (arrow). X 400.

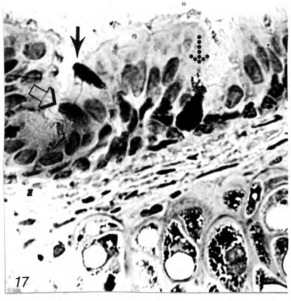

Fig. 17: Light micrograph of dorsal region of lower trachea of a peanut oil-treated, fasted hamster. Note cells containing AB-positive granules in apical cytoplasm (open arrow) or throughout the cytoplasm (broken arrow), and the PAS-AB positive granules throughout cytoplasm (solid arrow). X 400.

Fig. 18: Light micrograph of lateral region of upper trachea of CCl_4 treated hamster pretreated with DEM. Note cells containing PAS-positive material distributed homogeneously in the apical cytoplasm (solid arrow) or throughout the cytoplasm (open arrow). X 400.

Table 1: Effect of diethylmaleate (DEM) or fasting on CCl_4-induced injury in tracheal epithelial cells.*

	Po-fed	CCl_4-fed	Po-fasted	CCl_4-fasted	Po-DEM	CCl_4-DEM
Dorsal						
UT	0±0	11.08±3.88[ac]	2.59±1.30[bd]	16.49±3.98[ac]	38.55±5.31[ac]	25.72±11.58[ac]
LT	0±0	0.58±0.36	0±0	18.81±3.82[abc]	24.61±11.61[abc]	30.63±3.54[abc]
Lateral						
UT	0±0	2.25±1.29	0.19±0.18	8.66±3.71	19.13±6.87[ac]	5.48±2.91
LT	0±0	0.15±0.14	0	9.15±2.87[abc]	10.79±5.57[abc]	11.06±2.16[abc]
Ventral						
UT	0±0	0	0.16±0.16	12.52±3.12[abc]	19.83±3.09[abc]	4.56±1.84[abcde]
LT	0±0	0.20±0.19	0±0	4.26±0.49[abc]	9.25±3.94[abc]	7.75±3.31[abc]

Adult, male, Syrian golden hamsters were fed ad lib or fasted for 24 hrs or were pretreated with 0.5 ml/kg DEM for 30 min prior to administration of 1.0 ml/kg CCl_4. All hamsters were given CCl_4 or peanut oil vehicle. The fasted hamsters starved further after administration of CCl_4 or vehicle. Animals were killed 24 hrs later. Tracheal tissues were removed and subdivided into upper (UT) or lower (LT) levels and prepared for histological determination of numbers of damaged cells as described in Methods. Three hamsters were used in each group.

*Number of damaged cells in 1 mm length of tracheal epithelium. Values are mean ± standard error of the mean. Peanut oil treated groups are used for comparison.

a=Value is significantly different from that of the peanut oil treated fed (Po-fed) group ($P < 0.05$).

b=Value is significantly different from CCl_4-treated fed (CCl_4-fed) group ($P < 0.05$).

c=Value is significantly different from peanut oil treated fasted (Po-fasted) group ($P < 0.05$).

d=Value is significantly different from CCl_4 treated fasted (CCl_4-fasted) group ($P < 0.05$).

e=Value is significantly different from peanut oil treated DEM (Po-DEM) group ($P < 0.05$).

Table 2: Effect of diethylmaleate (DEM) or fasting on CCl_4-caused alterations in glycoprotein-containing cells (GP) of various levels of dorsal regions of tracheal epithelia.*

GP	Po-fed	CCl_4-fed	Po-fasted	CCl_4-fasted	Po-DEM	CCl_4-DEM
PAS-G-S						
UT	9.11±1.62	0.27±0.13^{ace}	13.08±5.45^{bde}	0^{ace}	4.07±1.52^{bd}	2.25±0.25^{bcd}
LT	4.02±0.68	0^{a}	9.49±2.45^{bd}	0.17±0.17	4.11±4.40	0.63±0.84^{c}
PAS-G-L						
UT	7.56±2.30	0^{a}	6.99±1.14^{bd}	0^{a}	1.62±0.75^{ac}	4.19±1.74
LT	2.48±0.32	0	7.93±4.19	0.40±0.18^{ac}	3.97±2.61^{d}	0.45±0.31^{c}
PAS-H-S						
UT	0	10.01±4.57^{a}	1.95±0.58	5.73±3.91	5.84±2.10	14.81±4.72^{a}
LT	0	8.08±2.83	2.92±1.10	4.51±1.37	2.62±1.16	9.80±5.01
PAS-H-L						
UT	0	8.67±4.01	2.16±1.27	10.07±8.61	6.99±0.58	19.65±5.13^{a}
LT	0	10.25±5.48^{a}	1.30±0.89	5.11±1.84	0.84±0.50^{bd}	9.39±4.54^{ae}
AB-PAS-G-S						
UT	0.76±0.23	0	0.14±0.13	0.6±0.59	0.90±0.81	4.11±2.40
LT	0.09±0.09	0	1.45±1.19	0.47±0.16	1.28±0.67	2.69±1.58
AB-PAS-G-L						
UT	0.12±0.12	0.08±0.08	0.04±0.03	1.00±0.99	0.22±0.22	0
LT	0.09±0.09	0.78±0.43	0.46±0.40	0.33±0.17	0	0
AB-G-S						
UT	0	0	0	0	0	0
LT	0	0	0.22±0.11	0	0	0
AB-G-L						
UT	0	0.08±0.09	0.04±0.35	0.07±0.06	0	0
LT	0	0.08±0.09	0.03±0.35	0.07±0.06	0	0

Adult, male, Syrian golden hamsters were fed ad lib or fasted for 24 hrs or were pretreated with 0.6 ml/kg DEM for 30 min prior to administration of 1.0 ml/kg CCl_4. All hamsters were given CCl_4 or peanut oil vehicle. The fasted hamsters starved further after administration of CCl_4 or vehicle. Animals were killed 24 hrs later. Tracheal tissues were removed and subdivided into upper (UT) or lower (LT) levels and prepared for histological determination of numbers of damaged cells as described in Methods. Three hamsters were used in each group.

*Number of glycoprotein-containing cells in 1 mm length of tracheal epithelium. Values are mean ± standard error of the mean. Peanut oil treated groups are used for comparison.
a=Value is significantly different from that of the peanut oil treated fed (Po-fed) group ($P < 0.05$).
b=Value is significantly different from CCl_4-treated fed (CCl_4-fed) group ($P < 0.05$).
c=Value is significantly different from peanut oil treated fasted (Po-fasted) group ($P < 0.05$).
d=Value is significantly different from CCl_4 treated fasted (CCl_4-fasted) group ($P < 0.05$).
e=Value is significantly different from peanut oil treated DEM (Po-DEM) group ($P < 0.05$).

Table 3: Effect of diethylmaleate (DEM) or fasting on CCl_4-caused alterations in glycoprotein-containing cells (GP) of various levels of lateral regions of tracheal epithelia.*

GP	Po-fed	CCl_4-fed	Po-fasted	CCl_4-fasted	Po-DEM	CCl_4-DEM
PAS-G-S						
UT	18.81±4.00	1.19±0.67[ace]	16.32±5.27[b]	0.73±0.35[ac]	8.67±2.00[bd]	4.88±1.99
LT	7.15±0.81	0.26±0.13[a]	17.32±5.58[b]	2.01±1.47[c]	5.78±2.47[b]	0.74±0.16[ace]
PAS-G-L						
UT	10.71±4.22	0.51±0.18[a]	9.84±0.74[b]	0.13±0.09[ac]	5.68±2.66[bd]	2.14±1.43
LT	4.06±1.25	0.15±0.14	8.95±3.80	0.64±0.42	5.06±2.60	0.10±0.06[ace]
PAS-H-S						
UT	0	12.86±3.64[a]	5.92±2.70[a]	9.95±1.71[a]	5.18±2.08	16.37±2.82[a]
LT	0	24.19±3.49[ac]	2.00±1.99	7.18±2.12[a]	2.14±0.61[b]	3.64±0.62
PAS-H-L						
UT	0	10.53±1.36[a]	4.88±2.74	8.58±1.54[a]	3.41±0.88[a]	5.52±1.17[a]
LT	0	12.21±3.49	0.97±0.88[b]	4.36±1.32	1.35±0.83	1.34±0.58
AB-PAS-G-S						
UT	0.56±0.44	0.58±0.33	0.17±0.09	0.67±0.27	1.05±0.35	1.05±0.65
LT	0.03±0.03	0.58±0.32	0.44±0.29	0.98±0.97	1.77±0.82	1.57±1.19
AB-PAS-G-L						
UT	0.28±0.14	0.19±0.18	0.12±0.06	0	0.36±0.17	0.03±0.04
LT	0.04±0.03	0	0.15±0.09	0.21±0.17	0.13±0.09	0.11±0.10
AB-G-S						
UT	0	0	0	0	0.26±0.04	0
LT	0	0	0	0	0	0
AB-G-L						
UT	0	0	0	0	0	0
LT	0	0	0	0	0	0

Adult, male, Syrian golden hamsters were fed ad lib or fasted for 24 hrs or were pretreated with 0.6 ml/kg DEM for 30 min prior to administration of 1.0 ml/kg CCl_4. All hamsters were given CCl_4 or peanut oil vehicle. The fasted hamsters starved further after administration of CCl_4 or vehicle. Animals were killed 24 hrs later. Tracheal tissues were removed and subdivided into upper (UT) or lower (LT) levels and prepared for histological determination of numbers of damaged cells as described in Methods. Three hamsters were used in each group.

*Number of glycoprotein-containing cells in 1 mm length of tracheal epithelium. Values are mean ± standard error of the mean. Peanut oil treated groups are used for comparison.
a=Value is significantly different from that of the peanut oil treated fed (Po-fed) group ($P < 0.05$).
b=Value is significantly different from CCl_4-treated fed (CCl_4-fed) group ($P < 0.05$).
c=Value is significantly different from peanut oil treated fasted (Po-fasted) group ($P < 0.05$).
d=Value is significantly different from CCl_4 treated fasted (CCl_4-fasted) group ($P < 0.05$).
e=Value is significantly different from peanut oil treated DEM (Po-DEM) group ($P < 0.05$).

Table 4: Effect of diethylmaleate (DEM) or fasting on CCl_4-caused alterations in glycoprotein-containing cells (GP) of various levels of ventral regions of tracheal epithela.*

GP	Po-fed	CCl_4-fed	Po-fasted	CCl_4-fasted	Po-DEM	CCl_4-DEM
PAS-G-S						
UT	7.56±2.87	1.92±1.92	15.88±2.05^{d}	1.32±1.06	14.62±3.43^{d}	5.81±3.49
LT	19.46±4.51	0^{a}	24.35±8.07^{bd}	0^{a}	3.67±2.67^{ac}	1.25±1.09^{ac}
PAS-G-L						
UT	5.96±1.95	0.61±0.61^{a}	21.75±7.81^{b}	0.18±0.18^{ac}	8.87±0.78^{bd}	3.43±1.71^{cd}
LT	7.95±0.75	0^{a}	11.22±5.47^{b}	0.87±0.78^{ac}	2.67±2.34	0^{ac}
PAS-H-S						
UT	0	12.64±6.07	0.43±0.43	7.19±3.16	5.42±2.63	11.81±0.56
LT	0	7.95±1.35^{a}	0.18±0.18^{b}	7.45±3.41	1.65±0.35	4.32±3.23
PAS-H-L						
UT	0	9.87±5.11	1.00±0.88	3.59±2.67	0.44±0.18	3.32±2.03
LT	0	6.24±0.83^{ac}	0	3.77±1.18	1.22±1.22	1.19±1.19
AB-PAS-G-S						
UT	0.06±0.06	0	0	1.67±0.68	0.42±0.25	1.15±1.14
LT	0.01±0.01	0.45±0.24	0.18±0.18	1.79±1.79	0.49±0.49	1.93±1.28
AB-PAS-G-L						
UT	0	0	0	0.26±0.22	0	0
LT	0	0.22±0.11	0	0	0	0
AB-G-S						
UT	0	0	0	0	0	0
LT	0	0	0	0	0	0
AB-G-L						
UT	0	0	0	0	0	0
LT	0	0	0	0	0	0

Adult, male, Syrian golden hamsters were fed ad lib or fasted for 24 hrs or were pretreated with 0.6 ml/kg DEM for 30 min prior to administration of 1.0 ml/kg CCl_4. All hamsters were given CCl_4 or peanut oil vehicle. The fasted hamsters starved further after administration of CCl_4 or vehicle. Animals were killed 24 hrs later. Tracheal tissues were removed and subdivided into upper (UT) or lower (LT) levels and prepared for histological determination of numbers of damaged cells as described in Methods. Three hamsters were used in each group.

*Number of glycoprotein-containing cells in 1 mm length of tracheal epithelium. Values are mean ± standard error of the mean. Peanut oil treated groups are used for comparison.
a=Value is significantly different from that of the peanut oil treated fed (Po-fed) group (P 0.05).
b=Value is significantly different from CCl_4-treated fed (CCl_4-fed) group ($P < 0.05$).
c=Value is significantly different from peanut oil treated fasted (Po-fasted) group ($P < 0.05$).
d=Value is significantly different from CCl_4 treated fasted (CCl_4-fasted) group ($P < 0.05$).
e=Value is significantly different from peanut oil treated DEM (Po-DEM) group ($P < 0.05$).

DISCUSSION

The present study demonstrated that CCl_4 caused alterations in glycoprotein-containing cells in hamster trachea. For example, numbers of glycoprotein-containing cells with PAS-positive granules decreased. In contrast, the glycoprotein-containing cells with homogeneous PAS-positive material increased in trachea of hamsters treated with CCl_4. This finding suggested that CCl_4 produced injury in membrane bound secretory granules, resulting in their disruption and subsequent formation of homogenous rather than granular staining. Alternatively, the homogenous material may arise from the presence of abnormal glycoprotein or abnormal proportions of normal glycoprotein that failed to form secretory granules.

Light and electron microscopy revealed that CCl_4 produced injury in secretory cells and in ciliated cells. Intracellular organelles, including mitochondria, rough and smooth endoplasmic reticulum, Golgi apparatus and secretory granules, were affected by this chemical. CCl_4 is known to be metabolized by cytochrome P-450-dependent monooxygenases to a free radical that is highly cytotoxic (Recknagel, 1967; Recknagel and Glende, 1973). This finding suggests that hamster tracheal nonciliated secretory cells, as well as ciliated cells have the potential of metabolizing this chemical.

Krishnan and Stenger (1966) found that fasting caused ultrastructural injury in rat hepatocytes. In my study in fasted animals, infrequent injury was observed in both nonciliated secretory

cells and ciliated cells. I found that the numbers of glycoprotein cells containing homogenous, PAS positive material were markedly increased in the lateral region of upper trachea. Electron microscopy revealed changes in intracellular organelles, including rough and smooth endoplasmic reticulum, mitochondria and Golgi apparatuses. Fasting potentiated CCl_4-induced injury in both secretory cells and ciliated cells. This is consistent with the finding of Krishnan and Stenger (1966), Diaz Gomes et al. (1975), and Harris and Anders (1980) who reported that fasting potentiated hepatotoxicity of CCl_4.

The mechanism by which fasting enhanced the cytotoxicity of CCl_4 is not completely understood. Several investigators reported that fasting depleted glutathione (GSH) Concentration in rat liver (Maruyama et al., 1969; Tateishi et al., 1974; Harris and Anders, 1980). It is generally agreed that one of the physiological functions of GSH is to protect cellular components from elctrophillic attack by chemicals or their metabolites that may cause tissue injury by reaction with macromolecules (Jakoby, 1978; Meister, and Anderson, 1983). CCl_4 is metabolized by a cytochrome P-450 dependent monooxygenase to a free radical which reacts with tissue macromolecules leading to cell injury (Recknagel and Glende, 1973). Substances such as GSH markedly reduce covalent binding of CCl_4 to liver microsomes (Corsini et. al., 1972). GSH also protects liver from toxic effect of CCl_4 (Gravela and Dianzani, 1970). Lindstrome et. al., (1978) found that addition of CCl_4 to isolated rat hepatocytes markedly depleted GSH concentrations in liver cells. Thus, it appears that GSH plays an important role in protecting the liver cell from the toxic effect of CCl_4. Harris and Anders (1980) found that fasting depleted GSH and potentiated the hepatotoxicity of CCl_4 in the rat liver.

However, the effects of fasting are not restricted to depletion of glutathione. For example, starvation has been shown to affect microsomal enzymes activities. Depending on the substrate examined, various authors have shown that fasting decreases, increases or causes no changes in hepatic microsomal monooxygenase activities (Dixon et. al., 1960; Kato, 1966; Gram et. al., 1970; Bock et. al., 1973; Marselos and Laitinen, 1975; Litterst et. al., 1977). Thus, it seemed likely that the potentiation of CCl_4-induced injury in trachea by fasting may be due to depletion of GSH and/or potentiation of the microsomal enzyme activity.

I observed injury in secretory cells of hamsters treated with DEM. Cytochemically, I found that DEM caused alterations in glycoprotein-containing cells. For example, glycoprotein-containing cells with homogenous, PAS positive material were observed in trachea of hamsters treated with DEM. Intracellular organelles, including mitochondria, rough and smooth endoplasmic reticulum and electron lucent secretory granules, were damaged by this chemical.

DEM also produced injury in ciliated cells. Brown et. al. (1974) and Docks and Krishna (1976) found that DEM did not produce injury in liver cells of rat. However, Anundi et al. (1979) and Casini et al. (1985) found injury in hepatocytes of rats treated with DEM. These authors reported that DEM depleted GSH and stimulated lipid peroxidation. Anundi et al. (1979) concluded that GSH deficiency per se could lead to lipid peroxidation. However, the effects of DEM are not restricted to depletion of GSH. Costa and Murphy (1986) showed that DEM inhibited protein synthesis in brain and liver of mouse. Anders (1978) found that DEM altered hepatic microsomal mixed function

oxidases of rat.

Several investigators reported that DEM potentiated the hepatotoxicity of many chemicals. For example, Harris and Anders (1980) found that DEM depleted GSH and potentiated hepatotoxicity of CCl_4 in rat liver. Boyd and Burka (1978) reported that pretreatment of rat with 0.6 ml/kg DEM strikingly increased pulmonary toxiciity of 4-ipomeanol. Originally, I used DEM to determine if it would enhance injury to tracheal epithelium caused by CCl_4. However, DEM itself produced marked injury to this tissue, rendering its effects on CCl_4 toxicity difficult to ascertain from these studies.

In summary, CCl_4 produced injury in secretory cells and ciliated cells of hamster trachea. This finding suggests that these cells may have the potential to metabolize CCl_4. Fasting potentiated the cytotoxicity of CCl_4 in hamster trachea. This might be due to the fact that fasting depleted GSH and/or induced microsomal enzyme activity. DEM itself produced injury in nonciliated secretory and ciliated cells, which masked any effect of DEM on CCl_4 toxicity.

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CHAPTER V

GENERAL CONCLUSION

Hamster tracheal epithelium comprises a mixed population of cells. One member of this population is secretory cells that contain abundant smooth endoplasmic reticulum (SER) and electron lucent, membrane bound, secretory granules. Cytochemically, most of the secretory cells contained neutral glycoproteins (PAS positive). A few contained acidic glycoproteins (AB-PAS positive). This finding indicated that hamster tracheal epithelial cells have the potential to synthesize both acidic and neutral glycoproteins.

The mixed function oxidases (MFOs) occur in smooth endoplasmic reticulum and provide a major pathway for metabolism of xenobiotic agents. The presence of SER in tracheal epithelial cells suggests that these cells may have the potential to metabolize xenobiotic agents.

It is generally agreed that carbon tetrachloride (CCl_4) is metabolized by MFO components to a free radical that is highly cytotoxic. CCl_4 produced injury in intrapulmonary airway epithelial cells. However, the effect of CCl_4 on tracheal epithelial cells has not been investigated previously. CCl_4 produced injury in nonciliated cells of various regions and levels of the trachea. For example, glycoprotein cells containing homogenous, PAS-positive material were absent in control animals, but they were present after CCl_4 treatment. This finding suggests that CCl_4 produced injury to membrane bound

secretory granules, resulting in their disruption and subsequent formation of homogeneous material rather than granules. Alternatively, the homogenous material may have arisen from the presence of abnormal glycoproteins or abnormal proportions of normal glycoprotein that failed to form secretory granules.

In control hamsters, the glycoprotein-containing cells that had granules positive for both AB and PAS were found infrequently. However, these cells were frequently seen in various regions and levels of trachea of hamsters treated 1 and 4 hrs previously with CCl_4. This finding suggests that CCl_4 altered the balance between synthesis and discharge of glycoproteins.

CCl_4 also produced injury in ciliated cells of hamster trachea. CCl_4-induced cell injury is dependent on metabolism of this agent by intracellular, NADPH-dependent cytochrome P-450 monooxygenases. Thus, it seemed likely that ciliated cells of hamster trachea may have the potential to bioactivate CCl_4. However, the extent of CCl_4-induced injury varied in different regions and levels of the trachea. This finding raised the possibility that some tracheal epithelial cells may be more capable than others of bioactivating CCl_4. Alternatively, cells of all regions and levels of the trachea might be capable of activating of CCl_4 to the same extent, but the cells which appeared to be less sensitive may detoxify the reactive CCl_4 metabolites more effectively. Other possibilities, however, cannot be ruled out at this time. For example, differences in blood flow among levels of trachea may result in related differences in delivery of CCl_4 to epithelium.

Several investigators have reported that fasting potentiates hepatotoxicity of CCl_4. The present study demonstrated that fasting by itself produced injury both in secretory cells and in ciliated cells. However, fasting enhanced CCl_4-induced injury both in secretory and in ciliated cells. The mechanism by which fasting enhanced toxicity of CCl_4 is not completely understood. Several investigators reported that fasting depleted glutathione (GSH) in liver cells. One of the physiological functions of GSH is to protect cellular components from attack by chemicals or their metabolites that may cause tissue injury by reaction with macromolecules. However, the effects of fasting are not restricted to depletion of GSH. Fasting has been shown to affect microsomal enzymes activities. Thus, the potentiation of cytotoxicity of CCl_4 in hamster trachea after fasting may be due to depletion of intracellular GSH and/or alteration of microsomal enzyme activity.

Several investigators reported that diethylmaleate (DEM) potentiated hepatotoxicity of CCl_4. However, the present study demonstrated that DEM itself produced marked injury in hamster trachea, rendering its effects on CCl_4 toxicity difficult to ascertain from these studies.