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Monocrotaline Pyrrole-Induced Pulmonary Hypertension: Effects on Pulmonary Vasculature In Vivo and on Pulmonary Vascular Cells in Culture

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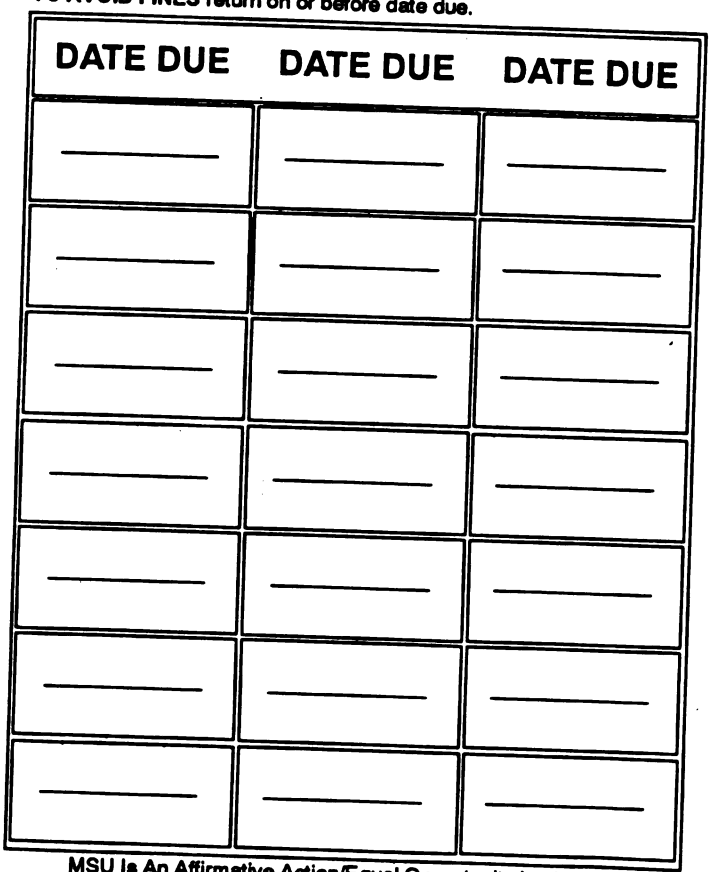

MONOCROTALINE PYRROLE-INDUCED PULMONARY HYPERTENSION: EFFECTS ON PULMONARY VASCULATURE *IN VIVO* AND ON PULMONARY VASCULAR CELLS IN CULTURE

BY

James Fredrick Reindel

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ABSTRACT

MONOCROTALINE PYRROLE-INDUCED PULMONARY HYPERTENSION:
EFFECTS ON THE PULMONARY VASCULATURE *IN VIVO*
AND ON PULMONARY VASCULAR CELLS IN CULTURE

By

James Fredrick Reindel

Monocrotaline pyrrole (MCTP), an unstable putative metabolite of the pyrrolizidine alkaloid monocrotaline (MCT), causes pulmonary vascular injury, pulmonary hypertension (PH) and right ventricular hypertrophy in rats. Many pathophysiologic features of advanced MCTP-induced cardiopulmonary disease resemble those of MCT-induced disease and certain forms of chronic pulmonary hypertensive disease of humans.

Early morphologic alterations of MCTP-induced lung disease in rats were defined in detail and related to alterations in other markers of cardiopulmonary injury and the occurrence of PH. Rats given a single low dose (3.5 mg/kg, I.V.) of MCTP had subtle interstitial pulmonary edema and pulmonary vascular leak 3 days after treatment. These changes slowly worsened thereafter and were associated with a progressive increase in pulmonary inflammation. Thickening of pulmonary arterial walls was apparent in small pulmonary arteries at 5 days posttreatment and in larger arteries by day 8. Wall thickness increased with time. Initiation of vascular remodeling preceded the occurrence of PH.

The reason for the delayed and progressive pulmonary alterations after a single administration of MCTP is unknown, but progressive pulmonary vascular leak and pulmonary edema suggested that MCTP caused toxic effects on vascular cells which were not manifest as overt injury until several days after treatment. Accordingly, the effects of MCTP were assessed in cells in culture. Concentrations of MCTP in the range of 5 to 50 μg/ml caused delayed but progressive deterioration of monolayers of transformed mammalian cells and bovine pulmonary arterial endothelial cells. Surviving cells underwent marked hypertrophy and atypical features developed in these cells. Bovine smooth muscle cells and porcine endothelial cells were resistant to the cytolytic effects of MCTP. Monolayers of these cells were minimally affected over the duration of the study. Lower concentrations of MCTP (0.5 μg/ml) caused inhibition of proliferation of all cell types without causing cell lysis.

In vivo, the delayed and progressive deterioration of endothelial cells could result in gradual disruption of the endothelial barrier and vascular leak, which may not be readily repaired by surviving cells that cannot proliferate. A consequence of the sustained vascular leak may be progressive pulmonary injury and inflammation associated with MCTP-induced lung disease.

DEDICATION

To my parents Oswald F. and Mildred O. Reindel,

my wife Amy, and the rest of the Reindel family.

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I would like to thank all individuals who have contributed to the completion of my research and this dissertation. Indeed the list of contributors is long. It includes many people from diverse departments; some as mentors, some as coworkers, some as technicians and some as research associates. I truly hope that the completion of this dissertation does not end my association with these individuals as I step into a new phase of my career.

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TABLE OF CONTENTS

TABLE OF CONTENTS (cont.)

TABLE OF CONTENTS (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

INTRODUCTION

Monocrotaline (MCT) is a toxic pyrrolizidine alkaloid (PA) found in several plants in the *Crotalaria* genus, including *Crotalaria spectabilis*. Consumption of the foliage of MCT-containing plants has been associated with human and animal disease throughout the world. MCT and other toxic PAs induces hepatotoxic and pneumotoxic effects in a wide range of animal species. They are also carcinogenic, capable of inducing a variety of tumors in laboratory animals.

Pulmonary lesions induced by these compounds are delayed in onset and progressive in nature and include pulmonary edema, inflammation and fibrosis. Associated with the pulmonary changes are progressive pulmonary vascular remodeling, pulmonary hypertension (PH) and right ventricular hypertrophy. Related to these changes, MCT-induced pulmonary disease has developed as an animal model of certain forms of chronic PH of people.

Evidence indicates that MCT and other toxic PAs are not toxic in themselves but are bioactivated by the cytochrome P-450 system of the liver to pyrrolic derivatives which are responsible for tissue injury. These pyrrolic derivatives are reactive electrophilic compounds capable of binding to cellular constituents. A portion of these pyrroles escape binding locally in the liver and travel to the pulmonary vascular bed where they bind to tissue and induce injury. How these pyrroles cause tissue damage is largely unknown.

Monocrotaline pyrrole (MCTP) is a putative metabolite of MCT. It is an unstable electrophile capable of inducing pulmonary injury when injected into the tail veins of rats. There are many morphologic similarities in the end-stage lung disease induced by MCT and that induced by low doses of MCTP. In addition, the time course of the diseases as assessed by biochemical and hemodynamic markers of lung injury are similar. These findings suggest that MCTP may indeed be the toxic principle resulting from MCT bioactivation.

Morphologic studies, detailing the time course of MCTP-induced lung injury have not been previously reported in great detail as they have with MCT-induced lung disease. In addition, morphometric assessment of vascular changes in the pulmonary arteries have not been made. It is important to determine if the morphologic changes induced by MCTP follow a similar time course to those caused by MCT, if we are to infer that MCTP is the proximate toxicant of MCT. In addition, it is critical to determine if and when vascular remodeling occurs in MCTP-induced pulmonary disease since this is hallmark of MCT-induced lung injury and of certain forms of chronic PH of man.

The delayed and progressive nature of MCT- and MCTP-induced lung disease, particularly in light of rapid metabolism and elimination of the parent alkaloid from the body and the short-lived reactive nature of pyrrolic metabolites, has puzzled investigators. Some investigators have suggested that MCT- and MCTP-induced disease may not be due to direct cytotoxic effects of the pyrrolic metabolites. For example, the injury might be due to triggering of an inflammatory cascade which ultimately results in progressive tissue injury. To date, no studies have detailed

the effects of MCTP on cells in culture. Indeed, it is necessary to determine whether MCTP is directly toxic to cells, particularly endothelial cells, if we are to understand the pathogenesis of pulmonary injury in this disease. In addition, if MCTP is toxic to cells, it is necessary to define dose-dependent responses of this short-lived toxicant on cells in culture. Findings from these studies may shed light on the pathogenesis of MCT-/MCTP-induced lung vascular disease and possibly certain forms of chronic PH of man.

The major hypothesis to be tested is that MCTP causes injury to cells of the lung vascular bed and that this injury is the result of direct interaction of these cells with MCTP. There are several major objectives of my dissertation research. The first major objective is to establish whether MCTP elicits a similar course of toxic effects on the pulmonary parenchyma and vasculature of rats as those produced by the parent alkaloid, MCT. Toward the achievement of this objective a detailed morphologic examination of the pulmonary vasculature and vascular cells will be made to define the time-related responses in these lung constituents after MCTP exposure *in vivo*. The second major objective of this dissertation research is to assess the toxicity of MCTP to cells in culture. If MCTP is toxic to cells in culture, additional objectives will be to establish dose-response relationships for certain toxic effects of MCTP on cells and a time course for the manifestation of these effects. Both established lines of mammalian cells and primary cultures of cells derived from pulmonary arteries of cattle and pigs will be used for these studies to determine if toxic effects induced by MCTP are dependent on cell type and if there are differences in response of vascular cells

derived from the same tissue source from different animal species.

Chapter I

LITERATURE REVIEW

A. Pyrrolizidine Alkaloids

1. General

Pyrrolizidine alkaloids (PAs) are structurally related chemicals found in hundreds of plant species distributed throughout the world (Bull *et al.*, 1968). Many are harmless, but a substantial number are toxic to livestock and humans. PA-containing plant species occur in 8 unrelated plant families. The majority are found in the families *Leguminosae*, *Compositae*, and *Boraginaceae* (Bull *et al.*, 1968). Genera in which a high proportion of these plants are found include *Crotalaria*, *Senecio*, *Heliotropium*, *Cynoglossum*, *Amsinckia*, *Echium*, and *Trichodesma*. PAs are found in the seeds, leaves, and stems of these plants, and many of these plants contain more then one PA (Bull *et al.*, 1968).

As a chemical group, PAs are defined by the pyrrolizidine nucleus they contain. This structure is composed of two fused five member rings with a nitrogen atom at the junction (Figure 1-1) (McLean, 1970; Mattocks, 1986). Variations in the functional groups attached to the ring structure give rise to the variety of PAs. To date, approximately 150 different PAs have been isolated and structurally characterized (Huxtable, 1979; Mattocks, 1986).

Approximately 30 of the structurally characterized PAs are known to be toxic. Structural features of toxic PAs which appear to be essential for toxic activity include: (1) ester linkages of branched chain acids to hydroxyl groups of the pyrrolizidine ring and (2) a double bond in the pyrrolizidine nucleus between carbons 1 and 2

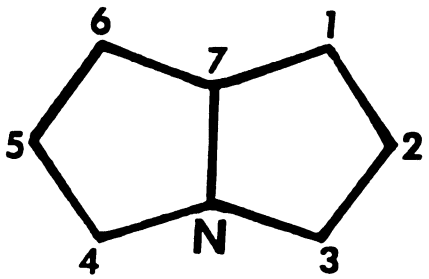


Figure 1. Structure of the Pyrrolizidine Nucleus

Monocrotaline-Containing Plants

Crotalaria spectabilis

C. retusa

C. mitchellii

C. novae-hollandiae

C. crispata

C. grahamiana

C. sericea

C. quinquefolia

C. mysorensis

Figure 2.

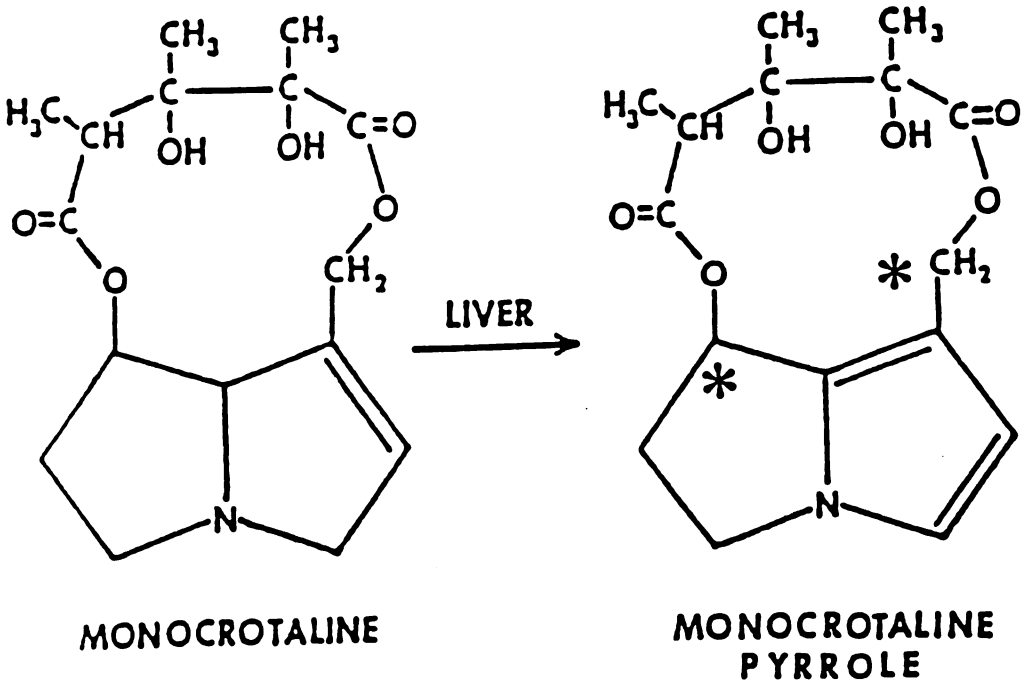


Figure 3. Structures of MCT and MCTP (Asterisks indicate potential alkylation centers)

(Schoental and Mattocks, 1960; McLean, 1970). Diesters are generally more toxic than monoesters (Culvenor *et al.*, 1976). Toxic PAs have many similarities in their metabolism, pharmacokinetics and organ toxicities which warrant discussing them as a group.

Monocrotaline (MCT) is a toxic PA derived from several plants of the *Crotalaria* genus in the *Leguminosae* family (Figure 1-2). Structurally, it is a macrocyclic diester consisting of the dicarboxylic acid, monocrotalic acid, esterified to the pyrrolizidine nucleus (Figure 1-3) (Adams and Rogers, 1939; Neal *et al.*, 1935) In the United States this PA is found principally in the toxic plant, *Crotalaria spectabilis*, also called "rattle box" or "rattle pod".

2. Toxicity to Animals and Humans

Animal intoxication due to ingestion of PA-containing plants had been suspected by farmers in Great Britain as early as 1787 (Bull *et al.*, 1968). A direct causal relationship between PA ingestion and animal disease, however, was not reported in the literature until nearly a century later. In 1884, Stalker established a direct association between PA-containing plant ingestion and a seasonally occurring disease of horses from the western Iowa-eastern Nebraska region of the United States (Stalker, 1884; Kay and Heath 1969). This disease, called Missouri River bottom disease, was experimentally reproduced in horses by intragastric administrations of infusions of *Crotalaria saggitalis*. Since that time, PA-induced disease has been recognized and reproduced in numerous species, including chickens (Allen *et al.*, 1960), turkeys (Allen *et al.*, 1963), cattle (Cushny, 1911; Bull *et al.*, 1961; Fowler, 1968), pigs (Emmel *et al.*, 1935; Harding *et al*, 1964), goats (Dickinson, 1980), sheep (Jago *et*

al., 1969; Bull *et al.*, 1956; Harris *et al.*, 1957), horses (Hill and Martin, 1951; Rose *et al.*, 1957a,b), fish (Hendricks *et al.*, 1981), dogs (Miller *et al.*, 1978, 1981), and laboratory animals (Schoental and Head, 1955; Hooper, 1974; Miranda *et al.*, 1981; Cheeke and Pierson-Goeger, 1983).

Animal exposure to toxic, PA-containing plants occurs worldwide. Field cases of animal poisonings by PAs occur under various situations. Intoxications occur in animals which graze pastures or rangeland contaminated with the toxic plants, particularly when pasture or range conditions are poor. PA-containing plants, including *C. spectabilis*, are not palatable but are ingested if suitable, palatable forage is not available (Piercy and Rusoff, 1945; Sippel, 1964). Poisonings also occur when animals are fed forage or grains harvested from fields contaminated with these toxic plants or when poultry are housed on litter contaminated with PA-containing seeds (Sippel, 1964).

In the United States, PA-containing plants responsible for substantial livestock losses include *C. spectabilis*, *Senecio jacobaea*, and, to a lesser extent, several other species of *Senecio*. *C. spectabilis*, a plant native to India, was introduced into Florida in 1921, as a leguminous cover crop for the improvement of fallow fields. The uncultivated plant now grows throughout much of the southern U.S. (Heath, 1969). *S. jacobaea*, a plant inadvertently introduced to North America from Europe, is now found in the western states and Canada in arid regions (Huxtable, 1980a). Other toxic, PA-containing plants grow in the southwestern and western United States, but their significance in producing livestock disease is relatively minor compared to that caused

by *C. spectabilis* and *S. jacobaea*. Nationally, losses related to PA intoxication of livestock cost millions of dollars per annum (Snyder, 1972).

Human intoxications with PAs have also occurred worldwide but are most common in underdeveloped, third world countries. They have occasionally been associated with inadvertent ingestion of PA-contaminated foodstuffs (Mattocks, 1986; McLean, 1970; Huxtable, 1980b). Vegetable oils and flour derived from cereal crops contaminated with seeds of PA-containing weeds have been incorporated into food for human consumption. Consumption of these products has been associated with outbreaks of human liver disease in India (Tandon *et al.*, 1976), Central Asia (McLean, 1970), Africa (Willmot and Robertson, 1920; Selzer and Parker, 1951), and Afghanistan (Mohabbat *et al.*, 1976).

Agricultural products from animals and insects exposed to PA-containing plants pose a potential threat to man (Eastman *et al.*, 1982). Milk from rats and mice given PA-containing plants has been shown to contain small quantities of PAs (Eastman *et al.*, 1982; Schoental, 1959), and nursing rat pups developed hepatic lesions, even when damage was not apparent in the lactating mother (Schoental, 1959). Cattle and goats administered very large doses of PAs also passed low concentrations of PAs into their milk (Dickinson *et al.*, 1976; Dickinson, 1980), and dried milk products from PA-exposed animals produced hepatic injury when fed to rats as a very high portion of the diet. In calves and goats fed milk from PA-intoxicated animals, however, hepatic lesions did not develop (Dickinson et al., 1976; Dickinson, 1980). Honey made from the nectar of PA-containing plants is also a potential source of human exposure, since

concentrations of PAs have been identified in honey from areas where PA-containing plants abound (Deinzer *et al.*, 1977). Fortunately, honey made from PA-containing plants is of low palatability and poor quality and is, therefore, not readily consumed. Contaminated milk and honey probably do not pose a major threat to humans, since the quantity of PAs present in these products is extremely low (Mattocks, 1986).

Human poisoning also occurs from voluntary ingestion of PA-containing plants. In many parts of the world, plants that contain toxic PAs are eaten as part of the diet or used deliberately or inadvertently in medicinals or folk remedies for a wide range of ailments (Mattocks, 1986). Schoental (1972) found that species of *Crotalaria*, *Senecio*, *Heliotropium*, and *Cynoglossum* are used in Africa as externally and internally applied medicinals. In the West Indies, plants from the bush are collected and used in "bush" teas among poorer populations. Many such teas are harmless, but others are made with PA-containing plants that have been associated with liver damage and death, particularly in infants (Bras *et al.*, 1961; McLean *et al.*, 1964). Ingestion of PA-containing teas and medicinals has been incriminated as a cause of the endemic liver disease in these areas, although ingestion of aflatoxin contaminated grains and a high incidence of viral hepatitis probably also contribute to the overall incidence of liver disease.

Although most human intoxications occur in less developed countries, well documented human cases of PA intoxication have occurred in the United States (Stillman *et al.*, 1977; Fox *et al.*, 1978). The PA-containing plant, *Senecio longilobus*, was mistakenly incorporated into medicinal teas

given to two young children. Ingestion of these remedies induced hepatotoxic and pneumotoxic effects and death in these infants.

3. Pharmacokinetics of Radiolabeled Pyrrolizidine Alkaloids

Lack of availability of radiolabeled PAs have precluded extensive pharmacokinetic studies with these compounds. The Ehrlich reaction has been useful in detecting the fate of pyrrolic products of PA metabolism but does not identify nonpyrrolic metabolites of PAs (Mattocks, 1968b).

Several studies in which various radiolabeled PAs were used have been reported. These studies indicate that labeled PAs disappear rapidly from the blood (McLean, 1970). They are also rapidly eliminated from the body. Sixteen hours after administration, only 7% of senecionine and 12% of seneciphylline remained in lactating mice (Eastman *et al.*, 1982). The liver contained the greatest amount of tissue-related radioactivity. Lesser amounts were present in the blood, kidneys, lung, and heart, and only small amounts were present in the brain. Radioactivity was detected in DNA isolated from the liver, kidney and lung of rats 6 hours after treatment with either of these 2 PAs, but only in liver DNA at 4 to 5 days posttreatment (Candrian *et al.*, 1985). PAs were rapidly excreted in the urine and feces of rats given senecionine and seneciphylline, with the urine being the major route. (Eastman *et al.*, 1982; Candrian *et al.*, 1985). Radioactivity was also detected in the skim milk fraction of lactating mice given these PAs (Eastman *et al.*, 1982).

The highest tissue radioactivity after intraperitoneal administration of radiolabeled MCT or lasiocarpine was also in the liver (Hayashi, 1966; Culvenor *et al.*, 1969). Within 3 hours after administration of radiolabeled MCT, 50 to 70% of the radioactivity

appeared in the urine, suggesting that urinary excretion was the predominant route of MCT metabolite excretion (Hayashi, 1966). Thirty percent of the radioactivity was recovered in the bile. By 72 hours, only the liver contained significant radiolabel. Other organs, including lung, showed very low activity at that time.

4. Metabolic Activation of Pyrrolizidine Alkaloids

It is generally recognized that PAs are not toxic themselves but are bioactivated to metabolic products which are responsible for the hepatotoxicity and pneumotoxicity (Mattocks, 1968a). PAs are relatively nonreactive with other chemicals *in vitro* (Mattocks, 1969; 1972). In addition, when administered to animals, PAs do not induce major tissue injury at the site of administration. They do, however, induce hepatic and pneumotoxic injury whether administered by oral, subcutaneous, dermal, or intravenous routes (Schoental and Head, 1955; Mattocks, 1972b). These findings are consistent with the idea that metabolic activation of PAs is necessary to induce tissue damage.

PAs could be metabolized by a variety of pathways in the liver to potentially reactive products. Reaction pathways of the mixed function oxidase system that could theoretically produce toxic products include N-oxidation, epoxidation or dehydrogenation of the pyrrolizidine nucleus (McLean and Mattocks, 1980). Epoxidation and N-oxide derivatives do not appear to be responsible for the toxic effects of PAs (Culvenor *et al.*, 1970, 1976). Pyrrolic derivatives, produced by dehydrogenation of the parent compound, are thought to be the proximate toxicants responsible for tissue injury (Mattocks, 1968a). One such pyrrolic derivative of MCT is the reactive, unstable electrophile, monocrotaline pyrrole (MCTP).

Numerous studies support the premise that primary pyrrolic derivatives of PAs (esterified pyrroles) are the metabolic products that induce tissue damage. Unlike the parent alkaloids, chemically synthesized, primary pyrrolic derivatives of toxic PAs are reactive chemicals (Mattocks, 1969) that readily bind to biologically important molecules *in vitro* (Mattocks and Bird, 1983a; White and Mattocks, 1972) and *in vivo* (Mattocks, 1972). In addition, they can induce localized toxic reactions at the site of application but do not induce major injury at distant sites (Mattocks, 1968a, 1969; Culvenor *et al.*, 1976; Butler *et al.*, 1970). Unfortunately, the unstable reactive nature of primary pyrroles precludes detection of the intact molecules *in vivo*; however, more stable hydrolysis products (metabolic or secondary pyrroles) which retain the pyrrolic pyrrolizidine nucleus can be detected colorimetrically by the Ehrlich reaction (Mattocks, 1968b, 1972).

The liver, more specifically the microsomal component, appears to be a principal site of PA bioactivation (Mattocks and White, 1971). Mattocks and coworkers, using the Ehrlich method for detection of pyrroles, confirmed that metabolic pyrroles are produced after incubation of the parent alkaloid with rat liver slices or liver microsomes *in vitro* (Mattocks, 1968a; Mattocks and White, 1971). In addition, Lafranconi and Huxtable (1984) demonstrated that the isolated perfused rat liver was able to convert MCT to pyrrolic metabolites, and such metabolites were able to cause reduced 5-hydroxytryptamine (5 HT) clearance when perfused through the isolated lung, suggesting that endothelial function may have been altered.

In vivo, metabolic pyrroles are detected in the liver, lungs and kidneys of animals given PAs, including MCT, with the highest concentrations found in the liver (Mattocks, 1968a; 1969; 1972; Hsu *et al.*, 1973). In the liver, these pyrroles are detected in the bound form in the solid debris and microsomal fractions of disrupted cells (Mattocks, 1968a). The pyrroles found in the kidneys largely represent water soluble, secondary metabolites of unstable toxic pyrroles in transit for urinary excretion (Mattocks, 1968a).

Metabolism of PAs to pyrroles appears to be restricted largely to the liver. When liver or lung slices were incubated with PAs (Armstrong and Zuckerman, 1970; Hilliker *et al.*, 1983), only the liver slices were able to convert these chemicals to pyrroles. Similarly, microsomes prepared from liver, but not lung, metabolize PAs to pyrrolic derivatives (Mattocks and White, 1971a; Guengerich, 1977). The bioactivation of PAs to pyrroles is dependent on the presence of NADPH and oxygen (Mattocks and White, 1971a). This conversion is inhibited by carbon monoxide, chloramphenicol, or SKF-525A and is enhanced in liver slices from phenobarbital-treated rats (Mattocks and White, 1971; Chesney *et al.*, 1974; Chesney *et al.*, 1974b; Lafranconi and Huxtable, 1984). *In vivo*, phenobarbital pretreatment of rats has been shown to enhance MCT toxicity, whereas toxicity is attenuated by chloramphenicol or SKF-525A pretreatment (Mattocks, 1972; Allen *et al.*, 1972; Bruner *et al.*, 1983). Since phenobarbital pretreatment increases hepatic, but not pulmonary, cytochrome P-450 monooxygenase activity, it is probable that the increase in toxicity is due to enhanced conversion of PAs to toxic metabolites in the liver (Mattocks, 1986).

5. Characteristics of Pyrroles

Primary pyrrolic derivatives of toxic PAs, such as MCTP, are reactive and short-lived in aqueous environments (Mattocks, 1969). Nearly all the reactivity of the MCTP is lost within the first minute of incubation in aqueous media (Mattocks, 1969; White and Mattocks, 1972; Bruner *et al.*, 1986). This loss of reactivity occurs as esterified pyrroles autopolymerize with other pyrrolic molecules, bind to other molecules in the medium, or are hydrolyzed to their corresponding alcohols (secondary pyrroles), which are much more stable and less toxic than their corresponding pyrrolic esters. Hydrolysis of MCTP produces the secondary pyrrole, dehydroretronecine (DHR) (Hsu *et al.*, 1973b). A single intravenous administration of this hydrolysis product to rats can induce injury in several organs, particularly the gastrointestinal tract, (Hsu *et al.*, 1973b) but does not damage the lung. Repeated daily administrations of DHR for 2-3 weeks can, however, induce pulmonary injury (Huxtable *et al.*, 1978). Reactive primary pyrroles induce injury at the site of administration or at the first vascular bed encountered after intravenous administration but not at distant sites, consistent with their short-lived nature (Butler *et al.*, 1970; Hooson and Grasso, 1976; Culvenor *et al.*, 1976). Conversion of PAs to corresponding pyrrolic derivatives creates instability in the molecule at the carbon-ester linkages of the mono- and diester PAs and this converts these molecules into unstable reactive electrophiles, which can alkylate other molecules (McLean, 1970).

Chemically synthesized pyrroles can interact with electron-rich centers such as sulfur, nitrogen, or oxygen atoms in other biological molecules, forming covalent bonds (Robertson *et al.*, 1977; Robertson,

1982; Mattocks and Bird, 1983a, Mattocks, 1986). The order of bond stability is greatest with the sulfur atom, followed by nitrogen, and finally by oxygen. O- and N-derivatives are less stable than S-derivatives and can themselves act as alkylating agents (Mattocks and Bird, 1983a; Mattocks, 1986). The chemical transfer of the bound pyrroles may maintain the reactivity of the pyrrolic metabolites from the point of generation (liver) to the site of toxicity (lung) as the transfer of the pyrrole eventually results in the more stable linkage to sulfur-containing tissue macromolecules (Mattocks and Bird, 1983a; Sun *et al.*, 1977).

The binding of pyrroles to cell macromolecules leads to cell injury by mechanisms that are only vaguely understood. Pyrroles such as DHR can inactivate enzymes *in vitro* by binding to sulfur containing amino acids in the active centers (Sun *et al.*, 1977). Similarly, PAs, when bioactivated in the liver, have been shown to reduce the function of some hepatic microsomal enzymes (Mattocks and White, 1971; Shull *et al.*, 1976b; Eastman and Segall, 1980; Miranda *et al.*, 1980a, 1981). PAs with diester linkages to branched chain acids can be bioactivated to bifunctional alkylating agents capable of electrophilic attack at two sites of the molecule (Figure 1-3) (Mattocks, 1969); therefore, they can crosslink biological molecules such as DNA (Mattocks 1969; White and Mattocks, 1972; Petry *et al.*, 1984). Indeed, interstrand crosslinking of DNA and DNA-protein crosslinks occur in hepatocytes shortly after MCT administration to rats (Petry *et al.*, 1984). Whether this crosslinking *in vivo* is due to the direct alkylation of these molecules or to some other alteration of cell function (inactivation of DNA repair enzymes or altered cell permeability and calcium homeostasis affecting cell metabolism) has not

been established (McLean and Mattocks, 1980). Pyrroles can react with other biologically important molecules, and alterations in cellular pools of these molecules or enzymes could have drastic effects on cellular metabolism (Mattocks and Bird, 1983a; Hsu *et al.*, 1975; Mattocks, 1986). Crossbinding of biologic molecules may not be critical to induce cell injury. In fact, monofunctional pyrroles can inhibit cell mitosis and induce cell injury *in vivo*, much like the bifunctional pyrrolic derivatives of PAs, even though they cannot crossbind molecules (Mattocks, 1978; 1986).

It is thought that, once PAs are bioactivated to pyrrolic derivatives in the liver, a portion of the pyrroles escapes binding locally and passes to the next vascular bed (ie., that of the lung) to induce injury (Barnes *et al.*, 1964; Mattocks, 1968a). Intravenous administration of several chemically-synthesized pyrroles, including MCTP, into the tail veins of rats produces lung injury similar to that induced by systemic administration of the parent compounds. (Mattocks and Driver, 1983; Butler *et al.*, 1970; Chesney *et al.*, 1974; Bruner *et al.*, 1983). The lung damage is not due to an organ-specific or selective feature of the compound. If pyrroles are administered into the inferior mesenteric artery, they induce necrosis of the walls of small portal veins with infarction of the liver served by the vessels (Butler *et al.*, 1970). When administered intraperitoneally, they produce hemorrhage and sloughing of the peritoneum (Mattocks, 1969; Barnes *et al.*, 1964). When administered subcutaneously, they induce localized chronic inflammation (Hooson and Grasso, 1976). Thus, reactive pyrrolic derivatives induce injury where they encounter tissue macromolecules.

Although most investigators believe that reactive primary pyrroles such as MCTP are responsible for lung injury following administration of PAs, others have proposed that relatively stable secondary metabolites of these pyrroles are more likely the proximate toxicants that induce the lung injury. Lafranconi and Huxtable (1984) found that perfusion of isolated lungs with effluent from MCT-treated isolated livers 15-30 minutes after collection of the liver effluent resulted in altered intrapulmonary serotonin clearance. This was thought to reflect damage to the endothelium; however, other factors could influence clearance of serotonin. Unfortunately, other parameters which reflect endothelial damage were not examined.

In the case of the PA, senecionine, Segall *et al.* (1985) reported that this PA could be metabolized to a reactive, electrophilic aldehyde, trans-4-OH-hexenal. This aldehyde may be a toxic metabolite responsible for inducing hepatic injury, since this and other structurally related trans-OH-alkenals can stimulate release of lactate dehydrogenase activity from primary cultures of rat hepatocytes (Griffin and Segall, 1986). Culvenor *et al.* (1970) also suggested that toxic aldehydes derived from metabolism of other PAs might be responsible for cell injury. Aldehydes derived from other PAs have not been examined for toxicity.

6. Other Routes of Pyrrolizidine Alkaloid Metabolism

Alternative routes of PA metabolism that appear to be important in inactivation and elimination of the molecules include ester hydrolysis, ring hydroxylation, and ring N-oxidation (Bull *et al.*, 1968; Jago *et al.* 1969; McLean, 1970, Mattocks and Bird, 1983b). These reactions, like pyrrole production, are affected by enzymes of the hepatic cytochrome

p-450 mixed function oxidase system. The levels of these inactivation enzymes influence the toxicity of the parent compounds. Indeed, the predominance of detoxification reactions over the dehydrogenation reaction may be one reason why some animal species, such as guinea pigs, are more resistant to the toxic effects of PAs (Chesney and Allen, 1973b; Cheeke and Pierson-Goeger, 1983; Mattock, 1972; 1986).

N-oxides of PAs are generally considered as detoxication products of PA metabolism (Mattocks, 1971, 1972; Chesney *et al*, 1974b); however, N-oxides of PAs have been shown to be toxic in some animal species, particularly when administered orally (Schoental and Magee, 1959; Barnes *et al.*, 1964). The toxicity of N-oxide derivatives of PAs appears to be due to metabolic reduction of these compounds to the parent alkaloids in the gut (Mattocks, 1971; Powis *et al.*, 1979; Brauchli *et al.*, 1982). N-oxide derivatives are much less toxic when administered by intravenous or subcutaneous routes. In addition, hepatic microsomes do not convert N-oxides of PAs to toxic pyrroles (Mattocks and White, 1971; Mattocks and Bird, 1983b; Jago *et al.*, 1970). These findings support the premise that N-oxides as such are nontoxic.

B. Organ Pathology Induced by Pyrrolizidine Alkaloids

1. General

Environmental and experimental exposures of animals to toxic PAs have caused damage to a number of organ systems. Organ sensitivity and selectivity depends on many factors, including: (1) the specific PA-containing plant or PA ingested, (2) the dose of PA received (Culvenor *et al.*, 1976), (3) the duration of the post-exposure period (Schoental and

Head, 1955), and (4) animal factors (McLean, 1970), including species (Sippel, 1964; Rose, 1945; Cheeke and Pierson-Goeger, 1983), age (Schoental and Head, 1955; Jago, 1970, 1971), sex (Goldenthal *et al.*, 1964), nutritional status (Hayashi *et al.*, 1979), and even the strain of animal studied (Lalich, 1964; Turner and Lalich, 1965).

Historically, PAs are best known for their hepatotoxic effects. Prior to the 1960s, reports of PA toxicosis focused chiefly on liver pathology, largely ignoring effects on other organ systems. Effects on other systems can be significant, however, and pulmonary disease has been responsible for animal death (Schoental, 1959; Valdivia *et al.*, 1967b).

2. Hepatic Pathology

Regardless of the route of administration (oral, dermal, subcutaneous, or intravenous), the liver is a major target in PA-induced disease (Schoental and Head, 1955; Schoental and Magee, 1957; 1959; Mattock, 1972). A variety of hepatic alterations have been described, ranging from acute to chronic effects and neoplasia. The nature and extent of the hepatic injury is greatly influenced by the dose of PA received and the duration of the survival period following exposure.

a. Acute Hepatic Effects

Acute experimental poisonings, at doses near the LD 50, cause hemorrhagic hepatic necrosis, beginning at 12 hours and peaking 24 to 48 hours after PA administrations. Necrosis is generally centrilobular to midzonal in distribution (McLean, 1970), although periportal necrosis has been described with selected PAs in some animal species, including mice, monkeys, and hamsters (Rose *et al.*, 1959; Wakim *et al.*, 1946; Ruebner and Watanabe, 1970; Harris *et al.*, 1942a). Fatty change develops in

hepatocytes that surround the necrotic zones (McLean, 1970). Morphologic changes in acute poisonings are similar to hepatic changes produced by a large variety of hepatotoxic chemicals requiring bioactivation by P-450 monooxygenases for their toxic effects.

b. Chronic Hepatic Effects

Chronic hepatic alterations are apparent within weeks of a single, sublethal dose or repeated small doses of PAs (Schoental and Head, 1955; Schoental and Magee, 1957, 1959; Turner and Lalich, 1965). These alterations can also be seen in rats surviving for up to 2 years after a single dose of alkaloid (Schoental and Magee, 1957), indicating that long term effects occur from a single administration of some PAs. Chronic liver alterations are not necessarily preceded by acute necrosis and hemorrhage. Grossly, the liver becomes darkened, congested, shrunken, and firm. The liver surface becomes granular to nodular in appearance, and liver lobes may be grossly deformed (Schoental and Head, 1955, 1957; Schoental and Magee, 1957, 1959).

The histologic changes of chronic PA-induced liver disease are characteristic for PA intoxication. Microscopic alterations include marked hepatocellular hypertrophy (megalocytosis), fibrosis, bile duct proliferation, nodular hepatic regeneration and veno-occlusion of central veins and small hepatic veins (Schoental and Head, 1955, 1957; Schoental and Magee, 1957, 1959). Marked endothelial proliferation in central veins and sinusoids, interpreted at the light microscopic level, was also reported in rats following administration of retrorsine, isatidine, or a mixture of alkaloids from *S. jacobaea* (Davidson, 1935; Schoental *et al.*,

1954). This interpretation was questioned by Selzer and Parker (1951), who concluded that these cells were more likely macrophages.

Megalocytosis of hepatocytes is a hallmark of chronic PA-induced liver alterations (McLean, 1970). Although it can be seen following administration of a variety of hepatotoxicants, including aflatoxins and chemotherapeutic (ie., antimitotic) agents, it is more dramatic in animals poisoned with PAs (Afzelius and Schoental, 1967; Bull, 1955). Megalocytotic hepatocytes are bizarre in appearance and are enlarged to 10 to 30 times normal size. They have gigantic, hyperchromatic nuclei and abundant basophilic cytoplasm. Cytoplasmic invaginations into nuclei are common (Svoboda and Soga, 1966). Cells have increased content of cytoplasmic organelles, including rough endoplasmic reticulum, enlarged golgi, and prominent cytoplasmic filaments, which suggest that these cells are synthetically active and not merely degenerative (Svoboda and Soga, 1966; Afzelius and Schoental, 1967). In addition, these cells have relatively few autophagic vacuoles and fat droplets characteristic of degenerating cells, supporting the belief that these cells are not strictly degenerative (Svoboda and Soga, 1966; Afzelius and Schoental, 1967; McLean, 1970).

Megalocytotic cells are thought to result from a block in cell mitosis. The rate of cell enlargement seems to be partially dependent upon a stimulus for cell turnover, since megalocytotic cells develop much more rapidly in young, growing animals (Jago, 1969; Culvenor et al., 1976) and can be enhanced by stimuli that enhance hepatocellular proliferation, such as partial hepatectomy or post-necrotic healing after carbon tetrachloride poisoning (Peterson, 1965; Downing and Peterson, 1968; Jago,

1969). Downing and Peterson (1968) found that the burst of mitosis in liver cells following partial hepatectomy is markedly inhibited by several hepatotoxic PAs given prior to the operation. This inhibitory effect on cell mitosis was prolonged in excess of 8 weeks. Cells that do begin mitotic division are thought to be incapable of completing the process, forming abnormal mitotic figures interspersed between the enlarged cells. Hepatocytes, although unable to divide effectively, continue to synthesize DNA, RNA, proteins, and constituents necessary for survival and cell enlargement (Afzelius and Schoental, 1967). The presence of multiple centrioles in megalocytotic cells, decreased mitotic figures in the liver parenchymal cells, and abnormal mitotic figures or cells with chromosomes that appear clumped, fragmented, or pulverized supports the premise of inhibited or abnormal cell division. Similar, bizarre megalocytotic cells have been described in the kidneys and lung following exposure to PAs or their pyrrolic derivatives (Hooper, 1974; Harding *et al.*, 1964; Kay *et al.*, 1969; Sugita, 1983b). These cells may be affected by the same antimitotic processes occurring in liver cells.

Veno-occlusion of central and sublobular hepatic veins of the liver is a common finding in PA-exposed cattle (Bras *et al.*, 1957), horses (Hill and Martin, 1958), nonhuman primates (Allen and Carstens, 1968,1969; Allen *et al.*, 1969), man (Bras and Hill, 1956), goats, and less frequently, rats (McLean *et al.*, 1964). The pathogenesis of the veno-occlusive lesion in monkeys caused by MCT appears to involve an initial fragmentation of endothelial cells, followed by subendothelial deposition of fibrin and subsequent organization of this exudate (Allen *et al.*, 1969). Allen and coworkers (1969) suggested that the small-and medium-sized hepatic veins

may be more susceptible to injury than larger vessels because of differences in wall structure. Smaller vessels have a limited cellular investment and supporting tissue, allowing for fluid and blood cells to move more freely into the muscular layer and adventitia of the vessel. Larger vessels, however, possess well-developed muscular walls with abundant collagen and elastic fibers, which could provide greater resistance to movement of blood cells and fluid. Similar principles may dictate which vessels in the arterial bed of the lung are subject to morphologic alterations following exposure to metabolic products of PA bioactivation. As in the liver, smaller vessels may be more prone to damage induced by reactive metabolites of the pneumotoxic or vasotoxic PAs.

In humans, veno-occlusive disease, likened to Budd-Chiari syndrome, is the predominant pathologic effect of PA intoxication (McLean and Mattocks, 1980; Mattocks 1986). Persistent fibrosis develops in the central venous areas and can progress to chronic cirrhosis, but bile duct proliferation does not occur and regenerative nodules are uncommon. (Tandon *et al.*, 1978; Bras and Hill, 1956). A curious feature of the disease in humans is that megalocytosis of hepatocytes has not been observed as it has in other animal species (Tandon *et al.*, 1978; McLean and Mattocks, 1980).

Hepatic fibrosis, bile duct proliferation and nodular regeneration of the liver are common repair responses following hepatic necrosis due to a variety of hepatotoxins and are not specific for PA toxicosis. The fibrosis generally develops in the centrilobular regions of the hepatic

lobule. The severity of fibrosis and bile duct proliferation differs between animal species (Kelly, 1985).

3. Renal Effects

Renal alterations have been reported in a few studies of animals poisoned with PAs, including MCT (Hayashi and Lalich, 1967; Turner and Lalich, 1965; Masugi *et al.*, 1965; Schoental and Head, 1955). They do not, however, appear to be as common as hepatic and pulmonary changes. Grossly, the kidneys of MCT-treated rats can be discolored dark brown and have petechial hemorrhages (Hayashi and Lalich, 1967). Microscopically, most renal changes are vascular in nature. Lesions of the small renal arteries and arterioles include desquamation of endothelial cells, disruption of elastic membranes, and edema of the vessel walls. Medial hypertrophy and intimal hyalinization develop in these vessels and larger arteries undergo fibrinoid necrosis (Hayashi and Lalich, 1967). Glomerular lesions include disrupted endothelial and mesangial cells, glomerular thrombosis, deposition of amorphous fibrillar material resembling basement membrane matrix between cellular components of the glomeruli, and focal to diffuse sclerosis of glomerular tufts (Carstens and Allen, 1970; Masugi *et al.*, 1965; Hayashi and Lalich, 1967; McGrath et al., 1975).

It is not known whether or not the kidneys are capable of bioactivation of PAs to toxic pyrrolic derivatives. Indeed, even if the kidney does possess this capacity, it does not seem likely that locally generated pyrroles would result in damage to the afferent vascular system of the kidneys. It is more likely that the renal microvascular effects are induced by small amounts of primary or secondary pyrroles that escape

binding in the lung and pass to the second vascular bed they encounter to induce damage. Similar appearing vascular lesions have also been occasionally identified in the brain and coronary vasculature of rats (Davidson, 1935; Blaustein *et al.*, 1965). Like the renal microvascular bed, the microvasculature of these organs represents the second vascular bed encountered by reactive species generated in the liver or secondary products produced by hydrolysis of primary pyrroles. Inflammatory mediators or thromboemboli released from the diseased lungs may also contribute to or cause the vascular lesions in these tissues.

Megalocytosis of cells of the proximal convoluted tubules has been described in kidneys of mice fed *Senecio jacobaea* (Hooper, 1974) and pigs fed *Senecio jacobaea* (Harding *et al.*, 1964), *Crotalaria retusa* (Hooper and Scanlan, 1977) or *C. spectabilis* seeds (McGrath *et al.* 1975). Renal megalocytosis may be due to metabolic activation of small amounts of the parent alkaloids to reactive metabolites in kidney cells, since these cells contain some of the same microsomal enzymes as the liver. Alternatively, secondary pyrroles excreted in the urine might cause effects on renal epithelial cells which are manifest as megalocytosis.

Renal hemosiderosis is evident after exposure to several PAs, including MCT (Bull *et al.*, 1968; Schoental and Head, 1955; Hayashi and Lalich, 1967). Hayashi and Lalich (1967) suggested that renal hemosiderosis was due to microangiopathic erythrocyte damage and intravascular hemolysis associated with microvascular damage in the lung and kidney.

4. Cardiac Effects

Cardiac hypertrophy and myocardial degeneration occur in several animal species environmentally or experimentally exposed to a variety of PAs or their pyrrolic metabolites. Cardiac hypertrophy is thought to arise as a consequence of an increase in pulmonary vascular resistance (PVR) and elevated pulmonary arterial pressure (PAP) (Turner and Lalich 1965; Hayashi *et al.*, 1967; Werchan *et al.*, 1989; Meyrick *et al.*, 1980) rather than as a direct effect of the PA or PA metabolites on the myocardium. Grossly, cardiac hypertrophy is restricted to the right ventricle and develops following the appearance of pulmonary alterations and increased PAP in animals administered *C. spectabilis*, MCT, and MCTP (Hislop and Reid, 1974; Meyrick and Reid, 1979; Meyrick *et al.* 1980; Roth *et al.*, 1981; Hilliker *et al.* 1982,1983c; Ghodsi and Will, 1981).

Histologically, cardiac changes are characterized by a mixed response of myocyte hypertrophy and degeneration, multifocal interstitial edema and small multifocal accumulations of mononuclear cells between myocardial fibers. The mononuclear cells are predominantly lymphocytes and monocytes (Lalich and Merkow, 1961; Hayashi and Lalich, 1967; Werchan *et al.*, 1989). These changes are limited to the right ventricular free wall and interventricular septum, sparing the left ventricular free wall (Hayashi and Lalich, 1967).

Coronary vascular alterations, including focal and diffuse atheromatous intimal plaques, were identified in Wistar rats treated with MCT (Blaustein *et al.*, 1965). These lesions were dose-related and were not reversible following withdrawal of MCT from the diet. Vascular

alterations have also been reported in the coronary circulation of monkeys following MCT administration (Chesney and Allen, 1973). These changes could be caused by small quantities of pyrrolic derivatives which escape binding in the first vascular bed. Alternatively, such vascular alterations in the heart and other nontarget tissues may reflect old-age changes which are accentuated in treatment-stressed animals (Greaves and Faccini, 1984). Subendocardial fibrosis in the right ventricle has been reported in monkeys (Chesney and Allen, 1973c).

Bruner *et al.* (1983) described a change in the electrocardiograms of rats treated with a single administration of MCTP. This consisted of a shift in the QRS complex mean electrical axis in the frontal plane and is a reflection of right ventricular hypertrophy (RVH). Although there is morphologic and physiologic evidence of PA-induced cardiac alterations, Werchan and coworkers (1986, 1989) indicated that right ventricular function of MCT treated rats was not impaired.

5. Effects in Other Organs

Lesions in other organs have been sporadically identified in animals with long-standing PA-induced disease. Some changes may be due to direct effects of PA metabolites, but others are thought to be secondary to prolonged portal hypertension related to PA-induced hepatic injury or the consequence of pulmonary damage.

Pancreatic islet cell enlargement occurred in pigs fed the PA-containing plant *C. retusa* (Hooper and Scanlan, 1977). In addition, the PA fulvine is toxic to pancreatic acinar cells (Putzke and Persuad, 1976). These findings, in conjunction with reports that indicate a carcinogenic action of some PAs on pancreatic islet cells (Schoental *et*

al., 1970; Hayashi *et al.*, 1977), suggest that some PAs can have a direct effect on pancreatic parenchymal cells.

Gastrointestinal lesions have been identified in several animal species exposed to PAs. Abomasal edema and gastrointestinal hemorrhage are common in ruminants with long-standing PA-induced disease and are associated with chronic diarrhea (Fowler, 1968; Sippel, 1964). The pathogenesis of these lesions has not been explored. Gastric mucosal erosions have been reported in rats given MCT (Turner and Lalich, 1965), and duodenal mucosal alterations and ulcerations have been reported in rats following administration of heleotrine (Bull *et al.*, 1968) and DHR (Hsu *et al.*, 1974; Allen and Hsu, 1974). Systemically administered, radiolabeled DHR has a specific affinity for cells in the gastric crypts (Allen and Hsu, 1974), resulting in direct injury and inhibited division of these cells. Heliotrine, when administered at doses near the LD 50, can also cause a severe reduction in cell mitosis in the duodenal mucosa of the rat (Bull *et al.*, 1968).

Splenomegaly and splenic hemosiderosis can occur in rats given lasiocarpine, retrorsine or MCT (Schoental, 1959; Schoental and Head 1970), and splenic atrophy can occur in rats given the pyrroles, DHR and dehydroheliotridine (Peterson *et al.*, 1972). Splenomegaly may have been due to portal hypertension and/or reticuloendothelial hyperplasia. Accumulation of hemosiderin in the spleen is likely due to destruction of red blood cells which were damaged by metabolites of the PA or by microangiopathic disruption of these cells passing through the altered vascular bed of the liver and lung. The cause of splenic atrophy is

unknown but could be due to endogenous glucorticoid effects on splenic follicles.

Mediastinal and abdominal lymph nodes may be enlarged and grossly discolored in PA exposed animals. These lymph nodes are edematous and congested and contain increased numbers of mast cells, increased amounts of hemosiderin, and evidence of erythrophagocytosis (Hayashi and Lalich, 1967; Turner and Lalich, 1965). This change is likely secondary to injury in target tissues served by the lymph nodes.

Neurologic signs have been reported in a number of animal species, including cattle, sheep, and horses, following intoxication with PAs (Mattocks, 1986). Hooper (1972) observed spongy degeneration of the brain of calves and sheep given PAs. These signs and morphologic changes may relate to hyperammonemia since they correlate directly with serum ammonia levels, and sheep given ammonium acetate had similar changes. The increased serum ammonia levels results from hepatic insufficiency due to chronic liver damage (hepatic encephalopathy). Vascular changes have been described in the brains of PA-treated rats (Davidson, 1935), but these changes are neither common nor widespread.

PAs can have an effect on germ cells in the testes of animals (Rosenfeld and Beath, 1945; Hsu *et al.*, 1973) and *Drosophila* (Bull *et al.*, 1968). Spermatogenesis is reduced in the testes of mice and rats exposed to heleotrine or its pyrrolic derivative dehydroheliotridine (Rosenfeld and Beath, 1945; Hsu *et al.*, 1973; Peterson *et al.*, 1972). The effects of several PAs on reproductive performance of organisms has been studied in *Drosophila* (Clark, 1959, 1960). Exposure to PAs resulted in an increase in sex-linked lethal traits in progeny of exposed *Drosophila*.

Many other changes associated with PA or pyrrole intoxication have been reported, but their incidence is infrequent. Increased incidence of mesenteric arteritis (McLean *et al.*, 1967), arterial lesions in the pancreas, and pancreatic edema (Schoental, 1959; Carstens and Allen, 1970) were seen in long-term studies in rats given MCT. These changes may have related to an increase in polyarteritis nodosa commonly seen in many strains of aged rats. Atrophy of hair follicles, thymus and bone marrow can occur in rats given dehydroheliotridine (Peterson *et al.*, 1972). In these rats tooth defects and necrosis of salivary gland ducts also occurred.

6. Carcinogenic Effects

The carcinogenicity of PAs was first described in 1950 by Cooke *et al.*, who produced liver tumors in rats treated with alkaloids of *Senecio jacobaea*. Since then, a number of PAs (Harris and Chen, 1970; Hooson *et al.*, 1973; Schoental and Head, 1954; Schoental *et al.*, 1954), including MCT (Newberne and Rogers, 1973) and one of MCT's pyrrolic derivatives, have been identified as carcinogens in animals. Shumaker *et al.* (1976) have shown that tumors in a variety of organs are increased in frequency following long-term treatment with MCT. These tumors included pulmonary adenocarcinomas and hepatocellular carcinomas (Shumaker *et al.*, 1976). A high incidence of insulinomas occurred in rats after a single administration of MCT (Hayashi *et al.*, 1977) and PAs extracted from *Amsinckia intermedia* and *Heliotropium supinum* (Schoental *et al.*, 1970).

Pyrrolic derivatives of some PAs are also carcinogenic. Intraperitoneal administration to rats of the pyrrole, dehydroheliotridine, induced a variety of tumors in rats, including

hepatomas and pulmonary adenomas (Peterson *et al.*, 1983). Basal cell and squamous cell carcinomas in mice and rhabdomyosarcomas in rats occurred at the site of injection of the pyrrole DHR. (Shumaker *et al.*, 1976; Allen *et al.*, 1975). Production of tumors at the site of application of pyrrolic derivatives supports the premise that pyrrolic metabolites of PAs are the proximate carcinogens. Parent alkaloids do not produce tumors at the site of administration but do induce tumors at distant sites. This supports the premise that PAs must be bioactivated before achieving their toxic and carcinogenic potential.

7. Teratogenic and Embryotoxic Effects

Skeletal and parenchymal malformations have appeared in fetal rat pups after feeding of heliotrine to pregnant rats during days 12-16 of gestation (Green and Christie, 1961). Skeletal malformations included dwarfism, deformed ribs, and hypoplasia of the lower jaws. Very high doses of heliotrine were embryotoxic, leading to fetal death. Fulvine was also teratogenic and embryotoxic when given to pregnant rats (Persaud and Hoyte, 1974). Fulvine caused dose-related fetal resorptions. The relatively stable pyrrole dehydroheliotridine is also teratogenic in pregnant rats (Peterson and Jago, 1980).

8. Pulmonary Effects

Several hepatotoxic PAs and some pyrrolic metabolites of toxic PAs cause delayed and progressive pulmonary damage in rats, monkeys, dogs, swine, and poultry (McLean, 1970; Culvenor *et al.*, 1976, Barnes *et al.*, 1964). Pulmonary disease in mice (Harris *et al.*, 1942a), cattle (Sanders *et al.*, 1936), sheep (Laws, 1968), and a human infant (Fox *et al.*, 1978) exposed to toxic PAs have been reported, although detailed morphologic

descriptions of the pulmonary alterations were not given. Pulmonary lesions induced by PAs are produced by the same or larger doses needed to produce minimal liver injury (Culvenor *et al.*, 1976).

Plants of the *Crotalaria* genus are most commonly associated with pulmonary disease (McLean, 1970), and *C. spectabilis* and MCT-induced lung injuries are by far the best characterized of the PA-induced lung diseases. Because of the morphologic similarities of diseases induced by these PAs to chronic cardiopulmonary disease with pulmonary hypertension (PH) of humans, PA-induced pulmonary disease has developed as a model of this disease. The pulmonary pathology resulting from MCT administration to laboratory animals is described in detail below (Section E).

C. Pulmonary Hypertension in Humans

Chronic PH in humans is caused by mechanical obstruction of blood flow through the pulmonary arterial circulation (Spencer, 1985). It has a variety of causes, including cardiac abnormalities, recurrent thromboembolic disorders and chronic interstitial or obstructive lung disease. It can also be sequela to chronic hypoxia, hyperoxia, treatment with various chemotherapeutic agents or adult respiratory distress syndrome (ARDS) (Reid, 1986; Snow *et al.*, 1982; Meyrick and Brigham, 1986). An additional disorder associated with chronic PH is primary or idiopathic pulmonary hypertension (PPH). Because of the similarities in the pathophysiologic features of PPH and MCT-induced pulmonary disease, the latter has been developed as a model of this hypertensive disorder of humans.

PPH is a disorder of unknown etiology. It can occur in either sex and at any age (Cotran *et al.*, 1989; Thurlbeck and Miller, 1988), although it may have a higher incidence in women in the third and fourth decades of life (Walcott *et al.*, 1970; Spencer, 1985). In cases of chronic pulmonary hypertensive disease, the diagnosis of PPH is made by exclusion, ie., by eliminating other known causes of PH (Walcott *et al.*, 1970; McLeod and Jewitt, 1986). The disease may in fact represent more than one disorder caused by different etiologic factors (McLeod and Jewitt, 1986). The pathophysiologic features of PPH, particularly in the early stages, are not known, partly because the disorder is clinically silent until lesions are advanced. Although the morbidity of this disease is low, mortality is high, and effective long-term treatment is not available. The disease is generally fatal within two to eight years (Spencer, 1985; Walcott *et al.*, 1970).

Many of the pathologic changes observed in patients with PPH are similar to those seen in rats exposed to modest doses of MCT (Roth and Ganey, 1988; Kay and Heath, 1969). PPH, as MCT-induced lung disease, is characterized by remodeling of the pulmonary arterial vasculature and pulmonary interstitial inflammation with interstitial fibrosis. Vascular remodeling involves thickening of the medial layer of muscular pulmonary arteries and muscularization of partially muscular and nonmuscular pulmonary arteries. In addition, intimal proliferations and adventitial changes are observed, as are necrotizing arteritis and vascular thrombosis (Walcott *et al.*, 1970; Watanabe and Ogata, 1976; Voelkel and Reeves, 1979; Reid, 1986). Plexiform lesions, or angiomatous tufts within vessel lumina, are also described in many, but not all, human patients and are

considered by some pathologists to be sufficient for a diagnosis of PPH (Walcott *et al.*, 1970; Cotran *et al.*, 1989). In rats administered MCT, plexiform lesions have been reported (Watanabe and Ogata, 1976) but are rare. Sequela to the pulmonary vascular changes are RVH and eventual right-sided heart failure (Voelkel and Reeves, 1979). Chronic PH can also develop in people as a sequela to adult respiratory distress syndrome (ARDS). ARDS can be initiated by a number of causes, including nonthoracic trauma, septicemia, shock and thermal injury. Many cases resolve with little residual lung or vascular damage, but other cases progress to widespread pulmonary fibrotic disease and vascular remodeling. Acute stages of ARDS are associated with noncardiogenic pulmonary edema, vascular thromboembolism and inflammatory cell sequestration in the lung (Tomachefski *et al.*, 1983; Snow *et al.*, 1982). Although similar changes do not develop acutely in MCT-induced disease, they are present within days to weeks of exposure. Morphologic alterations in late stages of the lung injury of ARDS closely resemble the changes induced by MCT and include muscularization of nonmuscular arteries and medial thickening of medial layers of muscular vessels (Snow *et al.*, 1982). In fact, some investigators suggest that MCT-induced cardiovascular disease may be an appropriate model for this late stage of ARDS (Langleben and Reid, 1985).

D. Models of Human Pulmonary Hypertension

MCT-induced lung disease has been developed as perhaps the earliest and one of the most widely investigated models of persistent PH in humans. A variety of other models of PH, however, do exist (Figure 2-4). Although each of these diseases presumably has some unique pathophysiologic

features, they generally appear to result in pulmonary vascular remodeling and increased PAP, which ultimately results in RVH (Coflesky *et al.*, 1988; Perkett *et al.*, 1986; Kirton and Jones, 1987; Meyrick and Reid, 1980; Hill *et al.*, 1984; Perkett *et al.*, 1988b; Wright, 1962; Herget *et al.*, 1981). In many of these models alterations in the arterial vascular bed have been detailed morphometrically, ie., hyperoxia, air embolization, endotoxin, MCT, indomethacin, radiation, hypoxia. It appears that an early change in vessel architecture is muscularization of small, nonmuscular pulmonary arteries. In addition, the medial layer of muscular arteries becomes progressively thicker as the disease develops (hyperoxia, air embolization, MCT, hypoxia) and this medial thickening occurs first in the smaller muscular arteries and later progresses to involve the larger vessels of the pulmonary trunk. In several of these models, venous changes have also been reported, but these are generally less pronounced than the arterial changes (eg., hyperoxia, MCT, endotoxin)(Hu and Jones, 1989; Will, 1981). Alpha-naphthalthiourea (ANTU)-induced PH appears to be the only exception to the axiom that PH is associated with arterial vascular remodeling. Hill *et al.* (1984) citing very limited morphometric studies, indicated that medial thickening of pulmonary arteries does not occur in this model.

Morphologic evidence of endothelial cell damage is an early feature of many models of PPH (ie., hyperoxia, radiation, hypoxia, MCT, endotoxin, ANTU, air embolization). Indeed, endothelial cells may be a major target of these toxicants. *In vitro*, several of these toxicants have been shown to be cytotoxic to endothelial cells or to alter endothelial cell function and morphology(radiation, hyperoxia, endotoxin, hypoxia)

Figure 1-4: Models of Persistent Pulmonary Hypertension

Monocrotaline . . . . . (Kay et al., 1966)

Hyperoxia . . . . . (Coflesky et al., 1988)

Hypoxia . . . . . (Meyrick and Reid, 1979b)

. . . . . (Rabinovitch et al., 1979)

Air Embolization . . . . . (Perkett et al., 1988)

Lung Irradiation . . . . . (Perkett, 1986)

. . . . . (Meyrick, 1987)

Repeated or Continuous . . . . . (Meyrick and Brigham, 1986)

Endotoxin Administration . . . . . (Kirton and Jones, 1987)

Carragean . . . . . (Herget et al., 1981)

ANTU . . . . . (Hill et al., 1984)

Indomethacin . . . . . (Meyrick et al., 1985)

(Bowman *et al.*, 1983; Rubin *et al* 1985; Meyrick 1986; Rosen *et al.*, 1989; Nawroth *et al.*, 1984). For some models, a single exposure to the toxicant may be sufficient to cause persistent or long-lived damage to endothelial cells and PH (MCT, radiation) (Hirst *et al.*, 1980). In others, however, intermittent or continual exposure may be necessary to elicit the chronic pulmonary hypertensive change (hyperoxia, hypoxia, endotoxin, air embolization, ANTU, indomethacin). Persistent or long-lived endothelial injury may be the critical event in this disease (Meyrick *et al.*, 1987).

Sublethal insults to endothelial cells with ineffective repair could result in persistent cell dysfunction and pulmonary vascular leak which do not readily resolve. Dysfunction of the endothelium may result in altered responses to endothelial-dependent vasoactive mediators and altered production of endothelial-derived growth factors and other molecules which alter smooth muscle cell behavior. Indeed it has been shown that hyperoxia alters endothelial-dependent relaxation and pharmacological sensitivity of pulmonary arteries to various agonists in rats (Coflesky *et al.*, 1988). In addition, exposure of endothelial cells *in vitro* to endotoxin or radiation stimulate cells to release enhanced amounts of prostacyclin (PGI_2)(endotoxin, radiation)(Meyrick, 1986; Rosen *et al.*, 1989; Nawroth *et al.*, 1984, Phillips *et al* 1988). Thus, endothelial cell dysfunction induced by these toxicants may alter endothelial-dependent, physiologic responses of the vasculature. Such alterations may be long-lived and may contribute to the hypertensive response.

Increased vascular permeability occurs following endothelial damage in many of these models (hyperoxia, radiation, MCT, ANTU, air

embolization, endotoxin). Prolonged vascular permeability and the resultant pulmonary edema could play a role in the development of PH (Sugita *et al.*, 1983; Perkett *et al.*, 1988; Coflesky *et al.*, 1988). Physical compression of small vessels and capillaries as the pulmonary interstitium fills with exudate may increase vascular resistance and ultimately lead to PH (Ngeow and Mitzner, 1983). The presence of exudate in the interstitium might also instigate an inflammatory reaction associated with the activation or release of inflammatory mediators. Many such mediators have vasoactive properties which could affect vascular tone and resistance.

Indeed, interstitial inflammation appears to a feature of most models of PH. In the caragean, air embolization, radiation, MCT, indomethacin and endotoxin models, interstitial inflammatory infiltrates occur which becomes more severe as the disease develops (Meyrick *et al.*, 1985). Inflammation and release of vasoactive mediators from inflammatory cells may contribute to an altered vasoactive response (Meyrick *et al.*, 1985; Coflesky *et al.*, 1988) and could be involved with the development of PH in some of these models (Meyrick *et al.*, 1987). The findings of two investigators question the role of inflammatory mediators in the development of PH. Hill et al. (1984) indicated that repeated ANTU administration caused acute sequestration of inflammatory cells in the lung but did not induce a sustained inflammatory reaction even though the PH was sustained. Also, Huxtable *et al.* (1978) has also reported that MCT, when administered in the drinking water at 20 mg/liter of water, does not induce pulmonary inflammation, but does induce vascular remodeling.

Thus sustained inflammation may not be required for the pulmonary hypertensive response.

There are apparent differences in some models of PH. A feature of the MCT model is that a single dose of the toxicant is sufficient to induce progressive pulmonary alterations and hypertension (Hayashi and Lalich, 1967), whereas in the other models long-term or multiple exposures are necessary to induce the hypertensive response. Radiation-induced lung injury may have some parallels to MCT-induced lung injury in that a single administration can result in long-term pulmonary injury and hypertension. This might suggest that MCT metabolites and thoracic radiation cause a more long-lived injury to the lung, particularly the endothelium. An additional difference is that in the MCT, hyperoxia, hypoxia, continuous air embolization and radiation models, the PH that develops is delayed in appearance for days to weeks after treatment and is progressive. In the ANTU and endotoxin models, PH can occur soon after administration of the toxins and is sustained. The reason for this difference is not entirely clear but may relate to acute sequestration of inflammatory cells and release of inflammatory mediators in the lungs of animals exposed to these latter insults.

Although there are some differences among these models, similarities in the pathophysiologic features of these diseases may provide some clue to common mechanisms of PH.

E. Monocrotaline-induced Pulmonary Disease

1. General

An enormous body of morphologic, physiologic, and biochemical information pertaining to the pulmonary disease induced by *C. spectabilis* and MCT has been reported. Although other potentially toxic PAs are present in *C. spectabilis*, results of these studies clearly implicate MCT as an important pneumotoxic principle of *C. spectabilis* (Lalich and Ehrhart, 1962; Turner and Lalich, 1965). MCT has been shown to induce virtually all of the pathophysiologic and morphologic abnormalities induced by feeding *C. spectabilis*. Discrepancies between studies can be largely attributed to differences in dosages of the toxicant, the routes and schedule of exposures, or animal factors. MCT and *C. spectabilis*-induced diseases will be considered one and the same.

Schoental and Head (1955) were the first to report consistent changes in the lungs of weanling and adult rats after exposure to MCT. They noted that hepatic and pulmonary damage occurred after dermal, oral, or subcutaneous administration of MCT. Their study focused largely on the hepatic abnormalities.

Systematic investigations of PA-induced pulmonary disease began in the early 1960s with the investigation of Lalich and Merkow (1961). These investigators observed that feeding seeds of *C. spectabilis* at a concentration of 0.013 to 0.2% of the diet induced a progressive and fatal pulmonary disease in rats. The disease was associated with vascular lesions that included pulmonary arteritis with periarterial and medial leukocyte infiltration. In an effort to identify the vasotoxic principle

of *C. spectabilis*, Lalich and Ehrhart (1962) found that nearly identical lesions were produced after prolonged feeding of MCT at 0.003% of the diet (30 mg/kg of food). In both of these studies, continuous exposure regimens were used, and pulmonary arterial lesions were found after the spontaneous death of the animals 4 to 6 weeks after initial exposure. Hayashi and Lalich (1967) reported that similar pulmonary alterations could be induced in rats after a single exposure to MCT.

Cardiac hypertrophy in conjunction with the pulmonary vascular abnormalities was noted in early experimental studies (Young, 1962; Turner and Lalich, 1965). Turner and Lalich (1965) demonstrated quantitatively that cardiac hypertrophy occurred in rats fed seeds of the plant and suggested that the rats had *cor pulmonale*. Kay *et al.* (1967) confirmed that PH developed in rats fed *C. spectabilis* seeds. The induction of pulmonary hypertensive disease by ingestion of a toxicant was a novel toxicologic response and suggested that PPH of man may have a dietary basis (Kay *et al.*, 1967).

2. Macroscopic Pulmonary Alterations

Grossly, the lungs of MCT-treated rats become congested, bulky, and edematous (Schoental and Head, 1955; Lalich and Merkow, 1961). In later stages, hemorrhagic foci are evident in areas of induration (Turner and Lalich, 1965; Hayashi *et al.*, 1984). Lesions become more pronounced with time (Hayashi *et al.*, 1984). Occasionally, entire lung lobes become consolidated. The pleural cavity frequently contains excess fluid that has modest increases in protein concentration (Mattocks, 1972).

3. Microscopic Pulmonary Alterations

a. Pulmonary Vascular Changes

A hallmark in both MCT and *C. spectabilis*-treated rats is increased thickness of the medial layer of pulmonary arteries (Turner and Lalich, 1965; Hayashi and Lalich, 1967; Ghodsi and Will, 1981; Kay *et al.*, 1982a; Hayashi *et al.*, 1984; Molteni *et al.*, 1984). Kay and Heath (1966) were the first to assess morphometrically changes in the pulmonary trunk and vasculature after prolonged feeding of *C. spectabilis* seeds. Rats fed *C. spectabilis* seeds for 5 weeks or more, had significant thickening of the pulmonary trunk, medial hypertrophy of small pulmonary arteries, and right ventricular enlargement in the heart.

The thickening of the wall of the pulmonary trunk was due in part to a proliferation of smooth muscle cells between the elastic laminae (Kay and Heath 1966), an increased number of elastic laminae in the medial layer (Kay and Heath, 1966) and an increase in vessel wall collagen content (Kameji *et al.*, 1980). There was also the appearance of smooth muscle cells in the adventitia (Kay and Heath, 1966). Intimal proliferations in thickened vessels were not seen by Kay and Heath (1970), but have been reported by other investigators (Merkow and Kleinerman, 1965; Hayashi and Lalich, 1967).

Small muscular arteries become thicker earlier and to a greater degree than larger pulmonary vessels (Turner and Lalich, 1965; Hayashi and Lalich, 1967; Meyrick and Reid, 1979a). The medial thickening of smaller pulmonary vessels is the result of hypertrophy of smooth muscle cells and possible proliferation of circularly oriented smooth muscle between the internal and external elastic laminae (Meyrick and Reid, 1979a, 1982;

Ghodzi and Will, 1981). Meyrick and Reid (1982) found that smooth muscle cells of the arterial medial layers had a small increase in ^{3}H-thymidine incorporation. The authors interpreted this as an indication of cell hyperplasia, although other factors could account for the increases observed, such as DNA repair or DNA synthesis without progression through the cell cycle. Turner and Lalich (1965) observed that cells, which appeared to be smooth muscle cells, developed outside the elastic laminae. They stated that these smooth muscle cells arose from premyocytes in the perivascular interstitium. Premyocytes were not defined as a particular cell type in this study, and no evidence was given that these cells in the perivascular interstitium had well defined features of smooth muscle cells.

In addition to thickening of the medial layer of muscular blood vessels, there is muscularization of small arteries which do not typically contain a muscular wall (Hislop and Reid, 1974; Kay and Heath, 1966). In these vessels, smooth muscle cells are present inside the internal elastic laminae. They are thought to arise from pericytes and intermediate cells in nonmuscular vessels (Meyrick and Reid, 1979a, 1982; Meyrick *et al.*, 1980).

Meyrick and Reid (1979a) examined the development of the arterial alterations and found that a trend toward thickening of the medial layer of muscular arteries was evident as early as 7 days following initiation of feeding seeds of *C. spectabilis*. The increased thickness reached statistical significance 10 days after feeding was begun. They also found that muscularization of nonmuscular arteries of the lung began as early as 3 days after exposure (Meyrick and Reid, 1979a). A comparable time

course for vascular alterations was reported by Ghodsi and Will (1981) using a single subcutaneous injection of MCT (60 mg/kg). Medial thickening of pulmonary arteries was evident at 1 week posttreatment and was more pronounced later. They did not examine rats at times earlier than 1 week. In both studies, vessel thickening preceded the development of PH and RVH. These structural changes in the pulmonary arteries and nonmuscular arteries reduced the cross-sectional area of the vessels by encroaching on the lumen (Meyrick, *et al.*, 1980).

Pulmonary arteritis occurs in animals exposed to MCT or *C. spectabilis* (Lalich and Ehrhart, 1962; Turner and Lalich, 1965; Kay and Heath, 1966), but it is generally not widespread. Frequently only one to several vessels of each rat lung are affected. Morphologically, pulmonary arteritis has been divided into acute and chronic phases (Turner and Lalich, 1965). In the acute phase, there is complete or partial destruction of the external elastic lamina. The vascular media appears necrotic and, along with the adventitia, is heavily infiltrated by inflammatory cells (Turner and Lalich, 1965; Kay and Heath, 1969). The internal elastic laminae of these vessels remain intact. In the healing or chronic stages, the adventitia and outer portion of the media are replaced by granulation tissue infiltrated by large numbers of cells (Kay and Heath, 1968). Acute and chronic stages of arteritis can be seen in the same lung lobe (Turner and Lalich, 1967). Lesions often start at the site of arterial branching and extend for variable distances along the length of the vessel (Turner and Lalich, 1967; Kay and Heath, 1969).

The incidence of pulmonary arteritis appears to vary markedly among studies. Lalich and Merkow (1961) reported finding arteritis in a high

proportion of rats from their study. In other studies, this lesion was only seen in a small fraction of the treated animals or not at all (Kay and Heath, 1966; Kay *et al.*, 1967; Meyrick and Reid, 1979; Meyrick *et al.*, 1980; Hislop and Reid, 1974; Turner and Lalich, 1965). The incidence of the lesion appears to depend in part on the duration of animal survival following exposure to the toxicant (Lalich and Merkow, 1961). Lalich and Merkow (1961) found that animals given more modest doses of *C. spectabilis* survived longer and had a higher incidence of arterial lesions than those given larger doses. Pulmonary arteritis develops relatively late in the course of the pulmonary disease (Hayashi and Lalich, 1967; Hislop and Reid, 1974). Animals that died or were killed at early stages of the disease prior to the occurrence of a substantial hypertensive response did not have this lesion.

Necrotizing arteritis has also been described in pulmonary arteries (Lalich and Merkow, 1961; Kay and Heath, 1966; Lalich and Ehrhart, 1962; Young, 1962). Necrotizing arteritis and pulmonary arteritis may in fact be the same lesion. Ultrastructural studies have shown that a true necrotizing response is not present (Merkow and Kleinerman, 1966). The vessel changes are more typical of fibrinoid vasculitis characterized by hyalinization of vessel walls. This hyalinized appearance was caused by PAS-positive material in the subendothelial intima, between smooth muscle cells of the media, and extending into the adventitia. This material is thought to be derived from plasma proteins that become trapped in the vessel wall. Necrosis of smooth muscle cells was not evident, and the ultrastructural appearance of these cells suggested heightened cellular activity.

It is of interest that the lesion develops at the site of arterial branching. Generally the toxicant affects areas of the vasculature where blood flow is slowed, thereby allowing interaction of the toxicant with the vessel walls (McLean, 1970). The branch points of vessels are areas of blood turbulence where the laminar flow of blood is disrupted. This disturbance of flow may deliver toxic pyrrolic metabolites directly to the endothelial surface. In addition, the turbulent flow in these areas demands a greater turnover rate of endothelial cells because of higher stresses on the cells. Toxic effects on cells and a block in cell proliferation could predispose these areas to vascular damage.

A decrease in the number of peripheral arteries has been reported in the chronic stages of lung disease caused by *C. spectabilis* (Hislop and Reid, 1974; Meyrick and Reid, 1979). These investigators also found "ghost" arteries (i.e. remnants of obliterated vessels) in late stages of the disease. In contrast to these findings, Kay *et al.* (1982a) did not find a decrease in the number of small pulmonary blood vessels in MCT-treated rats. The discrepancy between these studies could be attributed to differences in perfusion and fixation techniques.

Vascular thrombosis with platelet-rich or fibrin thrombi occurs in lungs of *C. spectabilis* and MCT-treated rats (Turner and Lalich, 1965; Lalich and Ehrhart, 1962; Kay and Heath, 1966; Merkow and Kleinerman, 1966; Valdivia *et al.*, 1967a,b; Smith and Heath, 1978; Meyrick and Reid, 1982). Thrombi are present in both the early (Valdivia *et al.*, 1967a,b) and late stages of *C. spectabilis* and MCT-induced lung injury (Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Hayashi and Lalich, 1967). Endothelium adjacent to these thrombi is often swollen or hypertrophied

(Turner and Lalich, 1965). Late in the disease, various stages of incorporation of fibrin thrombi into vascular walls can be found, resulting in a thickened intima and fibroelastosis. These thrombi and intimal lesions can cause significant luminal obstruction in damaged arteries (Turner and Lalich, 1965).

Small pulmonary veins are also affected following MCT (Will, 1981) and *C. spectabilis* ingestion (Kay and Heath, 1966; Heath and Smith, 1978). The predominant change is thickening of fibromuscular pads of these vessels. Ultrastructural studies by Smith and Heath (1978) revealed smooth muscle cell evaginations suggestive of a veno-constrictive response after feeding of *Crotalaria* seeds. In general, the venous alterations have not been scrutinized in the same detail as have the arterial changes. In fact, many reports either do not mention venous changes in their description of the pulmonary pathology or indicate that venous changes do not occur (Turner and Lalich, 1965).

Endothelial cell alterations occur in lung vessels of MCT- and *C. spectabilis*-treated rats and are particularly prevalent in the arteries and capillaries (Valdivia *et al*,. 1967a,b; Turner and Lalich, 1965; Kay *et al.*, 1969; Rosenberg and Rabinovitch, 1988). Endothelial damage is apparent as swelling and rarefaction of endothelial cell cytoplasm (Valdivia *et al.*, 1967a; Rosenberg and Rabinovitch, 1988) or altered staining intensity of the cytoplasm (Plestina and Stoner, 1972). Endothelial blebs or vesicles also occur in some cells (Valdivia *et al.*, 1967b; Kay *et al*, 1969). Kay and coworkers (1969) indicated that endothelial vesicles were the result of subendothelial accumulation of interstitial fluid. In addition, they reported that endothelial cells had

increased numbers of pinocytotic vesicles in the cytoplasm of these cells. Rosenberg and Rabinovitch (1988) found that these cells had swelling of mitochondria, a decrease in microfilaments and an increase in subcellular ground substance at 4 days posttreatment with MCT.

In later stages of the disease, endothelial hypertrophy and hyperplasia occur (Lalich and Merkow, 1961; Meyrick and Reid, 1982; Turner and Lalich, 1967; Hislop and Reid, 1974; Vincic *et al.*, 1989). Meyrick and Reid (1982) reported that hypertrophied endothelial cells had an increase in amounts of cytoplasmic organelles including endoplasmic reticulum and ribosomes and had prominent nucleoli. Endothelial hypertrophy and hyperplasia were reported to cause complete obstruction of some small arteries and capillaries (Turner and Lalich, 1965; Hislop and Reid, 1974). Meyrick and Reid (1982) found that in rats fed *C. spectabilis*, the number of endothelial cell nuclei per luminal surface of pulmonary arteries was increased above that of controls, but not until 21 days after initiation of exposure. This indicates that endothelial hyperplasia in these vessels may be a late response to injury.

The adventitia of pulmonary vessels and adjacent interstitial tissue of MCT or *C. spectabilis* treated rats generally contain an infiltrate of cells and increased numbers of fibroblasts and smooth muscle cells (Turner and Lalich, 1965; Lalich and Merkow, 1961; Meyrick and Reid, 1982; Molteni *et al.*, 1984). The infiltrate is predominantly mononuclear and includes monocytes, macrophages, mast cells, and lymphocytes.

Changes in the pulmonary vasculature and microvascular bed are a hallmark of MCT-induced disease. Both cellular and noncellular interstitial constituents are involved. The lesions are slow to develop

but progressive even after a single administration, suggesting a continuous effect of the toxicant on the vasculature long after the majority of toxicant is removed from circulation. Pulmonary arteritis occurs as a late response and involves leakage of serum products into the vascular wall and perivascular inflammation.

b. Pulmonary Parenchymal Alterations

Capillary congestion and interstitial edema of the alveolar septal walls and perivascular and peribronchiolar interstitium are seen early in the course of the disease (Valdivia *et al.*, 1967a,b; Hayashi and Lalich, 1967; Heath, 1969). The interstitial edema causes thickening of septal walls and widening of the perivascular interstitium. Interstitial lymphatics are distended with proteinaceous fluid and extravasated erythrocytes (Hayashi and Lalich, 1967; Valdivia *et al.*, 1967b). Interstitial edema tends to be an early and progressive lesion (Hayashi *et al.* 1984; Valdivia *et al.*, 1967a,b). The pulmonary interstitium is hypercellular and contains increased numbers of mononuclear cells, including lymphocytes and macrophages (Hayashi and Lalich, 1967). Mast cell hyperplasia is also apparent and is particularly prominent in the perivascular and peribronchiolar interstitium (Takeoka *et al.*, 1962; Hayashi and Lalich, 1967; Kay *et al.*, 1967b, 1969; Raczniak *et al.*, 1979).

Alveolar hemorrhage and flooding of alveoli with serous to serofibrinous exudate occur in later stages of the disease, and alveolar macrophages are increased in number. Intracellular edema and swelling occurs in type I epithelial cells (Kay *et al.*, 1969). In addition, metaplasia of alveolar epithelium to cuboidal cells has been described (Turner and Lalich, 1965). It is not known whether these cells arose by

metaplasia of the alveolar epithelium or proliferation of airway epithelium into the alveoli, but they were ciliated cells. Hyperplastic and hypertrophic changes in the bronchiolar epithelial cells have been seen in animals exposed to MCT (Lalich and Merkow, 1961; Lalich and Ehrhart, 1962; Turner and Lalich, 1965; Kay and Heath, 1966; Merkow and Kleinerman, 1966). The incidence of the bronchiolar alterations varies among studies but is generally not high (approximately 30% or less of rats per study). Interstitial fibrosis occurs late in the course of the disease (Kay *et al.*, 1969; Turner and Lalich, 1965).

A peculiar feature of PA- and MCT-associated lung disease is the appearance of large, bizarre cells in the pulmonary parenchyma (Barnes *et al.*, 1964, Valdivia *et al.*, 1967b; Kay *et al.*, 1969; Sugita *et al.*, 1983b; Stenmark *et al.*, 1985). Megalocytosis of septal and perivascular interstitial cells and the appearance of large atypical cells in alveolar sacs or attached to the alveolar walls have been reported in nearly all detailed descriptions of natural and experimentally induced lung disease. Based on morphologic studies, megalocytotic cells found free in the alveolar sacs or attached to the epithelium have been referred to as abnormal type II cells or granular pneumocytes (Kay *et al.*, 1969; Butler *et al* 1970). Sugita *et al.* (1983b), however, thought that these cells were abnormal macrophages. In actuality, both cell types may be involved. The megalocytotic interstitial cells may be of fibroblastic origin (Butler, 1970). The development of the abnormal megalocytotic cells in the lung has been equated with the development of the megalocytotic cells of the liver (McLean, 1970).

F. MCTP-induced Lung Disease

Several investigators have reported the effects of MCTP on pulmonary morphology (Chesney *et al.*, 1974; Butler *et al.*, 1970; Raczniak *et al.*, 1979; Lalich *et al.*, 1977; Plestina and Stoner, 1972; Hurley and Jago, 1975). The pulmonary lesions induced by MCTP are dose dependent (Plestina and Stoner, 1972). Large doses of MCTP (>10 mg/kg) induce acute, widespread pulmonary edema, pleural effusion, and animal death within 48 hours (Hurley and Jago, 1975; Plestina and Stoner, 1972). Lower doses (2 to 4 mg/kg) induce subacute to chronic pulmonary alterations similar to those induced by MCT (Chesney *et al.*, 1974; Lalich *et al.*, 1977; Raczniak *et al.*, 1979).

The early morphologic pulmonary alterations induced by MCTP have only been detailed for rats given large doses of the toxicant (Plestina and Stoner, 1972; Hurley and Jago, 1975). Although the endothelium is a proposed target of MCTP, widespread evidence of endothelial cell necrosis is not apparent as vascular leak is developing (Plestina and Stoner, 1972; Hurley and Jago, 1975). Large doses of MCTP cause congestion of the vasculature and an increase in lung weight by 6 hours after treatment. This is followed by leakage of circulating carbon particles from capillaries and venules at 9 hours after treatment (Plestina and Stoner, 1972; Hurley and Jago, 1975). The leakage of carbon particles does not appear to be associated with large defects in the endothelium (Plestina and Stoner, 1972). Ultrastructurally, carbon particles are evident within endothelial vesicles and beneath endothelial cells with intact, tight junctions.

Hurley and Jago (1975) stated that early injury to endothelial cells tends to resolve partially, with time. At 11 and 19 hours after treatment with high doses of MCTP, a few endothelial cells had abnormally dense or swollen cytoplasm containing large vacuoles. Most of the endothelium, however, appeared normal. By 44 hours, much vascular endothelium appeared normal and all small vessels were patent. The swollen or abnormally dense endothelial cells were no longer evident, but many of the endothelial cells of capillaries and venules had prominent nuclei and thickened cytoplasm (Hurley and Jago, 1975). These findings suggest that a single injection of a high dose of MCTP may not be acutely lethal to most endothelial cells, but it can alter vascular permeability.

Butler (1970) found that lower doses of MCTP also caused changes in endothelial cells by 1 week. He stressed that endothelial cells of capillaries and small vessels were markedly enlarged and caused partial vessel occlusion. The cytoplasm and plasma membranes near junctions of these cells were frequently stacked in multiple layers, and tight junctions were evident between leaves of adjacent cells. The cells contained increased amounts of rough endoplasmic reticulum, polysomes, Golgi bodies, mitochondria, and pinocytotic vesicles. Endothelial cells appeared more atypical with time. These changes are reminiscent of megalocytotic changes in other lung cells and the liver.

Morphologic studies employing MCTP, particularly at low, PH-producing doses, are few. They lack detailed descriptions of the early lesions (i.e. <7 days) induced by this compound. There is also controversy as to whether structural remodeling of arteries occurs after exposure. Butler *et al.* (1970) indicated that pulmonary arterial lesions

similar to those induced by *C. spectabilis* feeding were not present in rats in their study, and Mattocks (1986) stated that vascular remodeling does not occur. In contrast, Chesney *et al.* (1974) and Lalich *et al.* (1977) indicated that arterial lesions were present in rats 4 weeks after treatment. This controversy must be resolved if MCTP-induced PH is to be used as a model of PH in humans, since the arterial lesions are a hallmark of the human disease.

G. Progression of MCT/MCTP-induced Cardiopulmonary Lesions

Results of a number of morphologic studies detailing the time course of lung injury indicate that *C. spectabilis*, MCT, or low doses of MCTP induce delayed and progressive lung injury (Hayashi *et al.*, 1984; Meyrick *et al.*, 1980; Butler *et al.*, 1970). Valdivia and coworkers (1967a,b) were the first to examine the development of lung injury following MCT exposure. The earliest change they identified was a slight increase in interstitial edema 4 to 48 hours after treatment. This early injury was subtle and relatively minor compared to the extensive and severe changes observed at 7 days and thereafter. The earliest MCTP-induced lesions evident by light microscopy were evident 2 to 3 days posttreatment and consisted of edema and congestion of the alveolar septa and dilated interstitial lymphatics (Butler *et al.*, 1970). Later (i.e., 1 to 2 weeks), the alveolar walls were increasingly abnormal with edema of the interstitium, reduction of numbers of alveoli, and increased cellularity of the septa. Large abnormal cells were seen at later times in the septa at this time and there was measurable PH. The response of the lung to MCT or MCTP has been divided into three phases, based on morphologic studies:

1) a pulmonary edema phase, 2) an interstitial inflammatory phase, and 3) a pulmonary hypertensive phase (McLean, 1970).

Biochemical and gravimetric markers of lung injury are also not significantly altered until several days after a single administration of a low dose of MCTP (Bruner *et al.*, 1986) or MCT (Sugita *et al.*, 1983a; Hilliker *et al.*, 1982). Bruner *et al.* (1986) found that bronchoalveolar lavage (BAL) lactate dehydrogenase activity and protein concentration were not increased until after 3 days after treatment. Wet lung weight/body weight ratio and vascular leak, assessed as accumulation in lung of intravascularly administered ^{125}I-labeled albumin, were also not altered until this time and then progressively increased with the posttreatment interval. Alterations in these markers of lung injury preceded an increase in PAP, which was apparent by 7 days, and RVH evident by 14 days. A comparable delayed and progressive development was observed in studies employing a single administration of MCT (Sugita *et al.*, 1983a; Hilliker *et al.*, 1982). The delayed and progressive lung response following MCTP and MCT exposure is puzzling, particularly in light of the rapid elimination of MCT from the body and the reactive, short-lived nature of the electrophilic metabolite, MCTP.

H. Mechanisms of Action of MCT/MCTP in Pulmonary Hypertension

1. General

PH is a key feature of MCT- or MCTP-induced lung disease. The mechanism of PH and pulmonary vascular remodeling is the central focus of many recent investigations into MCT/MCTP induced lung disease. PH, assessed as an increase in PAP, first appears at approximately 2 weeks

after a single exposure to MCT (Hilliker *et al.*, 1982) or after continuous feeding of *C. spectabilis* (Meyrick *et al.*, 1980). RVH develops subsequently (Meyrick *et al.*, 1980) but has been reported as early as 2 weeks after exposure (Ghodzi and Will, 1981). MCTP can cause increased PAP slightly earlier (7 days), and RVH is apparent by 2 weeks posttreatment (Bruner *et al.*, 1983).

In this model, PH develops as a consequence of increased pulmonary vascular resistance (PVR). The mechanisms which cause the increased PVR are not presently known. It could be due to vasoconstriction of arteries or veins, vasoocclusion by platelet and fibrin thrombi, compression of microvascular lumina by perivascular exudate or vascular structural remodeling with lumen narrowing. Indeed, a combination of these factors could cause the increased PVR in this model, particularly in advanced stages of the disease.

2. Direct Effect on Vasoconstriction

One early theory put forth by Smith and Heath (1978) indicated that PAs or their pyrrole derivatives caused a direct vasoconstriction of pulmonary vessels, resulting in increased PVR and PH. This theory was based largely on morphologic evidence that the fibromuscular pads of pulmonary veins were thickened and the smooth muscle cells of these vessels appeared ultrastructurally constricted. This theory does not seem plausible, since in rats PVR and PH do not develop immediately after MCT feeding is initiated (Meyrick *et al.*, 1980). In addition, MCT is rapidly eliminated from the body and yet a delayed and progressive PH develops weeks after a single administration of the toxicant. PAs also do not cause an immediate, sustained increase in PAP in rats (Gillis *et al.*,

1978). In fact, Harris *et al.*, (1942a) found that extremely high concentrations of PAs administered intravenously to anesthetized cats actually caused rapid vasodilation of the pulmonary vascular bed rather than a vasoconstrictive response.

In contrast to findings with rats and cats, Miller *et al.* (1978) found that intravenous administration of MCT caused increased PVR within 30 minutes of administrations but did not cause a rapid, early increase in PAP in dogs. Czer *et al.*, (1986) also reported that intravenously administered MCT caused a rapid increase in both PVR and PAP. These responses in dogs are unlike those in rats or cats and may be related to species differences in response to PAs. The rapid increase in PVR could be due to mild constriction of the pulmonary vasculature, occlusion of vessels by platelet thrombi or increased viscosity (sludging) of blood. Increased viscosity of blood could occur as a result of hemoconcentration related to leakage of plasma across the endothelial barrier. Miller *et al.* (1978) noted that pulmonary interstitial edema occurred rapidly in these animals after treatment with MCT. Additional studies are necessary to clarify if other factors contribute to the increased PVR in the dog. The finding by Czer *et al.* (1986) of rapidly increased PAP differed from the findings of Miller *et al* (1978) although in both studies a similar treatment regimen was used. This discrepancy is difficult to resolve without additional studies.

3. Indirect Mechanisms of Vasoconstriction

Although it is unlikely that MCT metabolites cause a direct vasoconstriction of pulmonary vessels in rats, it is possible that these metabolites indirectly trigger vasoconstriction. This could occur by

several mechanisms. First, localized tissue hypoxia caused by patchy exudative changes might lead to hypoxic vasoconstriction of portions of the vascular bed. This could occur without systemic hypoxemia if blood is shunted to well-oxygenated lung parenchyma. Second, MCT metabolites, by mechanisms that are presently unknown, stimulate worsening parenchymal damage and pulmonary inflammation which could, in turn, promote a vasoconstrictive response. A host of mediators released from parenchymal or inflammatory cells could be involved in such a vasoconstrictive response. The role of some of these cells and their products in MCT-induced disease will be discussed.

4. Role of Inflammatory Cells

Pulmonary inflammation is a feature of MCT/MCTP-induced lung injury. This inflammatory reaction, although slow to develop, becomes more pronounced as the posttreatment interval increases. The inflammatory infiltrate, particularly prominent in the perivascular interstitial tissue and alveoli, is predominantly mononuclear, consisting largely of mast cells, macrophages, and small mononuclear cells (Takeoka *et al.*,1962; Sugita *et al.*, 1983a, b; Stenmark *et al.*, 1985). Neutrophils are sequestered transiently in lungs within the first few hours after MCT injection and are recovered in increased numbers in the bronchoalveolar lavage (BAL) fluid at days 7, 10, or 14 after treatment with MCTP or 21 days after treatment with MCT, but morphologic observations indicate they do not comprise a high proportion of the inflammatory cells in the lung (Stenmark *et al.*, 1985; Czer *et al.*, 1986: Dahm *et al.*, 1986; Ilkiw *et al.*, 1989). Small numbers of eosinophils are also recovered in the BAL

by day 10 (Dahm *et al.*, 1986) but their presences has not been reported in morphologic studies of MCTP or MCT treated animals.

To date, the specific role of neutrophils, macrophages, lymphocytes, and plasma cells or their products in this disease is largely unknown. Gillespie *et al.* (1988) have shown that the monokine, interleukin 1 (IL-1), a product of macrophages and other cells, is increased in the BAL fluid of MCT treated rats at day 4 posttreatment. Levels returned to control values at day 7 but then increased at days 14 and 21 posttreatment. Granulocyte-specific myeloperoxidase activity in the BAL was also increased by day 21, indicating that granulocyte activation had occurred. Administration of IL-1 to rats can cause increased vascular permeability and hyperreactivity to agonists which are seen in MCT-induced lung disease (Gillespie *et al.*, 1989). IL-1 and other granulocyte products may, therefore, play a role in this disease.

The role of toxic oxygen species from granulocytes in the generation of MCTP induced lung injury has been explored. Dahm *et al.* (1986) examined the ability of cells recovered from the BAL of MCTP treated rats to generate the toxic oxygen radical, superoxide anion. Superoxide production by these cells was suppressed in MCTP treated animals at days 7, 10 or 14 days after a single administration. The reason for this decrease in production is not clear, but it could mean that cells had released superoxide before or during the lavage procedure. Alternatively, superoxide production by these cells may be down-regulated at these times. Additional evidence that toxic oxygen species may not be important in this disease comes from the study by Bruner *et al.* (1987). They found that co-treatment of MCTP treated animals with drugs that

prevent the formation of toxic oxygen species or degrade or scavenge them did not affect MCTP toxicity, suggesting that oxygen radicals are not involved in MCTP-induced pulmonary injury. The role of other inflammatory cell products from monocytes, neutrophils and lymphoid cells has not been investigated comprehensively.

A role of mast cells and their products was proposed, since mast cells accumulate in the lungs of MCT-treated rats (Takeota *et al.*, 1962; Valdivia *et al.*, 1967a). Mast cells can release many vasoactive products, including 5-hydroxytryptamine (5HT), histamine, heparin, and a variety of arachidonic acid metabolites, including leukotrienes (LTs) D_4 and E_4, also referred to as the slow-reacting substance of anaphylaxis (SRS-A). Turner and Lalich (1965) proposed that 5HT, a potent vasoconstrictor and a product of mast cells, could be responsible for pulmonary vasoconstriction.

The increase in mast cells was later shown by Kay *et al.* (1967b) to correlate with the exudative changes in the lung rather than the degree of PH. In addition, although all treated animals developed RVH, only a portion of them showed an increase in the number of mast cells in the lung. Kay *et al.* (1968) also found that the levels of lung, plasma and platelet-associated 5HT were not different from those of control animals. Kay's findings do not preclude a contributing role of mast cells to the hypertensive response, since mast cells release other mediators of inflammation, such as histamine and SRS-A, which could contribute to the hypertensive response, but it appears that 5HT does not contribute to this response in a major way (Kay *et al.*, 1967b, 1968; Ganey *et al.*, 1986).

LTs, including SRS-A, are produced from inflammatory cells and mast cells via the 5-lipoxygenase pathway. These products can stimulate contraction of smooth muscle cells. SRS-A and LTB4 are increased in the lung lavage of rats treated with MCT (Stenmark *et al.*, 1985). In addition, treatment of the animals with the 5-lipoxygenase inhibitor, diethylcarbamazine (DEC), can ameliorate early MCT- and MCTP-induced pulmonary injury and MCT-induced PH (Stenmark *et al.*, 1985; Bruner and Roth, 1984). However, DEC does not inhibit the pneumotoxicity and PH observed later (14 days) in the course of MCTP-induced disease (Bruner and Roth, 1984). The disparate findings in relation to the effect of DEC pretreatment on the development of MCT- and MCTP-induced PH are difficult to resolve in the context of these studies. Additional studies are necessary to clarify the role of various LTs in MCT-/MCTP-induced lung disease.

5. Role of the Platelet

Hilliker *et al.* (1982; 1983b,c) proposed that platelets and their products may be important in generation of the pulmonary hypertensive response of MCT-induced lung disease. Their assertion was based on two observations: thrombocytopenia develops shortly after MCT exposure in rats and persists for several days (Hilliker *et al.*, 1982), and platelet-containing thrombi occur in the lung after treatment with MCT (Turner and Lalich, 1965; Valdivia *et al.*, 1967).

Platelets also appear to be important in the development of MCTP-induced disease. Although thrombocytopenia does not occur following MCTP treatment (Brunner *et al.*, 1983), platelets are sequestered in the lungs several days after treatment with MCTP (White and Roth, 1988) at a time

when major lung injury and PH is developing. In addition, platelet containing thrombi increase in number in the lung microvasculature as the posttreatment interval increases (Lalich *et al.*, 1977). Platelet depletion during critical stages of the developing MCTP-induced pulmonary disease with an antiplatelet antibody diminishes the hypertensive response and RVH (Hilliker *et al.*, 1984a). It does not, however, prevent development of lung injury (Ganey *et al.*, 1988). Platelets, therefore, apparently contribute to the development of PH but not the lung injury.

How platelets contribute to the hypertensive response is not known. It seems logical that the mere presence of large numbers of platelet-containing thrombi in the lung microvasculature could lead to an increase in PVR and PH. However, heparin treatment, which inhibits coagulation and the development of thrombi, did not diminish the increase in right ventricular pressure nor RVH in MCT-treated rats (Fasules *et al.*, 1987). It also did not alter vascular leak or the development of increased medial thickness of the pulmonary arteries. Thus, the mere physical occlusion of the vasculature by platelets does not appear to contribute significantly to the development of PH in this model.

Platelet products could contribute to the hypertensive response. Platelet granules contain serotonin, 5HT, ADP, platelet derived growth factor (PDGF), histamine, and other products capable of altering local vascular tone when released following platelet activation (Hilliker *et al.*, 1984a). In addition, thromboxane A_2 (TXA_2) is produced by enzymic metabolism of arachidonic acid in the platelet membrane (Cohen, 1980). TXA_2, PDGF, and ADP are potent vasoconstrictors. PDGF is also a mitogen for smooth muscle cells and fibroblasts (Ross and Vogel, 1978). To date,

the specific platelet factor or factors important in the development of the PH have not been identified.

The role of TXA_2 in the development of MCT-induced disease has been examined. Increased concentrations of thromboxane B_2 (TXB_2), the stable breakdown product of TXA_2, are present in the broncho-alveolar lavage of rats (Stenmark *et al.*, 1985) 3 weeks after treatment with MCT. Elevated levels of TBX_2 are also present in the effluent of isolated perfused lungs from rats treated with MCTP 14 days earlier (Ganey and Roth, 1987). TXA_2 does not, however, appear to be a major mediator of the hypertensive response, since inhibition of its production by pretreatment with a cyclooxygenase inhibitor (ibuprofen) or a thromboxane synthase inhibitor (Dazmegrel) or TXA_2 receptor antagonism (L-640,035) fails to attenuate the pulmonary hypertensive response or RVH (Ganey and Roth, 1987).

As mentioned above, 5HT also does not appear to be a major contributor to the hypertensive response. Kay *et al.* (1968) showed that serum and platelet levels of 5HT are not altered in rats administered *C. spectabilis* seeds. In addition, Ganey *et al.* (1986) found that the 5HT receptor antagonists, ketanserin and metergoline, do not protect rats against lung injury or RVH.

The role of other platelet products in MCT- or MCTP-induced lung disease development has yet to be examined in detail. It seems logical that PDGF may be an important mediator in PH. However, treatment of rats with an anti-PDGF antibody did not protect against the development of PH induced by MCTP (Ganey *et al.*, 1988) This does not preclude a role of PDGF in MCT-induced disease. It is possible that the circulating antibody did not inhibit PDGF activity at the site of release. Additional studies

are needed to clarify the role of other platelet products including PDGF in this disease.

6. Role of the Endothelial Cell

a. General

Endothelial cells are versatile cells with a complex role in many physiologic functions (Hammersen and Hammersen, 1985). They were first known for their role in maintaining a permeability barrier which selectively limits the flow of cellular and plasma constituents into the perivascular interstitial tissue. Compromise of this barrier results in extravasation of these constituents, which can incite inflammatory reactions. Endothelial cells metabolize circulating precursor molecules into vasoactive substances (eg., Angiotensin I to Angiotensin II). They remove other vasoactive substances from the circulation (prostanoids, bradykinin, serotonin). They also play an active, complex role in local hemostasis, inflammation, smooth muscle proliferation and adjustments of vasomotor tone in response to circulating substances (Hammersen and Hammersen, 1985; Vane *et al.*, 1987). Many of their functions in vascular pathophysiology are only vaguely understood. Endothelial products are far too numerous to discuss in detail, but many could play a role in the developing MCT-induced lung disease and the ensuing PH. For example, endothelial-derived relaxing factor (EDRF) is a product of healthy endothelial cells that affects vessel relaxation (Furchgott, 1983; Cacks *et al.*, 1985). Damage to endothelial cells could inhibit the production of EDRF and could, therefore, accentuate vasoconstrictive responses. A platelet-derived growth factor (PDGF) like molecule is produced by endothelial cells that have been damaged. It is a vasoconstrictor and

also a mitogen for smooth muscle cells and fibroblasts like the factor originating from platelets. This factor from damaged endothelial cells could, therefore, affect smooth muscle activity.

b. Morphologic Evidence of Endothelial Injury

The evidence that endothelial cells are damaged by MCT metabolites comes largely from morphologic studies of the pulmonary vasculature (Valdivia *et al.*, 1967a,b; Plestina and Stoner, 1972; Butler, 1970). Endothelial injury is initially apparent at 4 to 48 hours after treatment with MCT or high doses of MCTP as an increased density of cytoplasm or a thinning and thickening of endothelial cell profiles (Valdivia, 1967a,b; Plestina and Stoner, 1972). Endothelial blebs and vesicles are also seen after MCT treatment (Kay *et al.*, 1969). In late stages of MCT and MCTP induced disease, hypertrophy of endothelial cells occurs, which appears morphologically to cause occlusion of capillary and arteriolar lumina (Butler, 1970; Lalich and Turner, 1965; Hislop and Reid, 1974). Endothelial cell hyperplasia has also been observed in pulmonary arteries late in the progression of vascular alterations and could contribute to vascular occlusion (Turner and Lalich, 1965; Meyrick and Reid, 1982). Endothelial injury with MCT or MCTP is relatively minor compared to that occurring from other endothelial toxicants such as ANTU (Plestina and Stoner, 1972). In addition, the marked hypertrophic change in capillaries and small arteries in later stages of the disease is an unusual response in endothelial cells.

Valdivia *et al.* (1967a) and Raczniak *et al.* (1979) proposed that structural alterations in endothelial cells and capillary lesions may be sufficient to increase PVR by luminal occlusion. The increase in PVR

could subsequently lead to PH and vascular remodeling. Hypertrophic and hyperplastic endothelium often appear to occlude microvessels of the lung, particularly in advanced stages of the disease (Butler, 1970; Vincic et al., 1989). Other workers refute this proposal, arguing that endothelial structural alterations were not sufficiently widespread to affect PVR at a time when PH was apparent (Kay *et al.*, 1969).

c. Biochemical Assessment of Pulmonary Endothelial Function

1. General

Endothelial cells of the lung vascular bed are responsible for the metabolic activation or inactivation of a number of vasoactive molecules. These molecules include norepinephrine (NE), 5HT, bradykinin, angiotensin I, prostaglandins, and adenine nucleotides all of which can have profound effects on vascular physiology. The altered uptake and metabolism of these molecules have been proposed as contributing factors in the development of PH (Roth and Ganey, 1988). Enzymes involved in the metabolism of some of these molecules include angiotensin converting enzyme (ACE), 5'nucleotidases, and monoamine oxidases and are localized to endothelial cells.

Biochemical assessment of the functions of the pulmonary endothelium suggests that the endothelium is damaged after MCT and MCTP treatment; however, the altered functions are generally not apparent until days to weeks after treatment is initiated. In some studies, this injury is relatively specific for certain cell functions, while others are spared. That all endothelial functions are not equally altered by MCT or MCTP treatment suggests that certain functions are more sensitive indicators of endothelial damage. Alternatively, this could indicate that the

toxicant only modifies certain specific cell activities while sparing others. Effects of MCT/MCTP on some specific endothelial cell function in the lung are discussed in the subsequent subsections.

2. Altered Metabolism of Biogenic Amines

The uptake of 5HT or serotonin into endothelial cells is via a carrier-mediated system with receptors located on the luminal surface of endothelial cells. Once inside the cells, these molecules are deaminated by a cellular monoamine oxidase (MAO) to inactive molecules. It has been proposed that interference with the clearance of these molecules could lead to a localized increase in vasoactive 5HT, resulting in vasoconstriction (Ganey and Roth, 1988).

Although serum and platelet 5HT concentrations from *C. spectabilis* fed rats do not differ from those of control rats (Kay *et al.*, 1968), the uptake of 5-HT in isolated perfused lungs or lung slices of rats treated with MCT or MCTP is decreased (Gillis *et al.*, 1978; Huxtable *et al.*, 1978; Hilliker *et al.*, 1982, 1983). The depression in 5HT uptake by lungs of MCT-treated rats is delayed and progressive in that there was no effect at 2 days posttreatment, but uptake was depressed at 5 days posttreatment and depressed further as the posttreatment interval increased (Hilliker *et al.*, 1982). Metabolism of 5HT by the endothelium was altered by 14 days (Hilliker *et al.*, 1982) and 21 days (Gillis *et al.*, 1978) posttreatment. These findings are suggestive of a delayed and progressive toxic effect on the pulmonary endothelium.

The biogenic amine NE is also removed from circulation and metabolized by the endothelial cell. Reports of the effect of MCT on NE metabolism by the lung are disparate. Gillis *et al.* (1978) and Hilliker

et al. (1984c) reported that NE removal by the lung of MCT treated rats is decreased 14 days after treatment. Huxtable and coworkers (1978), however, indicated that NE removal was not altered in MCT treated rats. These conflicting results have not been adequately resolved.

3. Effects on Angiotensin Converting Enzyme

Angiotensin converting enzyme (ACE) is distributed on the luminal surface of endothelial cells in the calveolar pits (Ryan *et al.*, 1975). ACE is found in association with the microvascular beds of many tissues, but most of the ACE activity in the body is found in the lungs (Ng and Vane, 1967). The enzyme is an exopeptidase involved in converting angiotensin I, a decapeptide with little vasoactive function, to the octapeptide, angiotensin II, a potent vasoconstrictor (Keane *et al.*, 1982). It also inactivates bradykinin, which can act as a vasodilator in some vascular beds and increases vascular permeability. A number of studies have examined the effects of MCT on ACE activity of the lung. Interpretation of the disparate results of these studies is difficult since different dosing regimens and different methods for expression of activity were used.

Huxtable and coworkers (1978) found no change in ACE activity in lungs of rats treated with MCT (20 mg/liter of drinking water) for 3 weeks or DHR (4 mg/kg/day, SC) for 2 weeks when values were expressed on a whole lung basis. Molteni *et al.* (1984) found that the administration of MCT in drinking water (20 mg/l) to rats caused an initial increase in ACE activity of lung homogenate 7 days after treatment. By 2 to 3 weeks, activity returned to normal, but by 6 to 12 weeks activity in lungs had decreased. The decrease was apparent whether the activity was expressed

on the basis of whole lung, wet weight, or protein concentration. Hayashi *et al.* (1984) reported that lung homogenates from Sprague-Dawley rats treated with a single administration of 60 mg MCT/kg had decreased ACE activity at 7 days posttreatment, and ACE activity continued to decrease with time after exposure. Results were expressed as activity per mg of protein and per wet weight of lung. Keane *et al.* (1982) also reported that ACE activity in lung homogenates from Wistar rats was decreased 3 weeks after a single exposure to MCT (60 mg/kg). Lung ACE activity, determined in lung homogenates, was expressed as activity per milligram of protein. Lafranconi and Huxtable (1983) indicated that the decrease in ACE activity 3 weeks after treatment (20 mg MCT/liter drinking water) was likely due to an increase in protein content of the lung and, if ACE activity was expressed as activity per whole lung, there was no difference between treated and control animals. The discrepancies in these studies are difficult to explain. The duration of the studies was different, as were the dosing regimens. These factors make direct comparison of results difficult. If MCT exposure affects the level of lung ACE activity, results of Molteni *et al.* (1984) indicate it is a late term effect.

4. Other Markers of Endothelial Cell Function

The activity of 2 other endothelial cell enzymes in the lungs of MCT treated animals has been examined. 5'nucleotidase is an enzyme involved in salvaging nucleotides from the blood. It can cleave vasoconstrictive adenosine diphosphate (ADP) into the nucleotide, adenosine, which is not vasoconstrictive. Like ACE, 5'nucleotidase is located on the luminal surface of the endothelial cell. Huxtable *et al.* (1978) found that

activity of this enzyme was unaltered 3 weeks after multiple exposures to low doses of MCT.

Plasminogen activator (PLA), an enzyme produced by endothelial cells, is involved in generation of plasmin for fibrinolysis. PLA activity is decreased in the lung by 4 weeks after initiating daily administration of MCT in the drinking water (20 mg/liter) for 6 weeks (Molteni *et al.*, 1984). Captopril (an ACE inhibitor and antifibrotic agent) or penicillamine (an antifibrotic agent) ameliorated the MCT-induced depression in lung PLA activity (Molteni *et al.*, 1985), but had no affect on the depression of lung ACE activity. The decrease in activity of PLA could be related to damage to the endothelial cells which produce the enzyme (Molteni *et al.*, 1984) or to the continued consumption of this enzyme in fibrin clots. Why the antifibrotic agents altered lung PLA activity is unknown. Penicillamine is a chelating agent which is thought to inhibit fibrosis by binding copper, an important cofactor of enzymes involved in collagen metabolism. Perhaps metal cofactors are important in the activity of PLA and the chelation process reduces the activity of this enzyme.

5. Prostacyclin Production in MCT-treated Animals

Prostacyclin (PGI_2) is a product of arachidonic acid metabolism by the cyclooxgenase enzyme pathway of the cell membrane of endothelial cells. Physiologically, it functions as a vasodilator and an inhibitor of platelet aggregation. It may also serve as a marker of endothelial perturbation or damage. Stenmark *et al.* (1985) reported that 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable breakdown product of PGI_2, was increased in the BAL of rats by 1 week after treatment with a single

administration of MCT (30 mg/kg, I.P.). Molteni *et al.* (1984) also found that lung homogenates of rats administered a very low dose of MCT in the drinking water had increased levels of 6-keto-$PGF_{1\alpha}$ at 6 and 12 weeks after treatment. Ganey and Roth (1987), however, did not detect increased concentrations of 6-keto-$PGF_{1\alpha}$ in the effluent of isolated perfused lungs of MCTP-treated animals.

Although PGI_2 (6-keto-$PGF_{1\alpha}$)concentration is increased in the lungs of treated rats, Stenmark *et al.* (1985) reported that treatment with indomethacin, an inhibitor of the cyclooxygenase pathway, although inhibiting production of this prostanoid, had no affect on the development of lung injury and PH. Thus the release of this prostanoid into the lung lavage likely signals prolonged endothelial perturbation but the prostanoid does not appear to be important in modulating lung injury of PH.

6. Is Endothelial Injury the Direct Effect of Pyrroles?

The morphology and some biochemical functions of pulmonary vascular endothelial cells are clearly altered following MCT and MCTP treatment. What is enigmatic is that the biochemical markers of endothelial function are not altered until long after treatment is initiated. In addition, morphologic changes in endothelial cells are initially subtle and only later become pronounced. There are several possible explanations for this type of response. MCT metabolites such as MCTP may cause some direct and permanent or long-term dysfunction of endothelial cells, but the expression of this injury is somehow delayed in development. Alternatively, the injury to endothelial cells may be indirect. The delayed and progressive changes in endothelial function may be caused

largely by inflammatory or immunologic factors, the release of which being somehow triggered by MCT metabolites. In addition, some late term alterations could be responses to the gradual increase in PAP and not due to any direct effect of MCT or mediators.

It is difficult to distinguish from studies *in vivo* which of the morphologic and physiologic alterations of endothelial cells are a direct response to MCT metabolites and which are due to the influence of inflammatory mediators or the increased PAP. Many inflammatory mediators can influence endothelial permeability and leakage of vascular substances. These mediators can also alter the morphology of these cells. Some investigators have proposed that the injury induced by MCT metabolites is indeed due to an amplification of inflammatory cascades or to triggering of immunologic reactions that result in host injury, rather than a direct toxic effect of MCT metabolites on cells (Langlaben and Reid, 1985; Kay and Heath,1966).

7. Altered Responsiveness of the Pulmonary Vasculature

Altered responsiveness of the pulmonary vasculature to vasoactive substances occurs in MCT- and MCTP-treated rats (Altiere *et al.*, 1985; Gillespie *et al.*, 1986; Hilliker and Roth, 1985). Gillespie *et al.* (1986) found that isolated perfused lungs from MCT treated rats were hyperresponsive to hypoxia or angiotensin II induced vasoconstriction at day 4 but not at day 14 post-treatment. However, there was no difference in response to KCl-induced vasoconstriction at any time after treatment, suggesting that the hyperresponsiveness observed at 4 days was not due to smooth muscle cell alterations. They concluded that inappropriate pulmonary vasoreactivity may occur early in MCT-induced pneumotoxicity but

does not contribute to the later phase of sustained PH (Gillespie *et al.*, 1986)

Unlike the findings of Gillespie and coworkers, Hilliker and Roth (1985) found an enhanced vascular responsiveness to the vasoconstrictors angiotensin II and 5HT in isolated perfused lungs of rats treated with MCTP 14 days earlier. In addition, Ganey and Roth (1987) found exaggerated right ventricular pressure increases from the thromboxane mimic, U66419, in rats treated with MCTP. These differences in the vasoconstrictive response are difficult to resolve, but could be due partially to differences in the MCT and MCTP models.

The vasoactive responses of large pulmonary arteries to various pharmacologic agents are altered by MCT treatment. Altiere *et al.* (1985), using isolated segments of pulmonary vessels, found that vascular contractility was enhanced in the main pulmonary artery 4 days after treatment in response to NE and angiotensin II. At 7 days, however, contractility had returned to control levels, and at day 14 there was a decreased contractile response to angiotensin II but not to other vasoconstrictive agonists. Pulmonary arterial segments are also less responsive to the relaxant effects of isoproterenol and acetylcholine when compared to that of control at 14 days after treatment (Altiere *et al.*, 1985). The action of acetylcholine, but not isoproterenol, requires an intact endothelium. The decreased responsiveness to these chemicals would, therefore, suggest that the decrease in vasorelaxation is due to some alteration in nonendothelial components of the vessels. Increased collagen content of pulmonary arteries occurs at this time and may make vessels less compliant (Kameji *et al.*, 1980). Inability to respond

appropriately to vasodilatory effectors in these less compliant vessels could indirectly contribute to increased PVR and PH.

In the previously mentioned studies, the alterations in responsiveness of pulmonary vasculature to vasoactive agonists observed at 14 days occurred after the development of PH. It is uncertain whether such changes are a direct result of the toxic metabolites' effects on the vasculature or the detrimental effect of the increased PAP on the cells of the vasculature. Clinical studies of cases of PPH in people have shown that vasodilators provide only short-term beneficial effects in PPH, and therapeutic efficacy diminishes as the disease progresses (Weir, 1984). This is not unlike the responses seen with MCT-induced disease. This would suggest that some of the diminished sensitivity to vasodilators may be an indirect effect of the sustained increase in PAP.

I. Effects of PAs and Pyrrolic Metabolites *In vitro*

1. General

The effects of PAs have been examined in a number of assay systems *in vitro*. The focus of these studies and interpretation of results has largely reflected the concern of PAs being carcinogens. Toxic PAs have been identified as mutagens and/or genotoxins in mammalian and nonmammalian assay systems (Clark, 1959; Wehner and Theil, 1979; Styles *et al.*, 1980; Green *et al*, 1981; Mori *et al.*, 1985; Ord *et al.*, 1985). PAs differ markedly in their abilities to induce mutagenic effects, and the ranking of potency varies among different mutagenic and genotoxic assays.

2. Effects in Nonmammalian Systems

The PAs, heliotrine, lasiocarpine, and monocrotaline, are strongly mutagenic in adult males of *Drosophila melanogaster*, producing sex-linked lethal mutations (Clark, 1959, 1960; Cook and Holt, 1966). Some hepatotoxic PAs are mutagenic to *Salmonella* in the Ames mutagenesis assay, but others including MCT and heliotrine are not (Yamanaka *et al.*, 1979; Green and Muriel; 1975). However MCT and other PAs increase transformants in a repair resistant strain of *E. coli* in the presence of liver microsomes (Green and Muriel, 1975). Metabolic activation of PAs with an S-9 microsomal fraction is required for mutagenic effects in bacterial mutagenesis assays (Wehner *et al.*, 1979; Koletsky *et al.*, 1978; Mattocks, 1986). Several PAs, including MCT, can induce chromosome breakage in *Drosophila* and grasshoppers and in *Allium cepa* and *Vicia faba* root tips exposed to PAs in culture (Bull *et al.*, 1968; Avanzi, 1961, 1962). Generally, the concentrations of PAs used to induce these effects are extremely high (10^{-2} to 10^{-4} M) relative to the those experienced in tissues *in vivo*.

3. Effects on Mammalian Cells

There is little information available pertaining to the effects of PAs or their pyrrolic derivatives on mammalian cells in culture. In addition, results of studies are often conflicting. It should also be mentioned that in many studies concentrations of PAs were used which may not have been physiologically compatible with cells in culture. The toxic effects observed in these studies may be unrelated to the toxic activity of the compounds under physiologic conditions *in vivo*.

Available information indicates that PAs can be mutagenic, genotoxic and cytotoxic to mammalian cells. Bick and coworkers (1968, 1970) found that heliotrine suppresses cell division (2x 10^{-4} M) and induces chromosomal breaks (5x 10^{-5} M) in leukocytes from the marsupial *Potorus tridactylus*. Umedia and Saito (1971) found that MCT (without a bioactivation system) at high concentrations (10^{-2} to 10^{-3} M) was able to induce cytotoxic effects on HeLa cells and mixed cultures of lung and liver cells, evident as an increase in cell size and vacuolation of cell cytoplasm. Takanashi *et al.* (1980) reported that several PAs (10^{-2} to $5x10^{-4}$ M), without a metabolic activating system, caused damage to chromosomes of a Chinese hamster lung cell line (V79) and caused the nucleus and cytoplasm of these cells to become enlarged.

The relevance of the results from studies which did not used a bioactivating system in their preparations or cells which retain bioactivation capacity is questionable since it is known that PAs require bioactivation to induce relevant biological effects. Studies by Takanashi et al. (1980) showed the use of a bioactivation system increased potency of the toxic effects of several PAs and was necessary to increase the 8-azaguanine resistance mutational frequency above controls (Takanashi *et al.*, 1980). The PAs were able to induce chromosomal aberrations and interchromosomal exchanges. Even in this study the concentrations of PAs used were extremely high and the authors indicated some effects may have been related to alterations in the osmolality or pH of the incubation medium. In a DNA repair test employing primary cultures of rat hepatocytes, investigators have demonstrated that hepatotoxic PAs, including MCT, could induce unscheduled DNA synthesis (Williams *et al.*,

1980; Mori *et al.*, 1985). Styles *et al.* (1980) reported that several hepatotoxic PAs, including MCT, increased cell transformation of a mouse kidney cell line when incubated in the presence of a bioactivation system. The results of studies in which a bioactivating system was used are more pertinent to the *in vivo* situation. They also indicate that metabolites of PAs are genotoxic and mutagenic to mammalian cells.

Also pertinent to the *in vivo* situation are studies which used pyrrolic derivatives of PAs on cells in culture. The pyrrole DHR induced sister chromatid exchange in human lymphocytes (Ord *et al.*, 1985) and inhibited DNA, RNA, and protein synthesis in cultured fetal human liver cells (Armstrong *et al.*, 1972). After 24 hours of exposure, there were marked morphologic changes in the nuclei and nucleoli of liver cells, suggesting chromosome damage associated with alkylation of DNA. The pyrrole dehydroheliotridine, a derivative of heliotrine, was about 10 times more active in suppressing cell division and inducing chromosome breaks in leukocytes than the parent alkaloid (Bick and Culvenor, 1971).

Some PAs and their pyrrolic metabolites exert antimitotic activity in cultured mammalian cells. Lasiocarpine and retrorsine produce long-term inhibition of mitosis in cultures of human embryo liver cells (Armstrong *et al.*, 1972; Armstrong and Zuckerman, 1972). Lung cells were not affected by these parent alkaloids, but the pyrrole DHR was antimitotic in both lung and liver cell types. A criticism of the use of PAs with embryonic liver cells is that hepatic parenchymal cells have only very low activity of the cytochrome P-450 system that is needed to bioactivate the PAs. This activating system does not remain active in cells maintained in culture. In addition, the cell culture preparations

employed in these studies contained mixtures of parenchymal and mesenchymal cells. The specific cell types affected by PAs are therefore not clearly defined. It should be mentioned that at the time of these experiments the culture conditions necessary to maintain proliferation of hepatocytes *in vitro* were not known and hepatic parenchymal cells do not proliferate in cell culture, suggesting that the authors made an erroneous assumption in proposing an effect on hepatic parenchymal cells.

To date, no detailed studies of the effects of MCTP in cell culture systems have been reported. Details of the effects of pyrrolic metabolites on cell culture systems may be important in our understanding of the development of lung injury following MCT or MCTP exposure. Further investigations are necessary to elucidate the ramifications of cell-MCTP interactions and how they may relate to lung disease and the development of PH.

J. Research Goals

The major hypothesis tested is that MCTP causes injury to endothelium and other vascular cells of the lung and that this is the result of direct interaction of these cells with MCTP and ultimately results in progressive vascular remodeling of the pulmonary arterial vasculature.

The research project for my dissertation focused on 2 general areas. First, I assessed the time course of the early lung injury in rats after a single administration of a low dose of MCTP and correlated the morphologic alterations with biochemical and physiologic markers of pulmonary injury. Both light and electron microscopic techniques were

used to assess lung injury. In addition, morphometric techniques were used to assess changes in the relative wall thickness of the pulmonary arterial vasculature to determine whether medial thickening of arteries, similar to that reported for MCT and *C. spectabilis*-induced lung disease, occurs following exposure to a single dose of MCTP. The time course of the thickening of arterial walls was assessed to define the relationship between the development of PH and the restructuring of the arterial circulation.

The second major area of my research project focused on studies *in vitro*, in which I examined the effects of MCTP on cells in culture to identify if MCTP has direct toxic effects on cells, to determine the nature of some of these effects, and to establish dose-response relationships. Permanent cell lines were used for the initial studies to determine dose response relationships. In addition, after isolation and characterization of pulmonary arterial endothelial and smooth muscle cells, the effects of MCTP on these cells were determined in primary cultures of vascular cells as a more pertinent model of the *in vivo* setting. As a final portion of the studies *in vitro*, I compared the responses to MCTP of pulmonary artery endothelial cells of different animal species to determine whether differences in species' responses to MCT or *C. spectabilis in vivo* may be due in part to differences in cellular response to pyrroles or if these responses are solely due to variations in bioactivation and pharmacokinetics of the parent alkaloid.

CHAPTER II

DEVELOPMENT OF MORPHOLOGIC, HEMODYNAMIC AND BIOCHEMICAL CHANGES IN LUNGS OF RATS GIVEN MONOCROTALINE PYRROLE

SUMMARY:

A single, intravenous administration of a low dose of monocrotaline pyrrole (MCTP), a derivative of the pyrrolizidine alkaloid monocrotaline (MCT), induces progressive pulmonary hypertension (PH) and right ventricular hypertrophy (RVH) in rats. The temporal relationship between morphologic alterations, biochemical markers of lung injury and the development of PH, was determined during the developing pulmonary disease. Three days after a single i.v. injection of 3.5 mg/kg MCTP, small increases in bronchoalveolar lavage (BAL) fluid lactate dehydrogenase (LDH) activity and accumulation in the lungs of intravenously administered ^{125}I-bovine serum albumin (BSA) were associated with minimal to mild interstitial edema around large airways and blood vessels. By day 5, BAL fluid LDH activity and ^{125}I-BSA accumulation had increased further, and lung weight/body weight ratio (LW/BW) and BAL fluid protein concentration were greater than control. Interstitial edema was more pronounced and involved patches of alveolar septal walls. A mild increase in numbers of mononuclear cells, including hypertrophied interstitial cells, was evident in these areas. Walls of pulmonary arteries less than 60 microns in diameter, were mildly thickened. By day 8, scattered clusters of alveolar sacs contained serous exudate, and interstitial mononuclear infiltrates were more pronounced. Mild to moderate thickening of arterial walls was apparent in small and large vessels. By day 14 pulmonary arterial pressure was elevated and RVH was evident. Arterial walls were thickened and had hypertrophy of medial smooth muscle cells and intercellular edema,

which was particularly prominent in areas with perivascular interstitial inflammation. Large patches of interstitial tissue and alveolar lumens contained serous or serofibrinous exudate. In summary, a single, intravenous administration of MCTP induced a delayed and progressive pulmonary microvascular leak, interstitial inflammation and alterations in muscular blood vessels which resulted in PH within 14 days. These morphologic, biochemical and hemodynamic changes are nearly identical to alterations induced by the parent alkaloid, MCT.

INTRODUCTION:

Monocrotaline (MCT) is a pyrrolizidine alkaloid found in the seeds and leaves of the plant, *Crotalaria spectabilis* (Heath, 1969). When administered to rats, MCT or *C. spectabilis* seeds produce interstitial lung disease with arterial changes, pulmonary hypertension (PH), and right ventricular hypertrophy (RVH) (Hayashi and Lalich, 1967; Hislop and Reid, 1974; Lalich and Ehrhart, 1962; Meyrick et al., 1980; Meyrick and Reid, 1979). The development of the pathophysiologic changes associated with the lung disease is delayed and progressive. Signs of substantial lung injury are not apparent for several days after a single exposure to MCT, and thereafter the severity and extent of damage worsens with time. The earliest morphologic evidence of lung injury after exposure to high doses of MCT is mild swelling of endothelial cell cytoplasm (Valdivia *et al.*, 1967a). This is followed by the appearance of mild interstitial edema. The severity of the edema worsens with time and is associated with increasing interstitial infiltrates, thickening of the media of muscular pulmonary arteries, and muscularization of pulmonary arteries that do not

typically have muscular walls (Ghodzi and Will, 1981; Hayashi *et al.*, 1984; Hayashi and Lalich, 1967; Langleben and Reid, 1985; Meyrick and Reid 1979a; Wantanabe and Ogata, 1976). The pulmonary arterial pressure (PAP) is increased by 2 weeks after treatment (Hilliker *et al.*, 1982; Meyrick *et al.*, 1980). RVH develops after the appearance of PH and is thought to be a consequence of the sustained increase in PAP (Chesney *et al.*, 1974; Kay *et al.*, 1982a).

The mechanisms by which MCT causes pneumotoxicity and PH are unknown. However, it is generally considered that these changes are not caused directly by MCT but rather are due to one or more pyrrolic metabolites produced in the liver (Mattocks, 1968; Mattocks and White, 1971b). These toxic metabolites are released from the liver and pass to the lung to initiate injury. When administered intravenously, one putative metabolite, monocrotaline pyrrole (MCTP), produces lung injury and PH that is similar to that seen with MCT (Bruner *et al.*, 1983a; Butler, 1970; Chesney *et al.*, 1974). The pathophysiologic alterations caused by low doses of MCTP are delayed in development and progressive, following a time course similar to the disease produced by MCT (Bruner *et al.*, 1983a).

Many studies have been reported which detailed the morphologic alterations and the development of lung and pulmonary vascular changes after a single or repeated exposure to MCT or *C. spectabilis*. Few studies, however, have been reported which described the morphologic alterations in the lung of rats treated with low, PH-producing doses of MCTP (Butler, 1970; Butler *et al.*, 1970; Chesney *et al.*, 1974; Lalich *et al.*, 1977; Plestina and Stoner, 1972). In none of these studies was the

development of the pulmonary arterial thickening assessed by morphometric methods nor was there an attempt to correlate the development of biochemical and physiological alterations with histologic changes. The purpose of this study was to describe the time course of the morphologic changes that occur in the lung and pulmonary vasculature after a single injection of MCTP and to relate the observed structural changes with alterations in biochemical and hemodynamic markers of lung injury.

MATERIALS AND METHODS:

Male, Sprague-Dawley rats (CF:CD(SD)BR) (Charles River Laboratories, Portage, MI) weighing 230 to 280 g were housed on corncob bedding in animal isolators. Air in isolators was HEPA-filtered. Animals had free access to food (Wayne Lab Blox^{R}, Continental Grain Company, Chicago, IL) and water and were maintained in an alternating 12-hour light/dark cycle under conditions of controlled temperature and humidity.

MCTP was synthesized from MCT (Transworld Chemicals, Washington, DC) via an N-oxide intermediate (Mattocks, 1969). MCTP isolated by this synthesis procedure has Ehrlich activity (Mattocks and White 1971) and a structure consistent with MCTP as determined by mass spectrometry and nuclear magnetic resonance (Brunner *et al.*, 1986). MCTP was stored under nitrogen at -20°C.

On day 0 rats were given a single injection of either MCTP (3.5 mg/kg), its vehicle [N,N-dimethylformamide (DMF)], or saline via the tail vein and were killed 3, 5, 8, or 14 days later. Animals killed on a given day were divided into two groups. One group of treated and control animals was used for the determination of pulmonary injury and pulmonary

vascular leak. In a second group of treated and control animals PAP was measured. Lungs were subsequently fixed and processed for histological examination. RVH was assessed in all animals.

Pulmonary vascular leak was assessed as the accumulation of ^{125}I in the lungs after an intravenous injection of ^{125}I-labeled bovine serum albumin (^{125}I-BSA) as described previously (Ganey *et al.*, 1986). Rats were given an intravenous injection of ^{125}I-BSA and anesthetized four hours later with sodium pentobarbital (50 mg/kg, I.P.). Heparin (500 Units) was injected into the inferior vena cava, and one minute later 1 ml of blood was withdrawn for determination of radioactivity (^{125}I-blood). The trachea and the pulmonary artery were cannulated, and the lungs and heart were removed from the thorax enbloc. The lungs were inflated with air and deflated several times while 10 ml saline (0.9% NaCl) were perfused gently through the pulmonary arterial cannula to clear blood from the vasculature. The lungs were then lavaged twice with 10 ml of 0.9% NaCl solution as described previously (Roth, 1981), and the recovered lavage fluid was combined. The lungs were trimmed from the trachea and connective tissue, rinsed with saline, and placed in tubes for determination of radioactivity (^{125}I-lung). An increase in the ratio ^{125}I-lung/^{125}I-blood was considered to indicate vascular leak (Ganey *et al.*, 1986).

Lactate dehydrogenase (LDH) activity was determined in the cell-free bronchoalveolar lavage fluid according to the method of Bergmeyer and Bernt (1974). Protein concentration was determined by the method of Lowry *et al.* (1951). Wet lung to dry lung weight (WL/DL) ratios were determined after drying lungs to constant weight in an ashing oven. RVH was assessed

as an increase in the ratio of right ventricle weight to the weight of left ventricle plus septum (RV/(LV+S).

PAP was measured in anesthetized rats as described previously (Ganey and Roth, 1986). The distal end of a 3.5 French umbilical vessel catheter was bent to approximately a 45° angle. The catheter was introduced through the right jugular vein and advanced through the right ventricle into the pulmonary artery (Stringer *et al.*, 1981). Pressure was measured with a Statham P23ID pressure transducer and was recorded on a Grass Model 7 polygraph.

For histopathologic studies, rat lungs were removed from the thorax and perfused via the airway and the pulmonary vasculature with a modified Karnovsky's fixative (2% glutaraldehyde; 1% paraformaldehyde; 0.1 M cacodylate buffer, pH 7.4) for a minimum of 15 minutes. Tracheal and pulmonary arterial cannulae were attached to separate reservoirs of fixative, and lung airways and vasculature were simultaneously perfused under constant pressure (28 cm H_2O for airways; 32 cm H_2O for the vasculature). Lungs were sectioned and immersed in fresh, chilled fixative (4°C) for the remainder of a 4-hour period. Lung sections were then rinsed twice in cold cacodylate buffer (0.2 M, pH 7.4) and processed for histopathologic examination.

A standard longitudinal section of pulmonary parenchyma through the main stem bronchus of the left lung lobe was processed in paraffin for each rat. In addition, one or two sections from areas with obvious macroscopic lesions or, if no macroscopic lesions were noted, one or two areas selected at random were included. Paraffin-embedded tissue sections were cut at 4 μm and stained with hematoxylin and eosin (H and E) or

orcein stain for elastin. In addition, sections of lung with macroscopic lesions or sections from the dorsal surface of the right caudal lung lobe were processed for glycolmethacrylate plastic embedding. Plastic embedded tissue sections were cut 1-2 μm thick and stained with toluidine blue or a modified Gill's H and E stain. Histopathologic examination was performed on all tissue sections.

Morphometric assessment of pulmonary arterial wall thickness was performed on lung sections from 6 to 8 control and MCTP-treated animals from each day on which animals were killed. The external diameter (defined by the external elastic laminae) and internal luminal diameter of approximately 30 pulmonary arteries (20 to 200 μm diameter) per animal were measured in 2 (perpendicular) directions on orcein stained, paraffin embedded sections with the aid of a Joyce Loebl Magiscan Image Analyzer attached to a Nikon microscope. Average wall thickness/average external diameter ratios were calculated from the data and were expressed as a percent.

Five, 1.0 mm^3 tissue sections were selected from areas with lesions or from the dorsal surface of the right caudal lung lobe for each rat. Tissues for electron microscopy were post-fixed in 1% osmium tetroxide, stained enbloc with a 2% uranyl acetate solution, dehydrated in a graded alcohol series and propylene oxide and embedded in Epon-Araldite resin. Tissues from one saline control, one DMF control and two MCTP-treated rats were selected for each posttreatment interval for further processing. One micron-thick sections from each block were cut with a LKB Ultramicrotome 3, stained with toluidine blue and examined by light microscopy. Representative blocks were selected for thin sectioning, and gold-silver

sections were mounted on copper grids and stained with both uranyl acetate and lead citrate. Sections were examined with a Philips 301 transmission electron microscope.

Statistical Analysis. Data are presented as means + standard errors of the means (SE). Comparisons between the means of the control and treated groups on any given day were made using the Student's t-test. Data from the treated animals were also analyzed using a completely random analysis of variance (ANOVA). An arcsin $^{-1}$ transformation was performed on the morphometric data prior to application of the ANOVA. Individual comparisons among days for each treatment were made using the least significant difference (lsd) test for the biochemical and hemodynamic markers and Scheffe's test for the morphometric data (Steele and Torrie, 1980). The criterion for significance was $p < 0.05$.

RESULTS:

Controls

There were no differences in the biochemical markers of lung injury or in the gross or histologic appearance of lung between saline- and DMF-treated rats at any time after treatment.

Macroscopic Lesions

Macroscopic lesions were not detected in any DMF or saline-treated rats or in MCTP-treated rats killed at 3 days posttreatment. Patchy, irregular, reddened areas of parenchymal consolidation were detected in 1 of 8 MCTP-treated rats killed at 5 days, 6 of 8 rats killed at 8 days, and in all MCTP-treated rats killed at 14 days posttreatment. Lesions were more extensive at day 14 than at other days and commonly involved

entire lung lobes. Although the extent and distribution of the lesions were variable, dorsal anterior portions of the right caudal lung lobe were affected most consistently. The parenchyma at the hilar region of the right middle lung lobe and dorsal hilar portions of the left lung lobe also were commonly involved.

Day 3 Observations

Three days after treatment with MCTP, lavage fluid LDH activity was modestly elevated, and vascular leak was evident in treated rats (Table 1-1). There was a small but significant decrease in the WL/DL ratio in MCTP-treated rats compared to controls. The lung weight/body weight ratio (LW/BW) and lavage protein concentration were not altered by treatment with MCTP at this time, and PH and RVH were not present.

Characteristic histologic changes were subtle at this time and consisted of a mild accumulation of edema fluid within the interstitial tissue adjacent to bronchi, large bronchioles and pulmonary vessels located near the hilus of the lung lobe (Figure 2-1). There was mild to moderate lymphatic distention within these areas. There was no difference in the wall thickness of pulmonary arteries between control and MCTP-treated rats assessed morphometrically (Table 2-2).

Additional changes identified in some animals within the MCTP treatment group but not characteristic of the majority of animals in the group included small, localized areas of septal and interstitial edema. This was associated with a minimal to mild increase in the mononuclear cellularity of alveolar septal walls and adjacent perivascular interstitial tissue. Mild hypertrophy of perivascular interstitial cells

and of alveolar type II cells was restricted to these areas. Minimal to mild alveolar septal edema was detected ultrastructurally, but distinct morphologic alterations were not apparent in endothelial cells.

Day 5 Observations

By day 5, the WL/BW ratio, the WL/DL ratio, and lavage fluid protein concentration and LDH activity were elevated relative to controls (Table 2-1). The WL/BW and WL/DL ratios and lavage LDH activity at day 5 were significantly greater in treated rats than at day 3. Pulmonary vascular leak (lung ^{125}I-BSA accumulation) was increased compared to controls and was also more pronounced than at day 3. PAP was not elevated and right ventricular weights were normal.

The histologic changes were more pronounced and widespread in MCTP-treated rats killed at day 5 (Figure 2-2). Large, coalescing areas of altered parenchyma blended into areas with little or no microscopic changes. Interstitial edema was evident around blood vessels and airways of smaller caliber than at day 3. These areas of peribronchiolar and perivascular interstitial edema were contiguous with patchy zones of alveolar septal edema. The septal and perivascular cellularity was more pronounced compared to MCTP-treated rats at day 3 or to day 5 controls. This infiltrate comprised mononuclear cells predominantly but included lesser numbers of neutrophils with few eosinophils. Many perivascular and septal mononuclear cells and alveolar type II cells were distinctly hypertrophic. The increased perivascular cellularity extended to medium and small sized, muscular and nonmuscular blood vessels. Minimal to mild thickening of arterial walls was apparent in scattered muscular and partially muscularized vessels. Mild hypertrophy of endothelial cells was

apparent in arterioles and arteries. Morphometric evaluation of the wall thickness of pulmonary arteries revealed a slight but significant increase in vessel wall thickness for MCTP-treated rats compared to controls for vessels less than 60 μm in external diameter but not for vessels greater than 60 μm in diameter (Table 2-2).

Additional lesions noted in some, but not all, MCTP-treated animals at this time included mild hypertrophy of the bronchiolar epithelium with a localized reduction in size of apical domes of nonciliated bronchiolar epithelial cells, hypertrophy of lymphatic endothelial cells in dilated lymphatics, and accumulation of serous exudate in scattered alveolar sacs. Occasional mitotic figures and pyknotic nuclear debris were present in perivascular interstitial tissue and alveolar septa in regions where interstitial edema was pronounced. Scattered, necrotic and degenerate cells were also seen in the terminal bronchiolar epithelium.

Ultrastructural alterations included focal blebbing of capillary endothelial cells with an intracellular or subendothelial accumulation of granular precipitate (Figure 2-3). There was marked interstitial edema and increased cellularity of the perivascular and peribronchiolar interstitium and of some alveolar septal walls (Figure 2-4). A scant amount of fibrin was present within the alveolar septal interstitium. Pronounced alveolar type I cell swelling and degeneration were evident in some alveolar septa, and small amounts of granular exudate appeared in alveolar spaces in areas with severe alterations of alveolar type I cells. The increased interstitial and septal cellularity comprised hypertrophied interstitial cells (fusiform or spindle cells with abundant rough endoplasmic reticulum and cytoplasmic lipid vacuoles), activated

monocyte-macrophage type cells, small mononuclear cells and lesser numbers of granulocytes.

Day 8 Observations

LW/BW ratio, lavage fluid protein concentration and LDH activity, and vascular leak were greater in MCTP-treated rats compared to controls eight days after treatment (Table 2-1). The WL/DL ratio was not altered. Although in a previous study PAP was found to be significantly elevated by this time (Bruner *et al.*, 1983a), in this study group means for PAP were not significantly different at day 8. However, 3 of the 5 MCTP-treated rats had PAPs that were higher than any of the controls, suggesting the onset of PH. RVH was not apparent.

Histologic changes in MCTP-treated rats killed at day 8 were more extensive and severe than those at day 5. There was marked variation in severity of lesions among rats of the treatment group and within lung sections of individual rats. The parenchyma had large coalescing patches of septal and perivascular interstitial edema and hypercellularity. The perivascular cellularity was moderately increased and included numerous, extremely large mononuclear cells. Many of these hypertrophic cells were spindle shaped. Small mononuclear cells, granulocytes (including eosinophils) and mast cells were also increased in number.

In patchy areas of parenchyma, there was flooding of clusters of alveoli with serous exudate and a mild to moderate increase in numbers of alveolar macrophages. Many macrophages had abundant foamy cytoplasm and contained phagocytized cellular debris and proteinaceous fluid. Many alveolar type II cells were hypertrophic and had abundant, granular cytoplasm. These cells occasionally had bizarre, enlarged nuclei with

extremely prominent nucleoli or acidophilic globules in the cytoplasm. Nuclear debris and cells with mitotic figures were present in alveolar lumens, septal walls, perivascular interstitial tissue and occasionally in the bronchiolar epithelium.

Endothelial cell hypertrophy was evident in arteries and arterioles. Many partially muscular pulmonary arteries had thickened muscular walls with distinct hypertrophy of medial smooth muscle cells. These vascular changes were typically associated with marked perivascular interstitial alterations. The thickening of the medial layer of these vessels did not appear to compromise markedly the diameter of vascular lumens. Morphometric measurements indicated a moderate increase in vessel wall thickness of pulmonary arteries compared to controls and compared to MCTP-treated rats from 3 or 5 day groups (Table 2-2). Vessels in each size range measured were affected.

Other changes that occurred in some MCTP-treated animals but were not characteristic of the group included mild to marked hypertrophy of the terminal bronchiolar epithelium in areas with severe parenchymal disease. Apical domes of nonciliated bronchiolar epithelial cells were less pronounced than in controls. Bronchiolar epithelium extended into scattered alveolar ducts and alveolar sacs. Degenerate cells with swollen, rarefied cytoplasm ("clear" cells) were commonly noted near the basement membrane of many of these bronchioles. Localized goblet cell hyperplasia was present in larger airways. Fibrin and microhemorrhages were evident in the alveolar exudate in areas of severely damaged parenchyma.

Ultrastructural changes were also more extensive and severe than those at day 5. The marked cellularity of the perivascular and peribronchiolar tissue consisted predominantly of extremely hypertrophic interstitial cells and monocyte/macrophage-type cells. There was degeneration and necrosis of alveolar type I cells, and occasional necrotic alveolar lining cells were mixed with fibrin in accumulations of luminal exudates. Alveolar type II cells were hypertrophic, had large nuclei and prominent nucleoli and occasionally had cytoplasmic invaginations into the nucleus (Figure 2-5). Endothelial blebbing and focal rarefaction of endothelial cytoplasm were occasionally observed in endothelial profiles, but many profiles appeared normal. Within the hypertrophied columnar epithelium of bronchioles were increased numbers of immature goblet cells and Clara cells. Many Clara cells had degenerate changes, including rarefied cytoplasm and enlarged nuclei with loss of defined nuclear features. Changes evident in vascular walls of muscular vessels were mild and consisted of localized intercellular edema within the media in areas where the perivascular interstitial reaction was severe.

Day 14 Observations

On day 14, the LW/BW ratio, and protein concentration and LDH activity in lavage fluid remained elevated (Table 2-1). Pulmonary vascular leak was still evident in MCTP-treated rats, and the WL/DL ratio was again elevated. PAP was significantly higher in MCTP-treated rats, and RVH was apparent at this time.

Histologic changes in rats killed at day 14 posttreatment varied markedly among animals and among regions of pulmonary parenchyma in

individual animals, but they were generally more extensive and severe than at earlier times (Figure 2-6). Alveolar flooding was more extensive than at earlier times, and in many areas this exudate was more fibrinous in character and contained large numbers of alveolar macrophages and fewer granulocytes. Microhemorrhages were common in alveolar sacs. Alveolar septal walls were thickened and hypercellular (Figure 2-7). Capillary profiles in alveolar septa appeared to be reduced in number. Many alveolar type II cells and septal and perivascular interstitial cells were extremely hypertrophic and bizarre in appearance. Cells in mitosis and necrotic cellular elements were common in septal walls, perivascular interstitium and the alveolar exudate. The septal and interstitial infiltrate consisted of numerous monocyte/macrophage-type cells, lymphoid cells and hyperplastic spindle cells with lesser numbers of neutrophils and eosinophils. Mast cell numbers also appeared to be increased, particularly in areas with marked interstitial exudate. Marked hypertrophic and hyperplastic changes were noted in the bronchiolar epithelium (Figure 2-6) as described in rats in the day 8 posttreatment group, and proliferation of bronchial epithelium into alveolar ducts and sacs was more prominent.

Moderate alterations were evident in many muscular vessels (Figures 2-8 and 2-9). These included focal intercellular edema and moderate to marked hypertrophy of smooth muscle cells and endothelial cells. These changes were particularly prominent in areas where perivascular interstitial inflammation and edema were pronounced. Splitting of the external elastic laminae was evident in arteries with markedly thickened walls, and the medial muscular pads of many small veins were enlarged.

Morphometric evaluation of wall thickness of muscular and partially muscular arteries revealed that these vessels had thicker walls than at earlier times (Table 2-2). The histologic changes identified for the group were generally present in all animals of the group. The degree of lesion severity was, however, variable.

Ultrastructural alterations at day 14 were similar to those identified in the day 8 group. Serous to serofibrinous exudate filled alveolar lumens, septal walls were markedly thickened, and alveolar and interstitial cellularity was greater than in day 8 rats. There was marked hypertrophy of many of the interstitial mononuclear cells and alveolar type II cells. Many of these "bizarre" cells had irregularly shaped nuclei with cytoplasmic invaginations into the nucleus, prominent multiple and irregular nucleoli, little heterochromatin and abundant euchromatin. There was marked degeneration and necrosis of alveolar type I cells and, occasionally, alveolar type II cells (Figures 2-10 and 2-11). There was focal rarefaction and vesicular blebbing of a few capillary endothelial cells. Most endothelial cells did not have degenerative changes and few had thickened profiles suggestive of hypertrophic change. Capillary profiles in some thickened alveolar septal walls appeared to be reduced in number, and compressed capillary lumens were evident there interstitial exudate was marked. Vascular alterations of muscular arteries consisted of localized intercellular edema, hypertrophy of smooth muscle and endothelial cells and splitting of external elastic laminae (Figure 2-12). Hypertrophy of the bronchiolar epithelium was pronounced, and there was hyperplasia of immature goblet cells in some bronchioles.

DISCUSSION:

The nature of pulmonary injury after intravenous MCTP administration depends on dose. High doses (> 10 mg/kg) induce acute, widespread pulmonary edema and death soon after injection (Hurley and Jago, 1975; Plestina and Stoner, 1972). In contrast, lower doses (2 to 5 mg/kg) cause a delayed and sustained PH associated with progressive parenchymal and vascular alterations (Butler, 1970; Butler *et al.*, 1970; Chesney *et al.*, 1974; Plestina and Stoner, 1972) similar to those observed in certain types of chronic PH in humans. This study describes the morphologic alterations after a single injection of a low dose of MCTP and relates those findings to pulmonary injury assessed by biochemical and hemodynamic markers.

Results from this and previous studies indicate that marked alterations in certain indices of lung injury are not apparent until several days after a single administration of MCTP (Bruner *et al.*, 1983a,b; Hilliker *et al.*, 1982). In the present study, the only alterations detected at day 3 were small elevations in BAL fluid LDH activity and lung ^{125}I-BSA accumulation, suggesting a slow or delayed onset of pulmonary injury. These findings were consistent with morphologic observations at this time. For example, only subtle, perivascular and peribronchiolar interstitial edema with lymphatic dilation around large caliber airways and blood vessels was observed in rats 3 days posttreatment, consistent with mild vascular leak. Morphologic evidence of damage to lung parenchymal cells, including endothelial cells was not observed, at the light or ultrastructural level at this time. MCTP is a short-lived, reactive electrophile which binds covalently to nucleophilic

tissue constituents or is rapidly inactivated in aqueous media (Mattocks 1968; Mattocks and White 1971). The reasons why the development of lung injury from such an agent is delayed remain unknown. Possibly, the initial covalent binding of MCTP to lung cells initiates a sequence of events that results, after several days, in the expression and progression of overt injury.

Lavage fluid LDH activity and ^{125}I-BSA accumulation increased with time. By day 5, distinct morphologic damage to many types of cells was evident. Although vascular endothelium is presumed to be a major site of covalent binding of the MCTP electrophile, endothelial cell injury detected morphologically appeared relatively minor and was limited to occasional endothelial cell profiles. Cell degeneration and necrosis was evident in alveolar type I cells and few interstitial and bronchiolar epithelial cells. Proteins and enzymes released from these damaged cells and mild flooding of alveolar sacs with serous exudate observed in some animals likely contributed to the increased LDH activity and protein concentration in the lavage fluid.

Ongoing vascular leak was assessed as increased accumulation of circulating ^{125}I-BSA in lungs during a 4-hour period. This marker was slightly but significantly elevated at day 3. The progressively increasing values at days 5, 8 and 14 indicate that vascular leak became more severe with time. In addition, the WL/BW ratio was not increased until day 5 and subsequently increased further. This is consistent with the progression of the disease observed morphologically, insofar as larger areas of the lung interstitium were edematous and the interstitial distension was more severe as the posttreatment interval increased.

Sugita *et al.* (1983a) and Plestina and Stoner (1972) also found that MCT or MCTP induced vascular leak which was slower to develop but more persistent than with other substances that increase lung vessel permeability, such as a-naphthylthiourea (ANTU). The reason why the vascular leak is persistent and progressive in the MCT/MCTP model but resolves within a day or two after insult with other toxicants like ANTU remains unknown. The delayed and progressive vascular leak caused by MCT or MCTP might be the result of direct but slowly developing toxic effects on the endothelium, increased influence of mediators of inflammation and vasopermeability, increased hydrostatic pressure, decreased drainage of interstitial fluid via the lymphatic vessels or a combination of these factors.

Morphologic evidence of endothelial cell alterations at days 5 and 8 included cell hypertrophy as well as focal blebs in endothelial cell profiles. Mild, focal, cytoplasmic rarefaction and swelling in occasional endothelial cell profiles were also evident in some cells. The presence of increased amounts of fibrin in the alveolar and interstitial exudate by days 8 and 14 supports a progressive toxic effect on the endothelium. Fibrinogen is a larger plasma protein than those found in transudates or serous effusions; its leakage into an exudate thus suggests a vascular endothelial lining with defects large enough to allow passage of large molecules. Moreover, the ability of lungs of MCTP-treated rats to remove circulating 5-hydroxytryptamine (5HT) decreases following a single exposure to MCT or MCTP (Hilliker *et al.*, 1983b; Hilliker and Roth, 1985). With MCT exposure, this decrease in 5HT uptake is not apparent for several days after exposure and then becomes more pronounced. Since 5HT uptake

into lung is a carrier-mediated function of endothelial cells, the delayed and progressive decrease in 5 HT uptake by the lung suggests a progressive deterioration in endothelial function. Thus, both biochemical and barrier functions of pulmonary endothelium seem to be compromised in this model, and these changes coincide with morphologic alterations in endothelium.

By comparison to untoward changes wrought on other cell types by MCTP treatment, the changes in endothelium were mild. Indeed, most endothelial cell profiles appeared morphologically normal, even in regions where injury in adjacent parenchyma was pronounced. The subtle nature of the morphologic changes in endothelial cells is puzzling, inasmuch as they are presumed to be the cells that experience the highest concentration of the toxicant. Other investigators, using higher concentrations of MCTP or MCT, also indicated that early morphologic injury to the endothelium was not widespread (Plestina and Stoner, 1972; Ghodzi and Will 1981). Using carbon particles to determine the site of vascular leak, Plestina and Stoner (1972) found that particle deposition occurred between endothelial cells that were relatively normal in appearance. This suggests that MCTP may not be an acutely necrogenic compound but instead causes progressive derangement of cell functions, which leads to vascular leak and possibly altered vascular homeostasis.

Lung cell injury occurred at sites anatomically distant from the vasculature. Some alveolar type I cells and interstitial cells were necrotic, as were a few cells in the terminal airways. In addition, extremely hypertrophic, atypical, interstitial and alveolar type II cells were common. The causes of changes at these sites are unknown. Cells such as the alveolar type I cell are particularly sensitive to many

toxicants; perhaps low concentrations of the reactive electrophile reach these sites outside of the vascular bed. Alternatively, the leak of plasma constituents into the interstitium might invoke direct injury to these cells or might stimulate tissue phagocytes to release cytotoxic products. Indeed, numerous activated macrophages were seen in interstitial regions of the lung, and these might have been stimulated by the presence of plasma proteins and/or products of degenerating cells. Alveolar type I cell damage was associated with alveolar flooding, and this likely set the stage for type II cell proliferation and epithelialization of alveolar ducts.

Progressive remodeling of the microvascular bed occurred after MCTP administration. Changes in vessels were similar to, although occurred slightly earlier than, those described in MCT- and *Crotalaria spectabilis*-induced lung disease (Ghodzi and Will, 1981; Meyrick *et al.*, 1980; Meyrick and Reid, 1979a). In this study, morphologic alterations were evident in scattered muscular and partially muscular arteries by day 5 and were more frequent at day 8. These alterations included endothelial cell hypertrophy, intercellular edema in the medial layer, and mild hypertrophy of smooth muscle cells which resulted in a significant increase in wall thickness of small pulmonary arteries. By days 8 and 14, normally nonmuscular vessels were distinctly muscular, and there was moderate thickening of the medial layer of partially muscular and muscular arteries. Muscular veins had noticeable thickening of the muscular pads. Changes in the veins, however were less pronounced and less widespread than in arteries. Morphometric assessment indicated that smaller arteries ($< 60\ \mu m$) were significantly thickened as early as 5 days after MCTP

treatment, and larger vessels were affected by 8 days. By comparison, after feeding *C. spectabilis* seeds, Meyrick and Reid (1979a) did not detect thickening of pulmonary arteries until 10 days or more. Thus, the vascular remodeling that occurs after MCTP is similar to but may occur somewhat earlier than that caused by MCT or feeding of *C. spectabilis* seeds.

The changes in the media of arteries were preceded by an increase in interstitial edema and cellularity of the adventitia. The edema likely originated from the leaky microvascular bed and percolated toward the lymphatic channels in the perivascular interstitium. The vascular leak was persistent and progressive. The persistent leak of plasma proteins might activate resident inflammatory cells in the perivascular interstitium which could in turn influence arterial components. Mononuclear cells, including numerous monocyte-macrophage type cells, comprise the perivascular cellular infiltrate. These cells are capable of releasing mitogens that act on many other cell types, including smooth muscle cells, fibroblasts and endothelial cells (Gillespie *et al.*, 1985; Shimokado *et al.*, 1985), and such mitogens might promote the medial vascular thickening. Other potential sources of mitogens for smooth muscle cells include plasma proteins, platelets and damaged endothelial cells (Ross *et al.*, 1986).

Increased PAP was evident in 3 of 6 rats at day 8 and in all MCTP-treated rats at day 14. In previous studies, a significant increase in PAP was detected at day 8 and preceded the increase in RVH (Bruner *et al.*, 1983a). The vascular remodeling may have contributed to the increase in PAP. Marked infringement on lumens of pulmonary arteries by the

thickened vessel wall was not, however, apparent at these times. Other factors could also contribute to the observed PH. For example, vasoconstriction could be initiated by agents released locally from interstitial cells, inflammatory cells and/or platelets. Decreased production of endothelium-derived relaxing factor or of prostacyclin from endothelial cells might also contribute to increased vessel tone. Finally, the progressive increase in the exudate in the perivascular interstitium might impose mechanical limits on small vessel expansion, or even cause local vessel compression, and thereby contribute to elevations in vascular pressure. Presently, it is difficult to define which of these factors is most important in the genesis and maintenance of PH in this model.

The WL/DL ratio was elevated on days 5 and 14 but not on day 8. The WL/DL ratio has not changed reproducibly in studies in our laboratory which have used MCT or MCTP (Gillis *et al.*, 1978; Hilliker *et al.*, 1982; Roth *et al.*, 1981; unpublished observations). Serous exudate and cellular infiltration likely influence this ratio in opposite ways. That both of these responses occur together during MCTP toxicosis may explain why changes in the ratio are small and inconsistent.

Several histologic alterations described by others in MCT- or MCTP-induced pulmonary disease were not identified in this study or were not considered different from controls. Specifically, necrotizing arteritis (Merkow and Kleinerman, 1966), proliferative vascular intimal changes (Hislop and Reid, 1974; Turner and Lalich, 1965), vascular occlusion by hypertrophied, hyperplastic endothelial cells (Butler *et al.*, 1970), and "ghost arteries" (Hislop and Reid, 1974) were not observed.

In addition, vascular thrombosis was not widespread and was observed only occasionally in both treated and control animals. Organized thrombi and recanalized vessels were not seen, and changes in the vessel walls adjacent to platelet-rich thrombi were negligible, indicating that the thrombi had formed near the time of death. Pulmonary vascular thrombosis has been identified early in MCT intoxication (Hayashi and Lalich, 1967; Valdivia *et al.*, 1967a) and may represent thromboemboli originating in damaged liver. The absence of these changes in the present study indicates that they are not prerequisite to the development of PH. Increased frequency of fibrin thrombi was reported after MCTP treatment in studies of longer duration (i.e., 4 to 8 weeks) than the present study (Lalich *et al.*, 1977) and may represent further progression of the spectrum of lesions induced by these compounds. Progressive deterioration of the microvascular endothelium could account for increased thrombosis. Alternatively, factors such as age, strain and nutritional status of animals, the dose of compound to which the lung was exposed, and the choice of agent administered may have resulted in differences in the histologic appearance of the lesions.

Some histologic alterations observed in this study were not reported in other studies. For example, lymphatic endothelial cells of many dilated lymphatic vessels were markedly hypertrophic. Goblet cell hyperplasia was observed in larger bronchioles in which peribronchiolar edema was severe. These cells may have responded to mediators released from inflammatory cells and/or mast cells in the parenchyma or to plasma constituents in the edema fluid. In addition, "clear" cells and occasional necrotic cells were identified in the hyperplastic epithelium

of bronchioles. Because degenerate Clara cells were commonly identified in these bronchioles by electron microscopy when other cell types were normal or hypertrophic, we suspect that these "clear" cells represent degenerate Clara cells.

In summary, we examined morphologic, hemodynamic and biochemical changes during the development of injury in lungs of rats given a single, low dose of MCTP. The delayed and progressive nature of the morphologic lesions paralleled the changes in biochemical and hemodynamic markers of lung injury. Pulmonary alterations are first evident as mild vascular leak which persisted and became more severe as the posttreatment interval continued, suggesting a delayed or slowly developing injury to endothelial cells. Subsequent pulmonary reactions were complex, involving pulmonary inflammation and interstitial and pulmonary vascular remodeling. Whatever the causal factors, the vascular remodeling was associated with PH and RVH.

Table 2-1.

Summary of alterations in hemodynamic and biochemical markers of lung injury in rats after exposure to monocrotaline pyrrole.

Days After Treatment	Treatment[a]	WL/BW (x1000)	WL/DL	Lavage Protein (mg/ml)	Lavage LDH Activity (U/dl)	$\frac{^{125}\text{I-Lung}}{^{125}\text{I-Blood}}$	PAP (mmHg)	RV/(LV+S)
3	Control	4.0±0.1	5.6±0.2	0.12±0.01	1.8±0.2	0.18±0.01	20±1	0.274±0.008
	MCTP	4.1±0.1	5.3±0.1[b]	0.16±0.02	4.7±1.1[b]	0.26±0.03[b]	19±1	0.263±0.006
5	Control	3.7±0.2	5.3±0.2	0.14±0.01	2.3±0.3	0.20±0.02	19±2	0.273±0.008
	MCTP	5.4±0.4[b,c]	6.2±0.2[b,c]	0.54±0.12[b]	17.3±3.0[b,c]	0.40±0.03[b,c]	21±1	0.275±0.007
8	Control	3.6±0.1	5.6±0.5	0.10±0.01	1.4±0.1	0.21±0.04	17±2	0.258±0.010
	MCTP	5.5±0.5[b,c]	5.9±0.4	1.02±0.29[b]	17.9±3.8[b,c]	0.57±0.10[b,c]	24±4	0.283±0.007
14	Control	3.7±0.1	5.1±0.3	0.14±0.02	2.0±0.1	0.20±0.01	20±1	0.266±0.008
	MCTP	11.5±1.3[b,c]	7.3±0.5[b,c]	3.01±0.48[b,c]	11.4±1.6[b]	0.76±0.07[b,c]	41±5[b,c]	0.364±0.012[b,c]

[a]On day 0 rats received either MCTP (3.5 mg/kg) or DMF i.v. WL = wet lung weight; BW = final body weight; DL = dry lung weight; PAP = mean pulmonary arterial pressure; RV/(LV+S) = right ventricular weight/left ventricle plus septum weights. N = 5-8.

[b]Significantly different from DMF control on the same day.

[c]Significantly different from MCTP on day 3.

Table 2-2.

Summary of changes in wall thickness of pulmonary arteries in rats after exposure to monocrotaline pyrrole.

Days After Treatment	Treatment	External Diameter of Arteries		
		< 40 microns	40-60 microns	> 60 microns
3	Control	3.50±0.05	3.63±0.05	3.83±0.08
	MCTP	3.47±0.07	3.64±0.06	3.88±0.11
5	Control	3.61±0.08	3.58±0.07	3.70±0.16
	MCTP	4.56±0.12[b,c]	4.05±0.07[b,c]	3.81±0.12
8	Control	3.76±0.11	3.58±0.07	3.70±0.09
	MCTP	5.85±0.23[b,c]	5.06±0.12[b,c]	4.79±0.10[b,c]
14	Control	3.45±0.06	3.69±0.09	3.50±0.10
	MCTP	6.91±0.14[b,c]	5.81±0.10[b,c]	4.90±0.16[b]

Values represent $\frac{\text{average wall thickness}}{\text{average external diameter}} \times 100$. Numbers of vessels analyzed per group ranged between 49-126, 95-144 and 36-55 for vessels of < 40 μ, 40-60 μ and > 60 μ, respectively.

[b]Significantly different from control of same day.

[c]Significantly different from preceding day for group treated similarly.

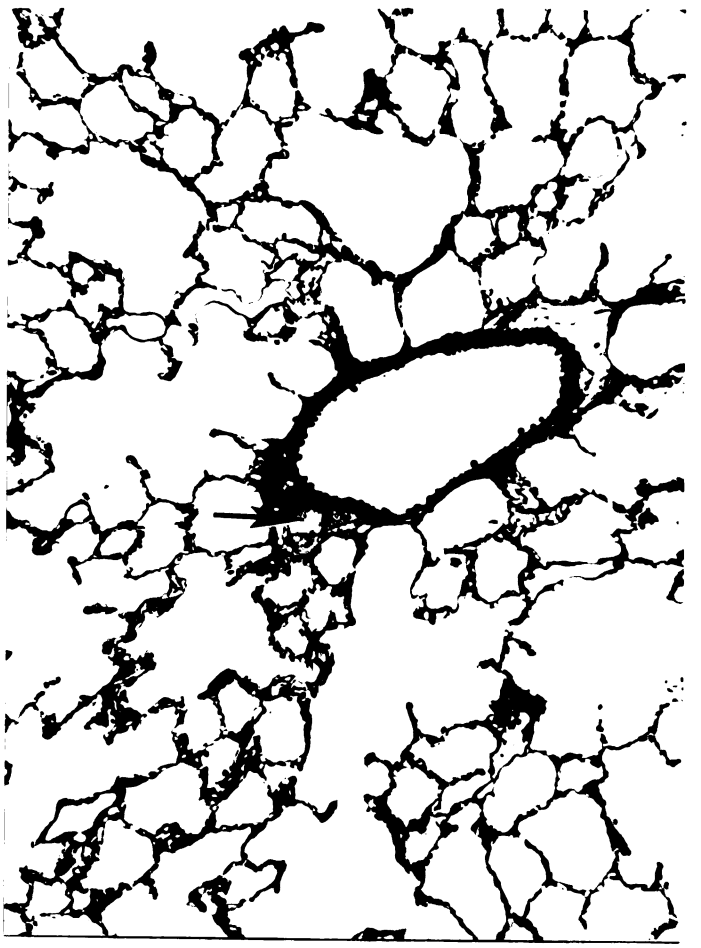


Figure 2-1. Photomicrograph (low magnification) of lung parenchyma of rat 3 days after treatment with MCTP. Interstitial edema is evident surrounding a medium-sized muscular vein, and the perivascular lymphatic is distended (arrow). Remaining parenchyma appears normal. This is a pronounced lesion relative to those apparent in other animals of this treatment group. Toluidine blue. 130x.

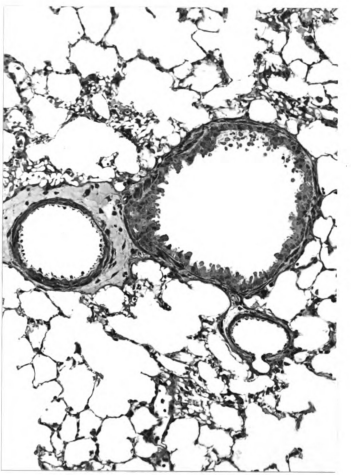


Figure 2-2. Photomicrograph (low magnification) of lung parenchyma of rat 5 days after treatment with MCTP. The perivascular edema and cellularity is more pronounced. Patches of alveolar walls are thickened and hyalinized. Toluidine blue. 130x.

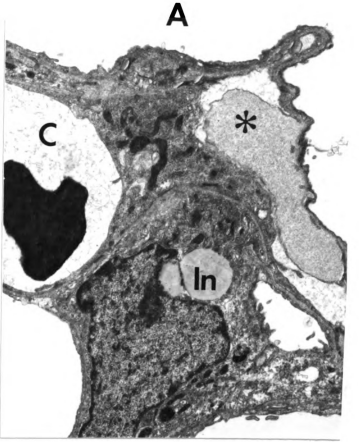


Figure 2-3. Electron micrograph of the alveolar septal wall 5 days after treatment with MCTP. Notice a large focal bleb in a capillary endothelial cell (asterisk). The endothelial cells in the adjacent capillary (C) appear normal. The alveolar space (A) is free of exudate and the interstitial cell appears normal (In). 7,100x.

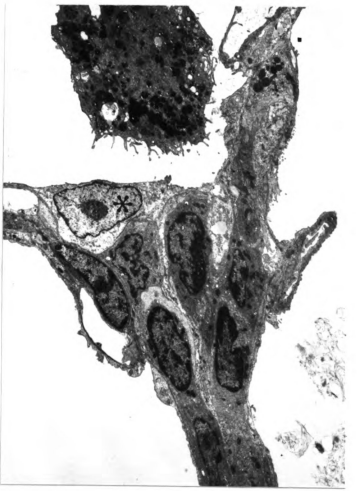


Figure 2-4. Electron micrograph of alveolar septal wall 5 days after treatment with MCTP. Notice an increase in septal cellularity and mild rarefaction of cytoplasm of an alveolar type I cell (asterisk). An alveolar macrophage is evident in an alveolar sac. 2,500x.

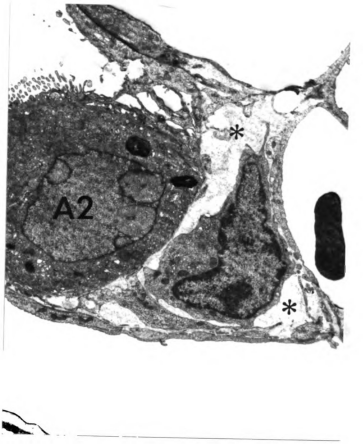


Figure 2-5. Electron micrograph of an abnormal alveolar type II cell (A2) evident at days 8 and 14 after exposure to MCTP. Septal interstitial edema is also evident (asterisks). 4,500x.

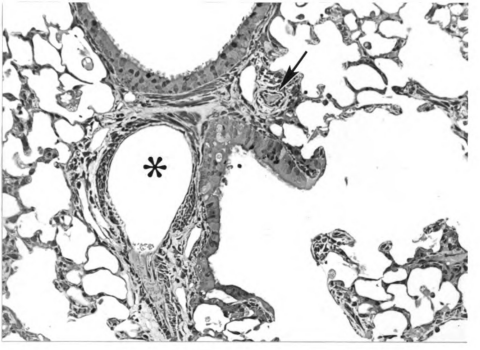


Figure 2-6. Photomicrograph of lung parenchyma of a rat 14 days after treatment with MCTP. There is an accumulation of exudate in the perivascular-peribronchiolar interstitium and thickening of the medial layer of muscular artery (asterisk). The epithelium of this terminal bronchiole is hyperplastic and hypertrophic. Alveolar septa are thickened and hypercellular, and alveolar sacs contain cellular debris. A small, poorly expanded, muscular artery is present near the transition between the terminal bronchiole and alveolar duct (arrow). Toluidine blue. 130x.

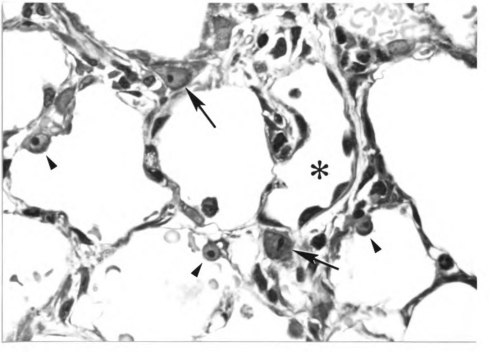


Figure 2-7. Photomicrograph of the alveolar septa of rat 14 days after MCTP administration. Alveolar septa are thickened and hypercellular. Scattered interstitial cells are hypertrophic (arrows) as are endothelial cells lining a nonmuscular vessel (asterisk) and alveolar type II cells (arrowheads). Toluidine blue. 700x.

Figure 2-8. Photomicrographs of a muscular pulmonary artery of an MCTP-treated rat (A) and a control rat (B) 14 days postexposure. For the muscular pulmonary artery of the MCTP-treated rat, the thickened vessel wall is surrounded by interstitial tissue distended by an accumulation of a cellular exudate. A perivascular lymphatic vessel (asterisk) is markedly distended. The epithelium of the adjacent bronchiole is hypertrophic and contains increased numbers of darkly stained goblet cells. Alveolar sacs contain serous exudate. Toluidine blue. 350x.

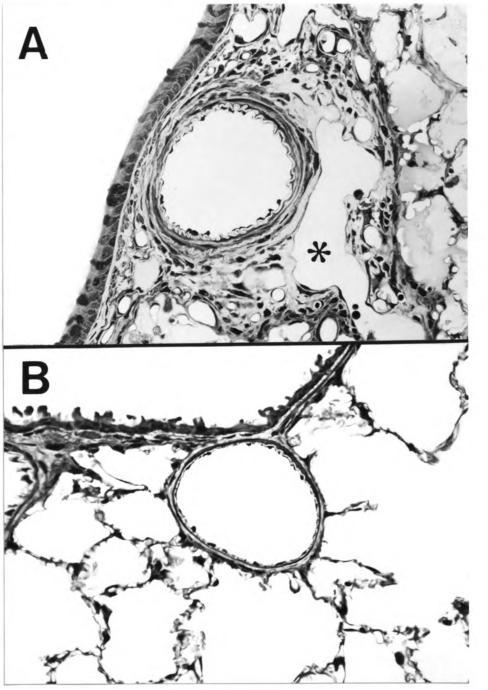


Figure 2-8.

Figure 2-9. Photomicrographs of a pulmonary artery along an alveolar duct from an MCTP-treated rat (A) and a control rat (B) 14 days posttreatment. For the small pulmonary artery of the MCTP-treated rat, the smooth muscle cells and endothelial cells of the vessel wall are hypertrophic. The perivascular interstitial tissue and alveolar septa are distended with exudate. Notice the hypertrophic interstitial cells (arrows). Endothelial cells and smooth muscle cells of the vessel from the control rat are inconspicuous. Toluidine blue. 437x.

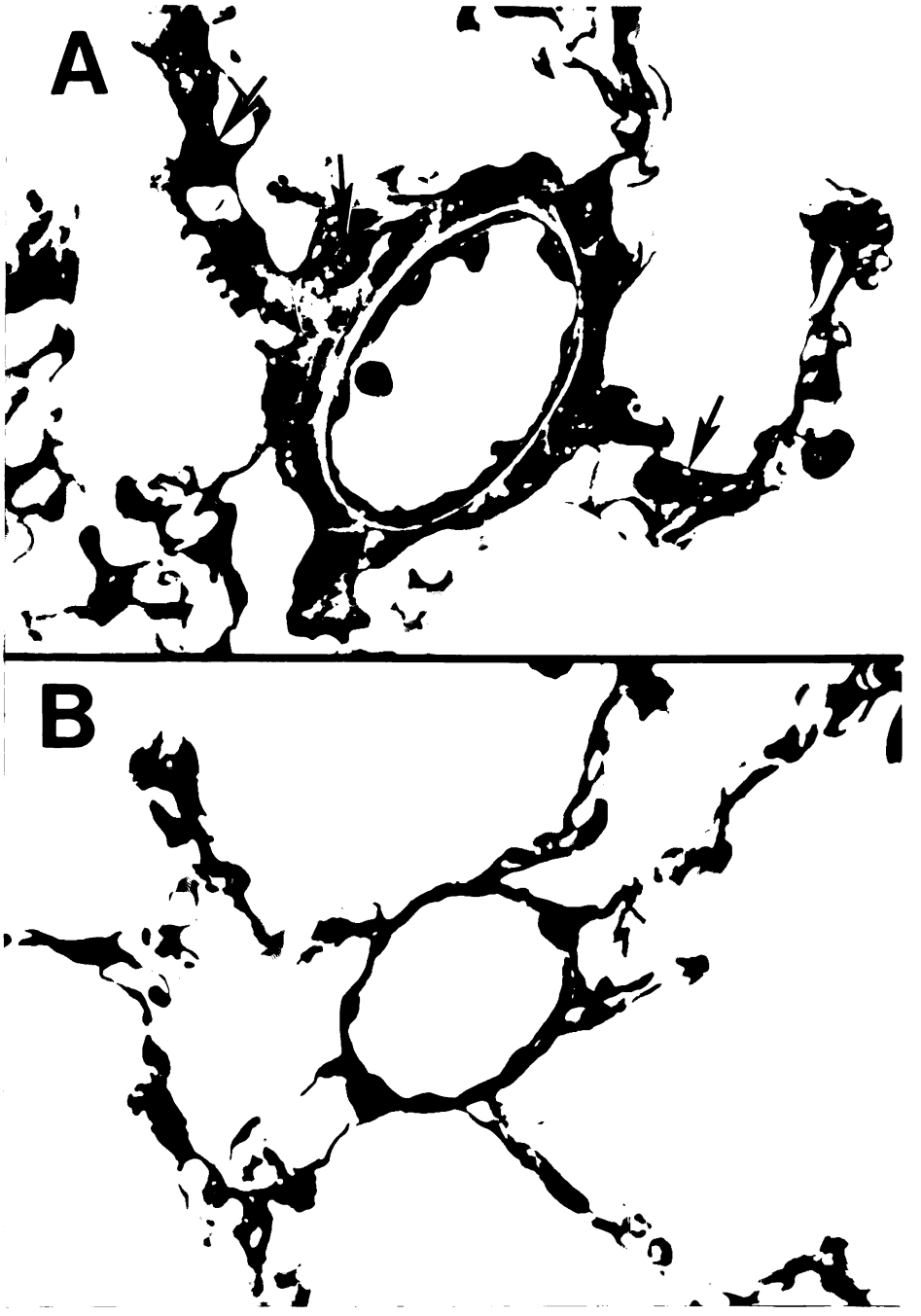


Figure 2-9.

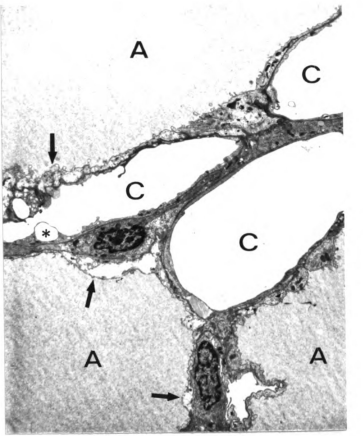


Figure 2-10. Electron micrograph of alveolar septal wall of a rat 14 days after treatment with MCTP. Notice showing alveolar type I cell rarefaction and necrosis (arrows) and serous exudate in alveolar sacs (A). Endothelial cell profiles of capillaries (C) appear normal except for cytoplasmic vesicles (asterisk). 1,900x.

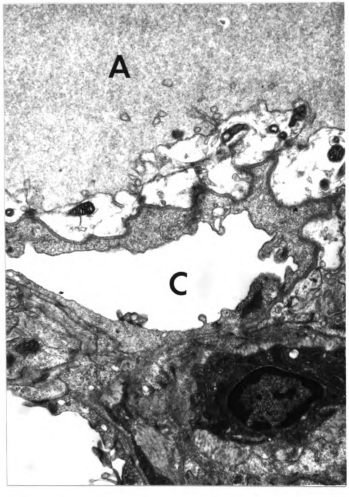


Figure 2-11. Electron micrograph of a portion of an alveolar septa (high magnification) to show severe damage to alveolar type I cell and finely granular exudate in alveolar space (A). Endothelial cell lining capillary (C) is minimally altered. 9,100x.

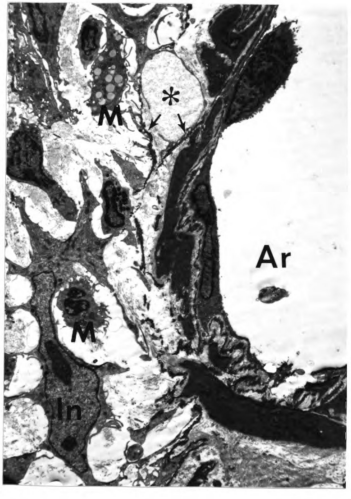


Figure 2-12. Electron micrograph of a wall of a small muscular artery (Ar) and perivascular interstitial tissue (adventitia) 14 days after MCTP treatment. There is an accumulation of intercellular edema (asterisk) in the medial layer and the surrounding interstitial tissue of the artery with splitting of the external elastic laminae (arrows). Perivascular cells included monocyte-macrophage type cells (M) and hypertrophied interstitial cells (In). Smooth muscle cells in the media of the vessel and endothelial cells lining the lumen were hypertrophic. 1,900x.

CHAPTER III

ALTERATIONS INDUCED BY MONOCROTALINE PYRROLE IN MAMMALIAN CELLS IN CULTURE

SUMMARY:

Monocrotaline (MCT), a pyrrolizidine alkaloid, induces hepatic and pulmonary disease that is slow to develop and progressive in rats. MCT is metabolized by the hepatic cytochrome P-450 monooxygenase system to pyrrolic derivatives that produce tissue injury. Monocrotaline pyrrole (MCTP) is one such putative metabolite. We determined the effects of a single exposure to MCTP on two lines of mammalian cells in culture. MCTP induced delayed and progressive injury to monolayers of Madin-Darby canine kidney cells (MDCK) and Crandel-Rous feline kidney (CRFK) cells that was not apparent until 24 hours or more after a single exposure and then worsened with time. The injury was evident as a slowly progressing increase in cell detachment from monolayers and an increase in lactate dehydrogenase (LDH) activity in the incubation medium. In addition, surviving cells underwent a progressive and marked hypertrophy and features of cellular atypia developed over time. At concentrations below those which caused LDH release and enhanced cell detachment, MCTP inhibited cell proliferation of both MDCK and CRFK cells. The delayed and progressive cell death, the progressive cell hypertrophy and atypia, and the inhibition of cell proliferation mimic changes observed *in vivo* in rats exposed to MCT or MCTP. These results are consistent with the premise that MCTP is the proximate toxicant of MCT bioactivation responsible for cell necrosis and cytomegaly.

INTRODUCTION:

Monocrotaline (MCT) is a pyrrolizidine alkaloid (PA) present in the foliage of several plants of the genus, *Crotalaria*. Ingestion of these plants is responsible for livestock and human disease throughout the world (McLean, 1970; Kay and Heath, 1969). Exposure to MCT is associated with injury to liver, lung and kidney in several animal species (Hayashi and Lalich, 1967; Hooper and Scanlan, 1977; Schoental and Head, 1955; Roth et al., 1981). MCT is also carcinogenic in some laboratory animals, inducing pancreatic, pulmonary and hepatic tumors (Newberne and Rogers, 1973; Shumaker *et al.*, 1976b; Hayashi *et al.*, 1977).

In vivo, hepatic alterations produced by moderate doses of MCT are slow to develop and progressive and result in long-lasting changes even after a single exposure (Schoental and Head, 1955). Morphologic alterations that occur in hepatocytes include cell necrosis, vacuolar change and a delayed, progressive cell hypertrophy (Schoental and Head, 1955; Allen *et al.*, 1970; Allen and Chesney, 1972). Typically, these alterations have a zonal distribution such that necrosis occurs in the centrolobular region, fatty change in the surrounding zone and megalocytosis in the peripheral portions of the lobule. The pattern of injury may reflect the lobular distribution of metabolizing enzymes and therefore the amount of reactive, toxic metabolites produced in these zones (McLean, 1970). In addition to morphologic alterations, MCT causes DNA crosslinking and biochemical changes in hepatocytes (Allen *et al.*, 1970; Petry *et al.*, 1984), and markedly inhibits cell proliferation (Jago,

1969, 1970). These changes likely result in altered cell function and inhibited organ repair following insult.

Pulmonary injury from MCT has features in common with the hepatic changes. Delayed and progressive injury can result from a single exposure to MCT (Hayashi and Lalich, 1967; Hilliker *et al.*, 1982; Hayashi *et al.*, 1984; Stenmark, 1985) and is associated with marked hypertrophy of parenchymal cells (Kay *et al.*, 1969; Molteni *et al.*, 1984; Sugita *et al.*, 1983). Unlike the liver, the lung does not metabolize significant amounts of MCT (Mattocks and White, 1971; Hilliker *et al.*, 1983). It is presumed, therefore, that lung alterations are not caused directly by the parent alkaloid or by metabolites produced locally but are induced by reactive metabolites which are produced in the liver and pass to the pulmonary vascular bed (Barnes *et al.*, 1964; Mattocks, 1968). The metabolite responsible for the toxic effects of MCT is not known with certainty. One putative metabolite of MCT that may be responsible for its lung and liver toxicity is monocrotaline pyrrole (MCTP), a reactive electrophile (Mattocks, 1968). Intravenous administration of chemically-synthesized MCTP to rats induces lung injury virtually identical to that induced by MCT (Bruner *et al.*, 1983; Chesney and Allen, 1974).

Studies in several mammalian and non-mammalian systems *in vitro* indicate that MCT in the presence of a bioactivating system is cytotoxic, mutagenic and genotoxic (Mori *et al.*, 1985). Studies employing other PAs indicate that mammalian cells undergo pronounced morphologic alterations not unlike megalocytotic changes observed *in vivo* (Armstrong and Zuckerman, 1972; Takanashi *et al.*, 1980). None of these reports present detailed information on dose-response relationships or the time course of

the injury. In addition, continuous exposure regimens with high concentrations of PAs and/or a bioactivation system have been used to elicit injury.

There are few studies in cell culture systems employing pyrrolic derivatives of MCT, particularly the primary pyrrole, MCTP. Large gaps remain in our understanding of cell responses to PA pyrroles and how these responses might translate into tissue damage. The purpose of this study was to define more precisely the responses of mammalian cells to MCTP. Madin-Darby Canine Kidney (MDCK) cells and Crandel-Rous Feline Kidney (CRFK) cells were used to define the cytolytic cellular response and subsequent cell monolayer disruption. Responses of these two cell lines were compared to assess whether cell types differ appreciably in response to MCTP.

MATERIALS AND METHODS:

MCTP was chemically synthesized via an N-Oxide intermediate from MCT (Transworld Chemical, Washington, DC) as previously described (Mattocks, 1969). MCTP was maintained as a stock solution in the organic solvent, N,N-dimethylformamide (DMF, Sigma Chemical Co.), at a concentration of 20 mg/ml of solvent and stored under nitrogen at -20°C. All dilutions of MCTP were in DMF solvent. MDCK cells and CRFK cells were a gift of Mr. Dan Taylor, Animal Health Diagnostic Laboratory, Michigan State University. Cells were maintained in tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) or Minimal Essential Medium (MEM) containing 2% fetal calf serum (FCS) supplemented with 2 mM glutamine and penicillin 100

U/ml, streptomycin 100 μg/ml and Fungizone 0.25 μg/ml (PSF, Gibco). Cells were split once per week at a 1:3 to 1:6 ratio by rinsing cells with Ca^{++}- and Mg^{++}-free Hank's balanced salt solution (HBSS) containing 0.2 mM EDTA followed by enzymic removal of cells with 0.025% trypsin-0.27 mM EDTA. All cell culture reagents were from Gibco Laboratories.

Cell and Monolayer Morphology

For experiments in which cell and monolayer morphology were monitored, equal volumes of a cell suspension were seeded in 12-well or 24-well tissue culture clusters (Costar) and allowed to proliferate to confluency. Cell monolayers were fed with fresh medium (2% FCS) at 2-3 day intervals. Confluent monolayers were exposed to 50, 5.0, 0.5 or 0 (DMF vehicle) μg of MCTP/ml of medium by rapidly mixing test compound with 1.0 ml of fresh medium and rapidly pouring the mixture into a well containing 1.0 ml of additional medium ("dilution technique"; total volume of medium = 2.0 ml). In some experiments, additional sets of wells received no treatment, and a set of wells received a localized bolus of 5 μl of test compound applied to the center of well containing 2 ml of medium (the final nominal concentration of MCTP was 50 μg MCT/ml). Cells were maintained for 2 weeks after the single exposure to MCTP. Monolayers in replicate plates were fixed with 2.0% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) on 3 to 4 day intervals. Cell monolayers were stained with hematoxylin and eosin or crystal violet (0.5%) stains.

For ultrastructural studies, confluent monolayers of MDCK cells grown on tissue culture cover slips (Thermanox, Miles Lab) were exposed to 50 μg MCTP/ml or DMF vehicle. Medium above monolayers was changed at daily intervals, and at 5 days post-treatment they were washed with 0.1

M phosphate buffered saline (PBS, pH 7.4) and fixed for 4 hours in 2.0% glutaraldehyde in 0.1 M PBS. After fixation, cells were washed twice with 0.1 M PBS. Cells were subsequently scraped from the coverslips, fixed in 1% osmium tetroxide and embedded in Epon-araldite resin. Sections of cell monolayers one micron thick were cut from blocks and stained with toluidine blue. Thin sections were cut from representative blocks with an LKB Ultramicrotome and placed on copper grids. They were subsequently stained with uranyl acetate and lead citrate and viewed with a Phillips 301 transmission electron microscope.

Cell Morphometry

Two-dimensional measurements of surface area of cells were made on hematoxylin and eosin-stained cell monolayers at days 5 and 14 posttreatment with a Joyce Loebl Magiscan Image Analyzer (Nikon) attached to an inverted Nikon TMS microscope. Approximately 150 to 200 cells were measured per treatment group per day.

Cell Proliferation Assay

CRFK cells were plated onto 60 mm^2 tissue culture plates at a density of 5,000 cells per plate and allowed to attach for 3 to 4 hours before being exposed to MCTP. At 7 and 14 days posttreatment, cells in plates were enumerated using a hemacytometer.

Colony-Forming Efficiency Assay

MDCK or CRFK cells were seeded in 100 mm^2 tissue culture plates(Costar) at 500 cells per plate. Cells were allowed to attach for a 3 to 4 hour period before being exposed to DMF vehicle or to MCTP at concentrations of 0.005 to 50 μg MCTP/ml medium. Medium was replaced with fresh medium 7 days posttreatment, and at 14 days plates were fixed with

10% neutral buffered formalin and stained with 0.5% crystal violet (Sigma). The number of cell colonies (>50 cells per colony) in triplicate or quadruplicate plates was determined, and results were expressed as plating efficiency relative to plates receiving 0 μg MCTP/ml (DMF vehicle).

Lactate Dehydrogenase Activity

Cell-free medium from above monolayers of exposed MDCK and CRFK cells was analyzed for lactate dehydrogenase (LDH) activity at daily intervals for five days after a single exposure to DMF or to MCTP at 50, 5.0 or 0.5 μg/ml. Medium from above the monolayers was removed from the wells and centrifuged to remove cells from the supernatant. LDH activity was determined in cell-free supernatants by the method of Bergmeyer and Bernt (1974). The monolayers were rinsed twice with 1 ml of HBSS, and 2 ml of fresh medium was applied to the wells. Cells of the monolayer were lysed with 15 μl of a 10% Triton X-100 solution for determination of total LDH activity in each well. Results are expressed as % release of total LDH as defined by the following formula:

$$\% \text{ release} = \frac{\text{LDH in medium}}{\text{LDH from lysed cells + LDH in medium}} \times 100.$$

Analysis of Data

Results were expressed as mean ± standard error of the mean (SEM). Percentage data were transformed by $\arcsin^{-1}$ transformation prior to analysis. Non-Homogeneous data were log-transformed to attain homogeneity prior to analysis. Data were analyzed by a completely random ANOVA. Tukey's omega-test was used for individual comparisons except for

morphometric data for which Bonferroni's correction for multiple comparisons was used. The criterion for significance was set at $p<0.05$.

RESULTS:

Cell and Monolayer Morphology

MDCK cell monolayers exposed to MCTP applied as a bolus developed a focal zone of cell detachment in the central portion of the wells; however, this zone of cell detachment was not apparent until approximately 24 to 36 hrs after exposure. The borders of the regions of cell detachment were well defined, and cells surrounding this zone appeared healthy and excluded trypan blue. By day 3, cells at the border of the detachment zone were distinctly hypertrophic compared to cells more distant from the zone of cell detachment or cells in control wells. By day 5, cells at the rim of cell detachment had enlarged further, and cells distant from the zone of cell detachment were also distinctly larger than controls.

In a related study in which MCTP was rapidly mixed with medium and applied immediately to the culture well ("dilution technique") a slightly different response of monolayers was apparent. Monolayers of MDCK cells receiving 50 μg MCTP/ml medium had no detectable increase in cell detachment until 24 or more hours after treatment. The increase in cell detachment at this time was subtle and distributed diffusely over the entire monolayer. Cell detachment increased over the next 3 to 4 days, but at no time were gaps in the monolayer apparent throughout the 14 days of the study. The cells in all areas of the monolayers were distinctly hypertrophic by day 3, and this response became more pronounced with time.

Despite cell loss due to detachment, monolayers remained intact because of enlargement of surviving cells. By day 14, the appearance of cells in the monolayers was virtually identical to that of cells in wells that received a bolus administration to the centers of wells.

Increased cell detachment in monolayers receiving 5 μg MCTP/ml was not obvious until slightly later times (48 to 72 hours) than in wells receiving 50 μg/ml. Again, cell detachment was initially subtle but became progressively more pronounced over a 3 to 4 day period. Cell detachment and cell hypertrophy were less pronounced than in cells exposed to 50 μg MCTP/ml. Distinct cell hypertrophy was apparent by 3 to 5 days after treatment and became more pronounced with time. The morphology of monolayers in wells exposed to 0.5 μg/ml did not appear different from vehicle controls.

The photomicrographs in figure 3-1 show the characteristic morphology of MDCK cells in each treatment group 14 days after treatment with various concentrations of MCTP. In the monolayer exposed to 5 or 50 μg MCTP/ml, all cells were tremendously enlarged, and the cytoplasm of few cells was vacuolated. Vacuoles in the cytoplasm were either numerous, small and diffusely distributed throughout the cytoplasm or few, large and located in the perinuclear cytoplasm. The large, hypertrophied cells had vesicular nuclei which were much larger than entire cells in the control wells. Multiple, large, irregularly shaped nucleoli were obvious in most cells. Multinucleated cells were common, and some cells had nuclei with irregular lobulation.

Similar responses of cell detachment and hypertrophy of surviving cells were observed in the CRFK cell monolayers after exposure to MCTP by

the dilution technique. CRFK monolayers were more severely disrupted at the 50 μg/ml dose by 3 to 5 days post-treatment than MDCK cell monolayers. Gaps between cells developed between 4 and 7 days in monolayers exposed to 50 μg MCTP/ml, but monolayers exposed to 5 μg MCTP/ml remained intact throughout the study. By day 14 most cells in wells exposed to 50 μg MCTP/ml and many cells in wells exposed to 5 μg/ml were markedly enlarged (Figure 3-2). Many of the CRFK cells in wells receiving 50 μg MCTP/ml and fewer cells in those receiving 5 μg/ml were multinucleated or had unusual, variably sized lobulations of nuclei. Some cells had 8 or more nuclei or nuclear lobulations bunched into the central portion of the cell.

The morphometric data of two dimensional surface area of MDCK cells 5 and 14 days posttreatment, are depicted in figure 3-3. Cells receiving 5 or 50 μg/ml MCTP underwent a dramatic, dose-dependent increase in size, which became more pronounced by day 14. Surface area of cells exposed to 5 and 50 μg/ml MCTP increased by 20 and 30-fold, respectively.

Ultrastructurally, the enlarged MDCK cells had markedly enlarged cell profiles compared to controls. The nuclei of cells had abundant euchromatin and contained multiple, prominent nucleoli compared to control cells, in which there was abundant heterochromatin and inconspicuous nucleoli. Nucleoli of MCTP exposed cells had separation of granular and fibrillar components, and some nuclei had invaginations of cytoplasm into the nucleus. The cytoplasm of a few cells had large perinuclear cytoplasmic vacuoles; however, most cells did not have evidence of distinct degenerative changes. Cytoplasmic organelle density or distribution did not appear to be altered, although there was a marked

increase in cytoplasmic area per cell, suggesting an overall increase in numbers of organelles per cell.

Cell Proliferation

The effects of MCTP on CRFK cell proliferation are shown in figure 3-4. Concentrations of MCTP greater then 0.05 ug/ml inhibited or prevented proliferation of CRFK cells.

Colony Forming Efficiency

MCTP induced a dose-dependent decrease in colony-forming efficiency in both MDCK and CRFK cells (Figure 3-5). No colonies were identified in any of the plates receiving 5 or 50 μg MCTP/ml. However, scattered, extremely large cells attached to the plate surface were evident in these wells 14 days after exposure. These cells were viable by the criterion of trypan blue exclusion. Colonies on plates receiving 0.5 μg MCTP/ml were fewer and smaller than colonies on plates receiving lower doses of MCTP or DMF vehicle. MDCK and CRFK cell colonies on these plates contained a mixture of large atypical cells, similar to those in wells receiving the higher concentrations of MCTP, and smaller, proliferating cells comparable to cells exposed only to the DMF vehicle.

Lactate Dehydrogenase Activity

MCTP induced a delayed and progressive increase in LDH activity in the incubation medium above monolayers of both MDCK and CRFK cells but not until after day 1 posttreatment (Figure 3-6). For CRFK cells, a difference in LDH activity release between 50 μg/ml wells and control wells was apparent on day 2. For MDCK cells exposed to 50 μg MCTP/ml, there was a trend toward increased LDH activity by day 2 although this did not reach statistical significance until day 3. CRFK cells were more

sensitive than MDCK cells to the cytolytic effects of MCTP, as indicated by release of a greater proportion of total LDH activity from monolayers and the more rapid increase in LDH release. This corresponded to the earlier and more extensive disruption of monolayers of CRFK cells as assessed morphologically.

LDH release from vehicle-treated MDCK cell monolayers increased slightly with time over the duration of the study. This was due to continued cell turnover in control wells after monolayers reached confluency, as evidenced by continued appearance of detached cells in the medium in the face of maintenance of monolayers with small, uniform cells. This turnover likely contributed to the small increase in LDH activity in control wells with time. The population of CRFK cells in control wells, however, was more stable after reaching confluency, and therefore the increased LDH release and increased numbers of detached cells were not evident in control wells.

DISCUSSION:

Our findings indicate that a single exposure of cells to MCTP causes delayed and progressive cytolytic injury to MDCK and CRFK cells. When MCTP was uniformly applied to monolayers of cells, injury was manifest as an evenly distributed, progressive detachment of cells from confluent monolayers that was not apparent until 24 hours or more. The appearance of enhanced levels of LDH activity in the medium above monolayers of cells signaled enhanced cell membrane permeability and lysis of at least some cells of the monolayer. Enhanced LDH activity in the medium above exposed

cell monolayers was not apparent at 24 hours posttreatment but was evident at 2 or 3 days and increased with time thereafter. CRFK cells seemed particularly susceptible to the cytolytic effects of MCTP, in that deterioration of the monolayers was more rapid and extensive than in MDCK cells. The delayed cell injury and progressive monolayer disruption of MDCK and CRFK cells are reminiscent of the time course of the tissue changes induced *in vivo* by low concentrations of MCTP, particularly the delayed and progressive lung injury observed after a single exposure to MCT or MCTP (Bruner *et al.*, 1983; Butler *et al.*, 1970).

MDCK cell monolayers exposed to a localized bolus of MCTP developed a well defined focus of cellular detachment. The cells in this area were presumably exposed to high concentrations of MCTP. That a well defined focus of cell detachment developed after exposure by this method is consistent with the short-lived, reactive nature of MCTP. These results emphasize that the exposure technique influences how monolayers respond to MCTP.

The zones of cell detachment induced by a bolus of MCTP were not readily repaired by surviving cells unless the foci were sufficiently small to be covered by gradual spreading and hypertrophy of cells which bordered these areas. Even when monolayer defects were covered by cell spreading and hypertrophy, only temporary repair of monolayer integrity was achieved. Repair by cell proliferation did not occur. Unexposed cells added to the well rapidly repopulated this zone and restored monolayer integrity, however, confirming that the growth surface was not altered by treatment (unpublished observations). These unexposed cells continued to repopulate areas of the plate where cells were lost by

attrition or mechanical removal if they had direct access to these areas along the plate surface. Parallels to these responses may occur *in vivo* in the liver parenchyma and pulmonary and hepatic vasculature. Unexposed or unaffected cells may be required for complete repair of the defective tissue. *In vivo*, the access of these cells to areas requiring repair may be limited because of contact inhibition from cells that are not reproductively viable but retain their metabolic viability. Perhaps it is only after the MCTP damaged cells are removed that unaffected cells can restore the damaged tissue.

MDCK and CRFK cells which survived the cytolytic effects of MCTP became markedly enlarged, and features of cellular atypia gradually appeared in these cells. The extreme cell enlargement maintained the confluency of MDCK and CRFK cell monolayers for days to weeks after a single exposure by the dilution technique. This occurred despite enhanced cell detachment. The extreme hypertrophy of MCTP exposed cells mimics responses of hepatic parenchymal cells and endothelial cells of the liver and lung after systemic administration of MCT or responses of the pulmonary endothelium after intravenous administration of MCTP. Megalocytotic cells develop in the hepatic parenchyma and help maintain organ size and function for months to years after a single exposure to MCT (Kelly, 1985). In the pulmonary vasculature of animals given MCT or MCTP, endothelial cell hypertrophy ultimately occurs which can cause microvascular occlusion (Butler *et al.*, 1970; Butler, 1970; Vincic et al., 1989). The enlargement and spreading of cells, particularly in the vasculature, could be a protective response following certain insults. This could explain in part why overt injury, evident morphologically as

perivascular interstitial exudation in the lung and central veins of the liver, is delayed or slow to develop. Compensatory enlargement and spreading of cells could limit vascular leak initially and delay the onset and progression of perivascular exudation.

The enlargement of MDCK cells was not attributed to degenerative changes, such as vacuolar or hydropic change, in most attached cells. In fact, many cells had ultrastructural features compatible with enhanced cellular activity: prominent, multiple nucleoli, enlarged nuclei, prominent endoplasmic reticulum and enlarged Golgi apparatus. These changes were similar to the megalocytotic changes which develop in cells of the liver and lungs of animals exposed to toxic PAs (Afzelius and Schoental, 1967; Butler, 1970). As *in vitro*, most enlarged cells *in vivo* did not have degenerative alterations but had cellular features which suggested enhanced cell activity as well as features of cellular atypia.

The rate of cell enlargement in cultured cells appeared to depend in part on the amount of room available for cell expansion. Contact inhibition with other cells appeared to limit cell enlargement. In wells exposed to a localized bolus of MCTP, cells near the border of the necrotic zone enlarged more rapidly than cells distant from this zone. Areas of detachment of cells provided a growth surface for expansion of surviving cells. In monolayers exposed by the dilution technique, cell enlargement was more uniform. However, if these monolayers had zones of cells removed mechanically, cells at the edge of this zone enlarged more rapidly and to a greater degree than cells distant from this zone. In addition, cells exposed in subconfluent monolayers enlarged more rapidly than cells of confluent monolayers (unpublished observations). These

findings suggest that the rate of cell enlargement is dependent not only on dose of MCTP but also on the room available for cell expansion.

In vivo, the rate of cell enlargement may also reflect the physical area available for cell expansion or a lack of contact inhibition. Hepatocellular megalocytosis develops more rapidly in young growing animals and in animals subjected to partial hepatectomy (Jago,1969; Peterson, 1965; Downing and Peterson, 1968). Prior administration of necrogenic hepatotoxicants, such as carbon tetrachloride, also caused more rapid cell enlargement (Jago, 1969). These conditions provide a stimulus for expansion of liver mass. Although the PA exposed hepatocytes can not divide, they respond to this stimulus by enlargement, which contributes to the maintenance of organ mass.

Concentrations of MCTP which did not cause release of LDH from monolayers inhibited MDCK and CRFK cell proliferation. Concentrations of 5 μg MCTP/ml blocked cell colony formation and 0.5 μg MCTP/ml caused a pronounced reduction in the ability of cells to proliferate and form colonies. As mentioned above 0.5 ug MCTP/ml did not cause enhanced lysis of cells in the monolayers. In addition, many MDCK and CRFK cells exposed to 50 and 5 μg MCTP/ml remained attached to the plate surface and were capable of excluding trypan blue, indicating that they were viable by these criteria. These findings indicate that although surviving cells were reproductively dead based on their inability to proliferative they were metabolically viable for days to weeks after exposure. MCTP can therefore induce direct cytolytic injury to some cells of a monolayer and can also inhibit proliferation surviving cells, thus ultimately hampering monolayer repair.

To our knowledge, only two reports have mentioned the effects of MCTP on cultured cells, and these do not present experimental details. Culvenor *et al.* (1970) indicated that MCTP was cytotoxic to KB cells with an ED50 of 17 μg/ml and the activity of MCTP was much more potent than the parent alkaloid, MCT (ED50 >100 μg/ml). Neither details of cellular responses nor the endpoint used for determination of the ED 50 were given in this report. However, the reported ED50 concentration was within the range of MCTP concentrations found to cause cell injury in our study. In another brief report, Hooson *et al.* (1973) indicated that MCTP was cytotoxic to an unspecified cell type. Exposure of cells to 30 μg MCTP/ml caused enhanced cytotoxicity within 10 minutes of exposure but not thereafter and it caused no inhibition of cell proliferation. These findings are unlike the findings in our study in that we did not observe cytolytic effects until much later after exposure, and MCTP caused a pronounced and long-lived inhibition in cell proliferation.

The MCTP-induced cellular responses observed in our study differ somewhat from those reported for the hydrolysis product of MCTP, dehydroretronecine (DHR). This secondary pyrrole had antimitotic effects like MCTP, but was not cytolytic to a line of rat hepatocytes (i.e., BL8L cells) (Mattocks and Legg, 1980). DHR also induced progressive cell gigantism in BL8L cells. Styles *et al.* (1980) found that this pyrrole inhibited proliferation of a line of mouse kidney cells and increased numbers of cell transformants. DHR therefore can inhibit cell proliferation, stimulate the development of cell hypertrophy and enhance mutational frequency in cells. It does not appear to induce cytolytic effects on cells.

The effects of the parent alkaloid, MCT, on cells in culture have also been reported. Styles *et al.* (1980) indicated that MCT, in conjunction with a bioactivating system, decreased survival (assessed as colony forming efficiency) of a line of BHK21/C13 mouse kidney cells at a concentration of 0.6 μg/ml. This concentration was virtually identical to the concentration of MCTP that inhibited MDCK and CRFK cell proliferation. This finding supports the contention that MCTP is a toxic metabolite of MCT.

MCT without a bioactivating system is far less toxic to cells in culture. Hirchinson and Hill (1960) reported that MCT at concentrations of 50 and 125 μg/ml caused a mild increase in nuclear size in human embryo liver (HuLi) cells by 3 days after exposure. Higher concentrations (250 to 500 μg/ml) induced megalocytosis and the appearance of bizarre mono- and multinucleated HuLi cells but had no effect on HeLa cells. Continuous exposure regimens were used in this study. Umeda and Saito (1971) reported that MCT was toxic to mixed cultures of primary cells from human fetal liver or lung. In contrast to the findings of Hirchinson and Hill (1960), they reported that MCT at the higher concentrations used in their study was also toxic to HeLa cells. These high concentrations of MCT (10^{-2} to 10^{-3} M) induced vacuolation and mild enlargement of these cell types. The mitotic index of the cells was not altered, however. This suggests that the toxic effects observed in that study were not due to toxic MCT metabolites, since changes in mitotic index have been seen in studies employing pyrrolic PA derivatives or PAs with a bioactivating system. Furthermore, hepatocytes of fetuses probably do not have a substantial capacity to bioactivate toxic compounds. Thus pyrrolic metabolites may

not have been formed in significant amounts. The concentrations of MCT used in the study of Umeda and Saito (1971) were extremely high relative to those employed by other investigators. The authors indicated the effects observed in the cells could have been caused by alterations in medium osmolality or pH. Some preliminary studies in our laboratory indicate that brief exposure of cells to MCT may not be sufficient to cause noticable cell injury; however, prolonged exposure can cause cellular alterations. We have observed that a single, 24- to 48-hour exposure of MDCK cells to 50 to 400 μg MCT/ml did not cause enhanced cell detachment or enlargement of cells, but continuous exposure to >100 μg/ml for 2 weeks stimulated mild cellular enlargement (unpublished observations). It thus appears that prolonged exposure to MCT is necessary to induce cell injury when a bioactivating system is not employed.

In summary, MCTP produces injury to MDCK and CRFK cells and cell monolayers that is delayed in onset and progressive. MCTP also causes progressive and pronounced cell enlargement and cellular atypia that is dependent on dose. Concentrations of MCTP below those which caused a cytolytic response caused a marked inhibition of cell proliferation. These dose dependent responses are similar to the responses of cells exposed to MCTP *in vivo*, particularly those of the hepatic and pulmonary microvascular endothelial cells. The similarities in cell response in our study with MCTP and in other studies with MCT with a hepatic S9 fraction support the contention that MCTP is an active pyrrolic metabolite of MCT which causes cell damage.

Figure 3-1. Photomicrographs of morphology of MDCK cell monolayers exposed to 0 (A), 0.5 (B), 5 (C) or 50 (D) μg MCTP per ml of medium 14 days after treatment. Hematoxylin stain. Phase contrast. 150x.

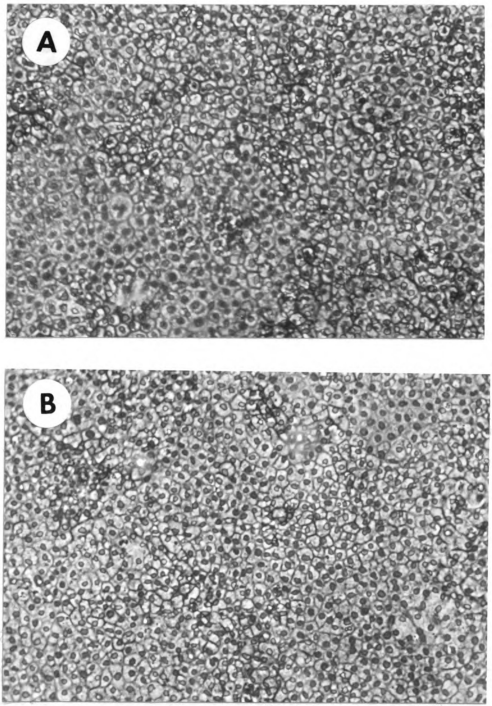


Figure 3-1

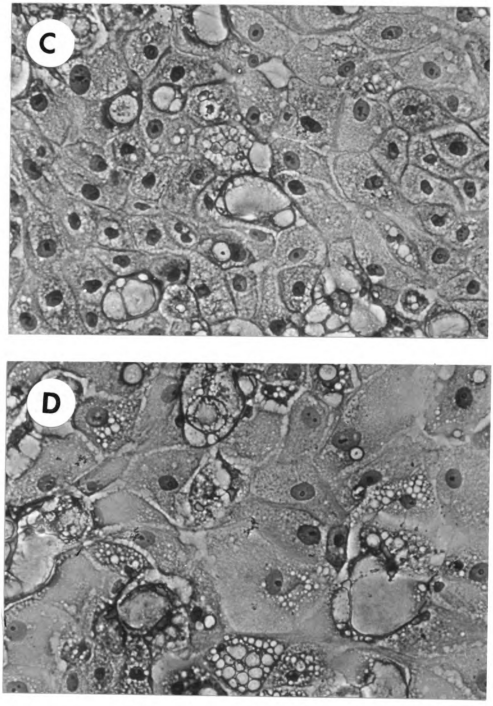


Figure 3-1. (cont.)

Figure 3-2. Photomicrographs of CRFK cells 14 days after a single exposure to 0 (A) or 50 (B) μg MCTP/ml of medium. Enlarged MCTP exposed cells have multilobulated nuclei and prominent nucleoli. Phase contrast. 300x.

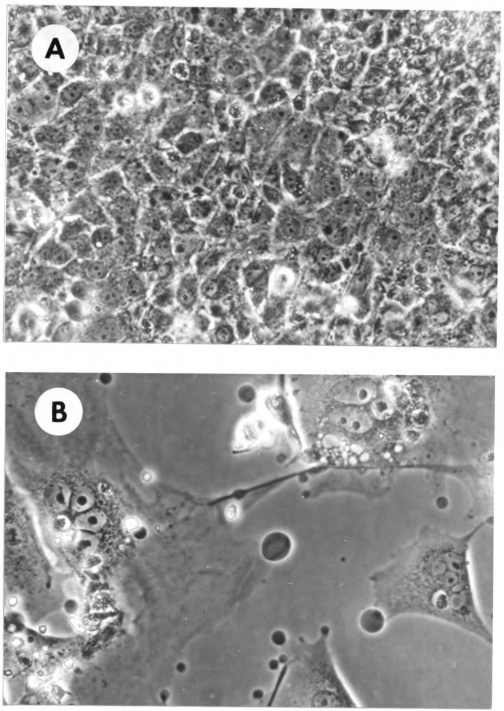


Figure 3-2.

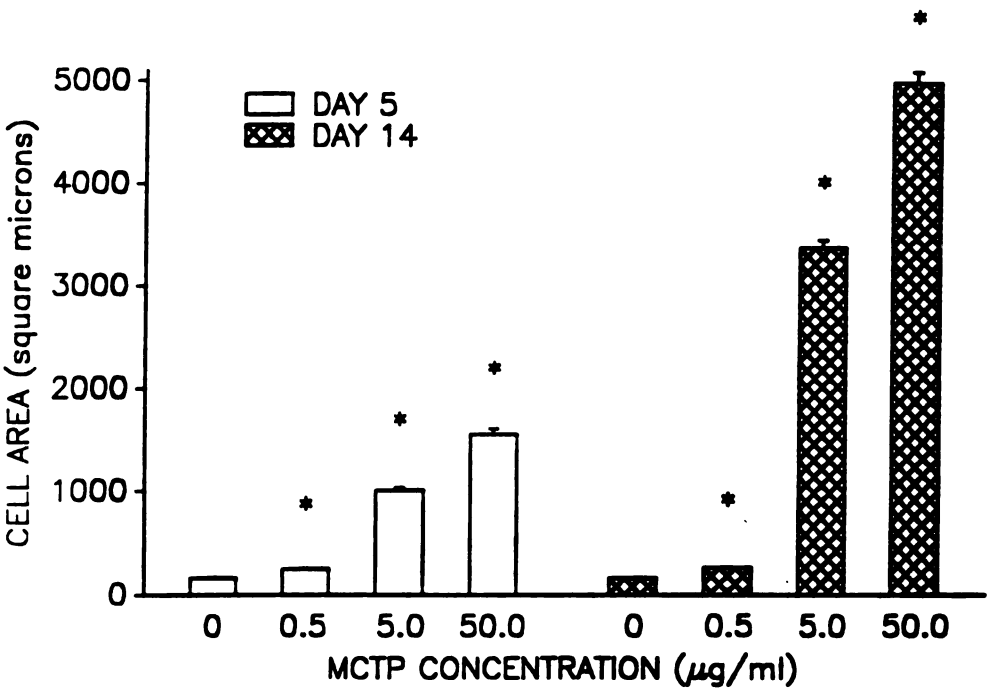


Figure 3-3. **Two dimensional surface area of MDCK cells 5 and 14 days after a single exposure to MCTP. Each values represents mean ± SEM for each treatment group (n=150-200). Asterisks indicate significant differences from control on the same day.** $p < 0.05$.

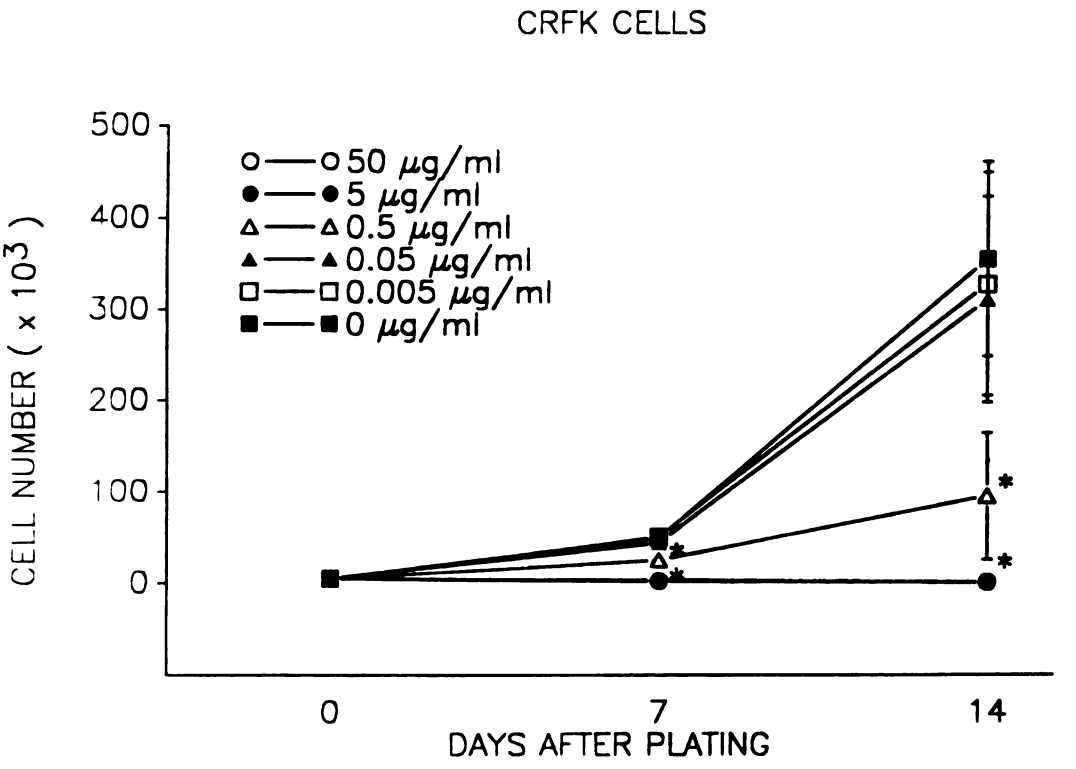


Figure 3-4. **Effects of MCTP on CRFK cell proliferation. Values represent** mean ± SEM (n=5). **The data points for the 5 and 50 µg MCTP/ ml dose** groups overlap. **Asterisks indicate significant differences from control** of the same day. $p < 0.05$.

Figure 3-5. Colony forming efficiency of MDCK (A) and CRFK (B) cells after exposure to MCTP. Values represent mean ± SEM as a percentage of controls (n=4 for CRFK cells; n=6 for MDCK cells). Asterisks indicate significant differences from control. $p < 0.05$.

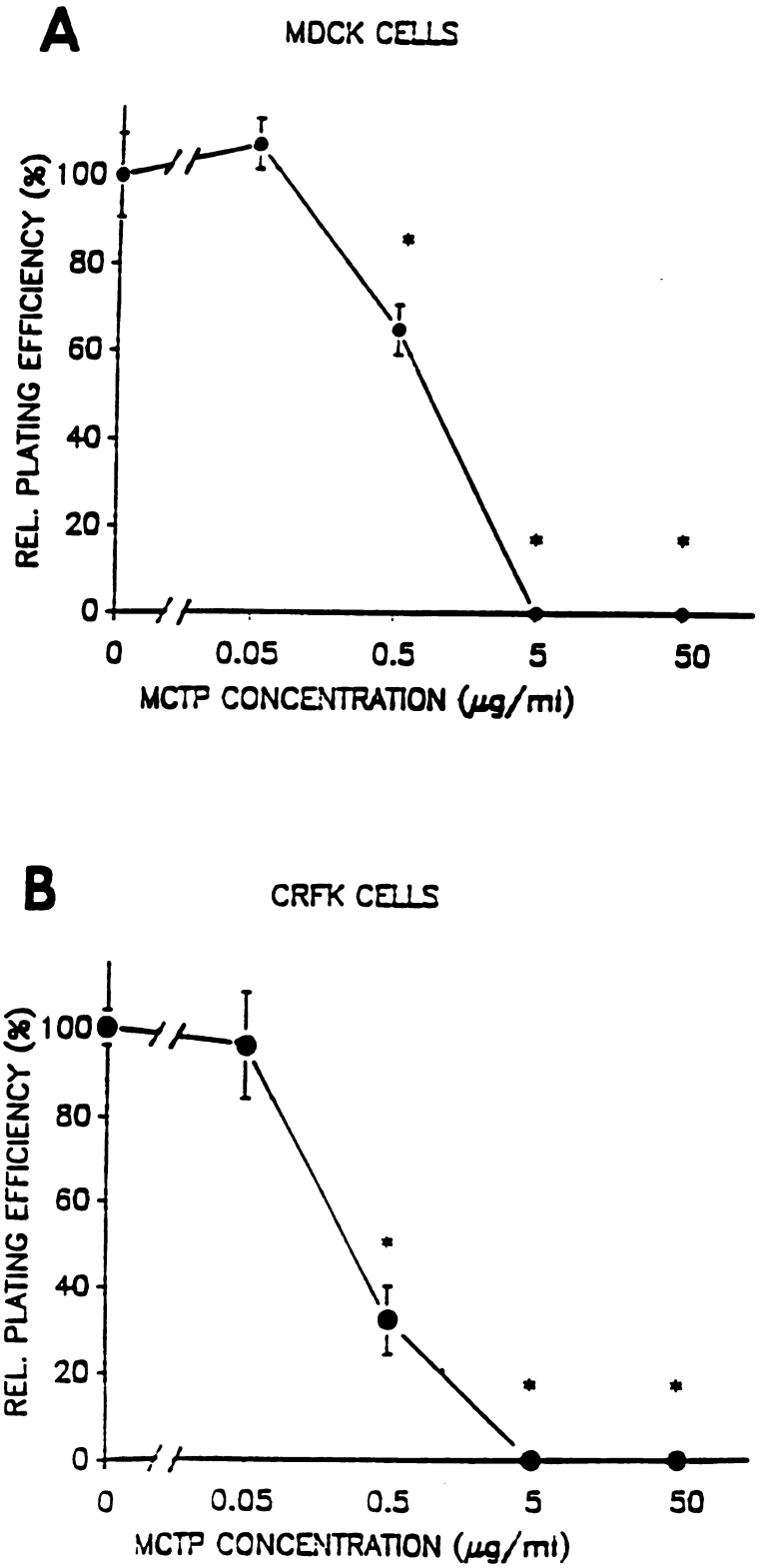

A
MDCK CELLS
REL. PLATING EFFICIENCY (%)
100
80
60
40
20
0
0
0.05
0.5
5
50
MCTP CONCENTRATION (μg/ml)
B
CRFK CELLS
REL. PLATING EFFICIENCY (%)
100
80
60
40
20
0
0
0.05
0.5
5
50
MCTP CONCENTRATION (μg/ml)

Figure 3-5.

Figure 3-6. Lactate dehydrogenase activity release from MDCK (A) and CRFK (B) cell monolayers after a single exposure to MCTP on day 0. Values are expressed as mean ± SEM (n=3). Asterisk indicates significant difference from control on the same day ($p < 0.05$).

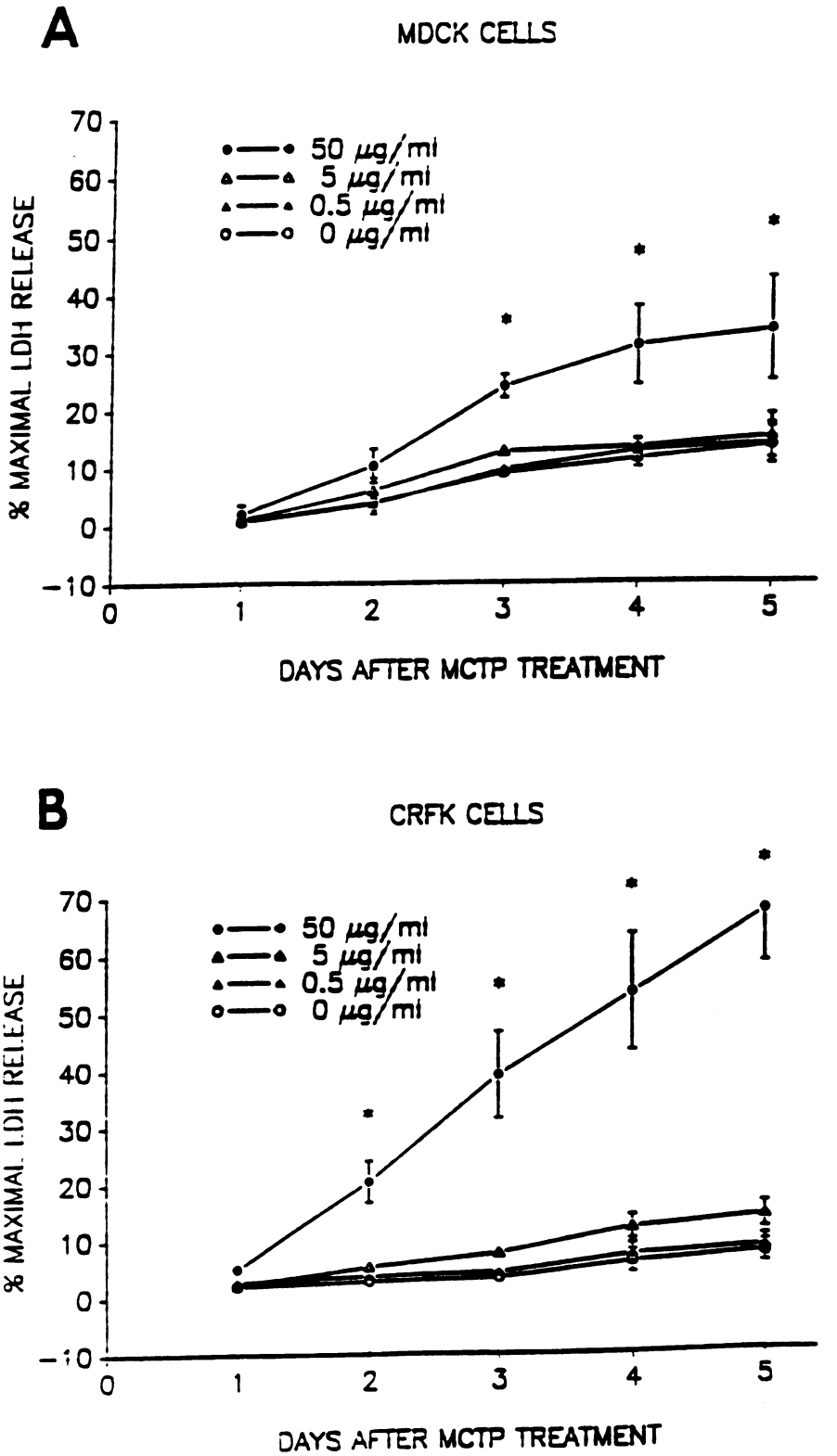

A
MDCK CELLS
50 µg/ml
5 µg/ml
0.5 µg/ml
0 µg/ml
% MAXIMAL LDH RELEASE
70
60
50
40
30
20
10
0
−10
0
1
2
3
4
5
DAYS AFTER MCTP TREATMENT
B
CRFK CELLS
50 µg/ml
5 µg/ml
0.5 µg/ml
0 µg/ml
% MAXIMAL LDH RELEASE
70
60
50
40
30
20
10
0
−10
0
1
2
3
4
5
DAYS AFTER MCTP TREATMENT

Figure 3-6.

CHAPTER IV

THE EFFECTS OF MONOCROTALINE PYRROLE ON CULTURED PULMONARY ARTERY ENDOTHELIAL AND SMOOTH MUSCLE CELLS

SUMMARY:

Monocrotaline pyrrole (MCTP), a reactive electrophile, induces delayed and progressive pulmonary edema, vascular remodeling and pulmonary hypertension after a single intravenous administration to rats. We examined the effects of a single exposure of cultured bovine pulmonary artery endothelial cells (BECs) and bovine pulmonary artery smooth muscle cells (BSMCs) to MCTP. MCTP caused a dose-dependent, delayed and progressive cell detachment and release of lactate dehydrogenase activity from monolayers of BECs but not BSMCs. Monolayers of BECs also released increased concentrations of 6-keto-prostaglandin $F_{1\alpha}$, the stable metabolite of prostaglandin as the post-treatment interval increased. Progressive and marked endothelial cell hypertrophy, assessed morphometrically, occurred after exposure to a nominal concentration of 5 or 50 μg/ml of MCTP but not after 0.5 μg/ml. Morphologic changes in monolayers of BSMCs were minimal, even up to 2 weeks after exposure. Ultrastructurally, the hypertrophic, MCTP-treated BECs had enlarged cell profiles with enlarged nuclei. The nucleoli were prominent, and occasionally multiple. Cytoplasmic microtubules and perinuclear intermediate filaments were prominent in some cells as were the golgi apparatus and endoplasmic reticulum. Degenerative changes were not prominent. MCTP inhibited proliferation of both cell types at concentrations (0.5 μg/ml) which were not cytotoxic. These findings indicate that MCTP induces direct, dose-dependent injury to cells in culture that is delayed and progressive, and the expression of this injury depends in part on the cell type.

INTRODUCTION:

Monocrotaline (MCT) is a pyrrolizidine alkaloid (PA) found in the foliage of *Crotalaria spectabilis* (Adams and Rogers, 1939). A single administration of MCT to rats results in pneumotoxic changes that are delayed in onset and progressive (Ghodsi and Will, 1981; Hilliker et al., 1982). These include pulmonary vascular leak, pulmonary arterial vascular remodeling and pulmonary hypertension (PH). Pulmonary damage is not caused directly by MCT but by its metabolic products. MCT and other pneumotoxic PAs are converted to reactive pyrroles by the mixed function oxidase system of the liver, but not of the lungs (Hilliker *et al.*, 1983a; Mattocks, 1968a; Mattocks and White, 1971). It is presumed that small quantities of these pyrrolic metabolites escape binding in the liver and travel via the blood stream to the pulmonary vascular bed, where they bind covalently to cellular macromolecules (Barnes *et al.*, 1964). The dynamics of the responses of pulmonary vascular cells after this initial binding are largely unknown, and how this binding relates to the delayed and progressive pneumotoxicity and vascular remodeling is not well understood. For example, it is unknown whether pyrrolic derivatives of MCT produce their effects by direct, cytotoxic interactions with cells of the lung or whether cell injury requires secondary factors, such as cellular release of inflammatory mediators or activation of the immune system.

Monocrotaline pyrrole (MCTP) is an unstable, electrophilic, putative metabolite of MCT (Mattocks, 1968a). When a single injection of a low dose of chemically synthesized MCTP is injected into tail veins of rats, delayed and progressive pulmonary injury, PH and right-sided myocardial

hypertrophy results that is similar to that induced by MCT itself (Bruner *et al.*, 1983a). Associated with these changes is remodeling of the pulmonary arteries and microvasculature (Chesney *et al.*, 1974; Butler *et al.*, 1970; Lalich *et al.*, 1977; Raczniak *et al.*, 1979).

Circulating blood cells and pulmonary vascular endothelial cells are presumably among the cells first exposed to MCTP after intravenous injection. Indeed, endothelial cells of the pulmonary arterial vasculature and capillary bed are likely exposed to higher concentrations of the short-lived pyrrole than are other lung cells. In morphologic studies of MCT- or MCTP-induced lung disease, endothelial cell changes are evident as the pulmonary disease progresses (Valdivia *et al.*, 1967; Butler, 1970; Hurley and Jago, 1975; Rosenberg and Rabinovitch, 1988). These changes are relatively subtle early in the course of the disease but become more apparent with time. Smooth muscle cells of the blood vessels and cells of the alveolar septal interstitium might be exposed to lower concentrations that escape binding in the vascular compartment and survive passage into the interstitial fluid.

The response of endothelial cells and vascular smooth muscle cells *in vitro* may aid in understanding the delayed and progressive pathologic changes which occur *in vivo* in the pulmonary parenchyma and microvasculature after exposure to MCTP. For example, such responses may help to define which of the effects observed *in vivo* are direct consequences of MCTP-cell interactions and which ones may be responses to endogenously-synthesized mediators, the release of which might be triggered by MCTP or by the pulmonary hypertensive response. Accordingly, we tested the hypothesis that cells of the pulmonary vasculature are directly injured

after a single exposure to MCTP and that this injury is delayed and progressive. Toward this end, we examined the cytotoxic and proliferative responses to MCTP of bovine pulmonary artery endothelial cells (BECs) and pulmonary artery smooth muscle cells (BSMCs) grown in culture. Our finding of delayed and progressive alterations in these cells is reminiscent of the response to MCTP *in vivo*.

MATERIALS AND METHODS:

Preparation of Endothelial Cells.

Bovine endothelial cells were isolated from segments of pulmonary artery by the modifications of the methods of Jaffe *et al.* (1973) and Booyse *et al.* (1975). Segments of the mainstem pulmonary artery were aseptically removed from young calves, rinsed with Hank's balanced salt solution (HBSS) containing 1% penicillin (100 units/ml)-streptomycin (100 μg/ml)-fungizone (0.25 μg/ml) (PSF; Gibco) and the adventitia and external elastic laminae were removed. The 5 to 6 cm segments of vessels were opened and placed luminal surface down into 60-mm petri dishes containing a 3 to 5 ml of collagenase solution (0.1%) (Sigma Chemical Co.). The abluminal surface of the vessels was briefly exposed to ultraviolet light to kill remaining adventitial fibroblasts, and the surface layer of smooth muscle cells. The tissue was incubated for 3 to 5 minutes at 37°C, and sheets of endothelial cells were gently brushed from the luminal surface with a rubber policeman. These brushings were dispersed into 100 mm petri dishes containing HBSS. Individual small sheets or clusters of the suspended endothelial cells were collected with a Pipetman® pipetter using

200 μl pipet tips and the aid of a TMS Nikon inverted microscope. Cells were transferred to wells of 12-well tissue culture cluster plates (Costar) containing 0.5 ml of cell culture medium. Cells were allowed to attach to the well surface for 3 to 4 hours. Wells were then rinsed vigorously with HBSS to remove unattached cells, and fresh medium was placed into the wells. Plates were incubated until individual colonies developed (4 to 7 days), and selected wells free of fibroblasts and smooth muscle cells (spindle cells) were identified. Cells from healthy colonies were gently scraped from the culture surface and replated onto 60 mm tissue culture petri dishes or 6 well tissue culture clusters (Costar). Once cells in these wells had proliferated to confluency, endothelial cells were removed from selected plates with trypsin-EDTA solution (0.025% trypsin, 0.27 mM EDTA in Ca^{++}-free HBSS) and seeded onto 100 mm tissue culture plates for expansion of the cell population. Endothelial cells were identified by the characteristic cobblestone morphology of monolayers, ultrastructural features, positive staining for factor VIII antigen and negative staining for desmin antigen.

Preparation of Smooth Muscle Cells.

BSMCs were isolated from small sections of pulmonary artery from which the external elastic laminae was removed. The tissue was rinsed vigorously, and the luminal and adventitial surfaces of the vessel were exposed to ultraviolet light to kill surface smooth muscle and endothelial cells. The vessel intima and internal elastic lamina was removed from the vessel, and fragments of the wall, approximately 1.0 mm^3, were excised from the muscular layer. Each fragment was placed in a tissue culture well with sufficient medium to immerse the lower portion of the tissue.

Within 4 to 7 days, outgrowths of bipolar spindle cells spread from the tissue sections on the plastic surface of the plate. Tissue fragments were then removed from the plate, and the attached cells continued to proliferate to form monolayers.

BSMCs isolated in this manner stained positively for desmin antigen, negatively for factor VIII antigen, and were spindle-shaped in appearance. These cells grew in a hill-and-valley, whorling pattern. They did not form a uniform monolayer but grew in overlapping layers.

BECs and BSMCs were grown in Dulbecco's Modified Eagles Medium (DMEM; Gibco) containing 10% fetal bovine serum (Gibco) and with an additional 2 mM glutamine and 1% PSF solution (Gibco). Both BECs and BSMCs were passed using trypsin-EDTA solution and were used at passages less than 10.

Preparation of MCTP.

MCTP was prepared from MCT (Transworld Chemicals, Washington, DC) via an N-oxide intermediate by the method of Mattocks (1969). MCTP isolated by this synthesis procedure has Ehrlich activity (Mattocks and White, 1971) and a structure consistent with MCTP as determined by mass spectrometry and nuclear magnetic resonance (Bruner *et al.*, 1986). MCTP was maintained in N,N-dimethylformamide (DMF) vehicle as a stock solution of 20 mg/ml under nitrogen at -20°C. All dilutions of MCTP were with DMF solvent immediately prior to use. A 2.5 μl volume of MCTP solutions or DMF (control) per ml of medium was used in all studies to achieve the nominal concentrations of MCTP (0.005 to 50 μg/ml) used in the study.

Cell and Monolayer Morphology.

BECs and BSMCs were plated into 12 or 24 well tissue culture clusters and allowed to proliferate to confluency. Five microliters of MCTP solution were added to test tubes containing 1.0 ml of media, the tubes were rapidly shaken, and the mixture was poured immediately into wells already containing 1.0 ml of medium. Monolayer morphology was examined at daily intervals using phase contrast microscopy. At selected intervals, monolayers in replicate plates were fixed with 2.0% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4) or 10% neutral-buffered formalin. Cells were stained with hematoxylin and eosin or with crystal violet (0.5%) stains.

Morphometric assessment of cell size (two-dimensional surface area) was determined with the aid of a Joyce Loebl image analysis system attached to an inverted Nikon TMS microscope. The two-dimensional cell surface area of approximately 200 cells per dose were measured on 1, 5 and 15 days after exposure to MCTP.

Transmission Electron Microscopy.

Confluent monolayers of BECs were grown on Thermanox tissue culture coverslips (Lux, Miles Laboratories) and exposed to 0, 5 or 50 μg/ml MCTP. Five to 7 days after exposure, monolayers were washed twice with HBSS and fixed for at least 4 hr with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Monolayers were subsequently washed twice with 0.1 M phosphate buffer. Fixed cells were scraped from coverslips and embedded in Epon Araldite resin. Ultrathin gold sections were cut from blocks with an LKB ultramicrotome, stained with uranyl acetate and lead citrate and viewed with a Phillips 301 transmission electron microscope.

Cell Proliferation Assays.

Five hundred BECs or BSMCs were plated onto 100 mm^2 tissue culture plates containing 10 ml of medium. Triplicate or quadruplicate plates were used for each treatment group. Cells were allowed to attach for 4 to 6 hours prior to exposure to the test compound and were given fresh medium 7 days post-treatment. At 14 days, cells were fixed with 10% neutral-buffered formalin (pH 7.4) and stained with crystal violet (0.5%). Colonies of cells (>50 cells per colony) were enumerated for each plate, and results were expressed as percentage of control.

Cellular Release of Lactate Dehydrogenase.

Cytolysis of BECs and BSMCs was assessed as the appearance of lactate dehydrogenase (LDH) activity in the medium above monolayers of cells. Replicate plates were used for analysis of LDH activity daily for 5 days after MCTP treatment. Medium from triplicate wells for each treatment and post-treatment interval was analyzed for LDH activity. After removal of medium, monolayers in these wells were rinsed with HBSS, and the cells were lysed with 15 μl of 10% Triton X 100 (Sigma) in 2 ml of fresh medium to determine cell-associated LDH activity. Percent release of LDH for each well was defined by the following formula:

$$\% \text{ release} = \frac{\text{LDH in medium above monolayers}}{\text{LDH from lysed cells + LDH in medium above monolayer}} \times 100.$$

The mean of triplicate wells for each study was considered a replication for statistical analysis (n=5 replications for BECs; n=4 replications for BSMCs).

Determination of 6-keto-prostaglandin $F_{1\alpha}$.

BEC monolayers in 24 well tissue culture clusters were exposed to 50, 25, 5, or 0.5 μg/ml MCTP or to vehicle in a total volume of 2.0 ml of medium. At daily intervals, medium from above monolayers was collected in polystyrene tubes containing 15 μg of indomethacin. Tubes were centrifuged at -20°C, and supernatant fluids were collected and stored at -20°C until analyzed.

Unextracted samples of medium were analyzed for 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable breakdown product of prostacyclin (PGI_2), by radioimmunoassay (RIA). Standard curves were constructed using DMEM as diluent. Specific antibodies and antigen were purchased from Seragen (Boston, MA). The crossreactivity at 50% maximal binding of antigen (B/Bo) for 6-keto-$PGF_{1\alpha}$ antibody was 7.8% for $PGF_{1\alpha}$, 6.8% for 6-keto-PGE_1, 2.2% for $PGF_{2\alpha}$, 0.7 for PGE_1, 0.6% for PGE_2 and <0.1% for PGD_2, PGA_2, PGA_1, PGB_1, PGB_2, TxB_2, 15-keto-PGF_2, 15-keto-PG and $F_{2\alpha}$, $DHKE_2$, or $DHKF_{2\alpha}$ (Seragen).

Quadruplicate wells were used at each dose on each day, and 6-keto-$PGF_{1\alpha}$ accumulation in medium above exposed monolayers was determined for 4 or 5 days after a single exposure to MCTP.

Enumeration of Attached Cells.

After removal of medium for 6-keto-$PGF_{1\alpha}$ determination, monolayers were washed twice with 1.0 ml of HBSS to remove non-adherent cells. Adherent cells were detached by treatment with 0.025% trypsin - 0.27 mM EDTA solution (Gibco) in Ca^{++}-free HBSS and were enumerated from quadruplicate wells using a hemacytometer.

Analysis of Data.

Results were expressed as mean ± standard error of the mean (SEM). Percentage data were transformed by $\arcsin^{-1}$ transformation. Data were analyzed by a completely random ANOVA. Non-homogeneous data were log-transformed to attain homogeneity prior to analysis. Tukey's omega-test was used for individual comparisons except morphometric data for which Bonferroni's correction for multiple comparisons was used. The criterion for significance was set at $p<0.05$.

RESULTS:

Cell and Cell Monolayer Morphology.

Morphologic evaluation indicated no evidence of distinct injury to monolayers of BECs until 20 to 48 hours after a single exposure to a dose of MCTP of 5 or 50 μg/ml. The injury was first evident as a subtle increase in cell detachment which became progressively more pronounced over the subsequent 2 to 3 days. The cells remaining attached to the plate enlarged so that the plate surface remained covered as adjacent cells were lost. Cellular enlargement was first evident two to three days post-treatment and became progressively more pronounced as the post-treatment interval increased. By 3 to 4 days, small gaps in the monolayers were apparent between the enlarged endothelial cells of the 50 μg/ml dose group but generally not at the lower doses.

Cytoplasmic vacuolation was evident in occasional endothelial cells after 5 days post-treatment. Photomicrographs of endothelial monolayers 14 days after a single exposure to 0, 0.5, 5 and 50 μg MCTP/ml of medium

are shown in figure 4-1. Nuclei of cells in the 5 and 50 μg/ml wells enlarged as the post-treatment interval increased, and nucleoli were prominent. As cells enlarged, filamentous strands or stress fibers radiating from the perivascular region often became evident in the cytoplasm. Morphology of cells and cell monolayers exposed to the 0.5 μg/ml dose did not differ appreciably from control.

The morphology of monolayers of BSMCs was not markedly altered after MCTP administration. There was no distinct evidence of enhanced cell detachment from monolayers at any dose. In monolayers exposed to 50 μg/ml, there appeared to be a slight decrease in cellularity of BSMC monolayers compared to control and a mild enlargement of nuclei in some scattered cells after the first 5 to 7 days post-exposure, but these changes were subtle (Figure 4-2). By day 14, monolayers of BSMCs remained intact, and cell degeneration (i.e., cytoplasmic vacuolation, nuclear pyknosis) was unremarkable.

Morphometric assessment of BEC size revealed a dose-dependent increase in cell surface area at day 5 post-exposure but not at day 1 (Figure 4-3). The cell surface area increased further at the 5.0 and 50 μg/ml doses as the post-treatment interval increased. By day 15, the mean surface area of BECs exposed to 5 and 50 μg/ml MCTP were 5.9 and 7.9 times that of control, respectively.

Electron Microscopy.

Endothelial cells 5 to 7 days after exposure to 5 and 50 μg/ml had larger cross sectional profiles than control cells (Figure 4-4). The cell enlargement was not due solely to a thinning and spreading of cytoplasmic processes but consisted of an increase in nuclear and cytoplasmic area.

MCTP-treated cells had large, oval, elongate nuclei with little or no heterochromatin and abnormally prominent, often multiple, nucleoli. Cell cytoplasm contained moderate amounts of rough endoplasmic reticulum, numerous polysomes, prominent golgi and numerous microtubules and pinocytotic vesicles. Calveoli were common at the cell surface. Occasional MCTP-treated cells had increased perinuclear intermediate filaments and cytoplasmic microtubules. Intercellular tight junctions were evident between cells, and lamellar like processes of plasma membrane overlapped at the cell boundaries. Occasional, partially detached MCTP-treated cells had small vesicular dilations of endoplasmic reticulum. The proportion and density of the cytoplasmic organelles appeared similar to control, although the increased amounts of cytoplasm in MCTP-treated cells indicate that these cells have increased quantities of organelles. Some MCTP-treated and control cells had variably sized, perinuclear autophagic vacuoles.

Lactate Dehydrogenase Release.

LDH activity in the medium in which BEC monolayers were incubated was not significantly elevated at any dose at day 1, but thereafter it increased in a dose-dependent manner (Figure 4-5). At the two higher MCTP doses, a tendency toward increased LDH activity in the medium occurred at day 2, and this increase became statistically significant and progressively greater with time. The magnitude of the LDH release from the BECs was dose-dependent. The lowest MCTP concentration tested (0.5 μg/ml) did not result in increased LDH release at any time during the 6-day experiment. In contrast to the BEC, LDH activity in medium from BSMC did not increase at any time or dose as a result of MCTP exposure.

Prostacyclin Production.

At day 1 post-treatment, the concentration of 6-keto-$PGF_{1\alpha}$ in media from BEC monolayers exposed to MCTP was not different from controls at any dose (Figure 4-6). Thereafter, it increased in wells exposed to MCTP at concentrations of 5 μg/ml and greater. The magnitude of the increase was largely dose-dependent, although monolayers exposed to 50 μg/ml MCTP consistently produced slightly less 6-keto-$PGF_{1\alpha}$ than did monolayers exposed to 25 μg/ml. The four replications in this study yielded similar findings, although the magnitude of the increase in 6-keto-$PGF_{1\alpha}$ varied considerably among studies.

Monolayer Cellularity.

Enumeration of BECs in monolayers from which PGI_2 release was determined showed that monolayer cellularity was not altered by MCTP at day 1 post-treatment (Figure 4-7). Thereafter, the number of cells remaining in the BEC monolayers exposed to an MCTP concentration of 5 μg/ml or greater decreased with time. The cellularity of monolayers exposed to 25 μg/ml MCTP was not significantly different from that of monolayers exposed to 50 μg/ml. The cellularity of BEC monolayers receiving 0.5 or 0 μg/ml increased slightly after day one, with the number of cells in the 0 μg/ml group consistently being greater than 0.5 μg/ml group.

Colony Forming Ability.

Despite the differences in morphologic and cytotoxic responses between BECs and BSMCs, the effect of MCTP on colony-forming efficiency was similar (Figure 4-8). Both cell types exposed to 0.5 μg/ml MCTP had significantly reduced ability to proliferate. No colonies developed at

concentrations of 5 and 50 μg/ml, although scattered, extremely large individual cells were attached to the plate surface. These cells were viable as judged by Trypan blue exclusion. Many of them had vacuolated cytoplasm and large nuclei with prominent nucleoli which became increasingly evident as the post-treatment interval increased.

DISCUSSION:

A single I.V. administration of a low dose of MCTP produces pulmonary injury, pulmonary vascular remodeling and PH in rats (Bruner *et al.*, 1983; Chesney *et al.*, 1973). Pronounced lung injury, however, is slow to develop and is not prominent until several days after MCTP administration (Bruner *et al.*, 1983; Butler *et al.*, 1970). This injury is initially apparent as a slight vascular leak and mild pulmonary interstitial edema. Thereafter, lung edema becomes more widespread and severe and is accompanied by progressive interstitial inflammation and arterial vascular remodeling.

The delay in development of major lung injury in MCTP-induced disease is enigmatic since MCTP is an unstable, reactive electrophile which binds covalently to tissue macromolecules or is rapidly inactivated in aqueous environments (Mattocks, 1968; White and Mattocks, 1972; Bruner *et al.*, 1986). The delayed and progressive nature of lung injury has led some investigators to suggest that MCTP or other pyrrolic metabolites of MCT may not cause cytotoxic injury directly, but rather that binding of metabolite(s) may trigger indirect mechanisms that lead ultimately to overt lung injury (Langleben and Reid, 1985; Kay and Heath, 1966; Bruner

et al., 1983; Meyrick *et al.*, 1980). Our findings *in vitro* indicate that endothelial cells and cell monolayers are damaged directly by a single exposure to MCTP and, as *in vivo*, the damage is delayed in onset and progressive.

The injury caused by MCTP was dependent upon the dose that the endothelial cells received. Higher doses caused enhanced BEC detachment and lysis and resulted in progressive deterioration of the endothelial cell monolayers over a period of several days. The lowest concentration (0.5 μg/ml) suppressed BEC proliferation but was not associated with overt cell injury or monolayer disruption. Even in monolayers exposed to higher concentrations of pyrrole, many cells did not die immediately after exposure but survived for two weeks or longer, maintaining a substantial degree of monolayer integrity. Cell-spreading and hypertrophy of the surviving endothelial cells tended to maintain monolayer confluency, thereby compensating to a large degree for the loss of cells through detachment from the monolayer. BEC monolayers in wells exposed to 5 μg/ml MCTP appeared intact throughout the two-week period of these studies, despite enhanced cell loss. If a similar response occurs *in vivo*, then the slow cell death with spreading and hypertrophy of surviving endothelial cells could allow for substantial, albeit temporary, maintenance of vascular intimal integrity, thereby retarding development of interstitial edema. This may, in part, explain why major lung leak and overt lung injury appears to be delayed after MCT or MCTP is given *in vivo* (Bruner *et al.*, 1983; Hilliker *et al.*, 1982).

The hypertrophic effects of MCTP seen in endothelial cells in culture are indeed seen *in vivo*. Hypertrophied endothelial cells have

been described in several morphologic studies of pyrrolizidine alkaloid- or MCTP-induced lung disease (Butler, 1970; Chesney *et al.*, 1974; Turner and Lalich, 1965; Meyrick and Reid, 1982). Some investigators have reported occlusion of the pulmonary microvasculature by extremely enlarged endothelial cells, particularly in animals in late stages of the disease (Turner and Lalich, 1965; Butler, 1970; Chesney *et al.*, 1974). Others have described thickened profiles of endothelial cells (Valdivia *et al.*, 1967). The findings in this study suggest that the hypertrophic change is a direct effect of MCTP on these cells.

The surface area of endothelial cells gradually increased to approximately eight times the size of control cells by day 15 posttreatment. This gradual increase in cell size is reminiscent of the megalocytotic response of hepatocytes after treatment of animals with PAs. In the liver, the rate of PA-induced hepatocyte enlargement appears to be determined in part by a stimulus for liver growth or regeneration (Peterson, 1965; Downing and Peterson, 1968; Jago, 1969). Young, growing animals and animals subjected to partial hepatectomy or to the post-necrotic reparative phase that follows carbon tetrachloride injury develop the megalocytotoxic response early. In our study, the rate of enlargement of endothelial cells in culture seemed to depend in part on cell density. Cells exposed when monolayers were subconfluent enlarged at a faster rate than cells exposed in confluent monolayers. In addition, cells in confluent monolayers exposed to 5.0 μg/ml MCTP attained the size of endothelial cells exposed to higher MCTP concentrations if portions of the monolayer were mechanically removed to allow room for cell expansion (unpublished observations). For exposed endothelial cells, at least in

the culture dish, there is likely a maximal size that cells can achieve. When cells approach this limit, gaps between cells such as we observed would be expected to develop.

In the vascular system, replacement of damaged endothelial cells by cell replication is critical to maintain an intact permeability barrier. After exposure to certain endothelial cell toxicants, such as endotoxin, a wave of cell loss is followed by proliferation of surviving cells, which maintains an intact endothelium (Reidy and Schwartz, 1983; Evanson and Shepro, 1974). MCTP, however, causes a marked inhibition of cell proliferation. Were this to happen *in vivo*, it might limit the repair or replacement of damaged endothelium and thereby promote vascular leak. In this regard, compromised compensatory responses of endothelium may in part explain two issues that have puzzled investigators. First, very young animals appear to be more susceptible to PAs or their pyrroles than are mature animals (Schoental, 1968), and, secondly, diet restriction protects against the pneumotoxicity of MCT and MCTP (Hayashi *et al.*, 1979; Ganey *et al.*, 1985). These and other observations suggest that growing animals are more sensitive to PAs. Animal growth requires an integrated proliferation of endothelial cells and other parenchymal cells. A block of endothelial cell proliferation in the face of a stimulus for general lung growth would predispose the animal to vascular intimal defects and to vessel leak as endothelial cell proliferation fails to keep pace with that of other lung cells. Diet restriction and perhaps other pharmacologic treatments may restrict animal growth and therefore provide an indirect protective effect that reduces vascular leak.

Endothelial cell spreading may compensate somewhat for limited cell loss and may maintain integrity of the intima initially, but, when the limit of this compensatory response is reached, an endothelium limited in proliferative capacity may become leakier, resulting in passage of plasma into the interstitium and exposure of the subendothelial matrix. *In vivo*, exposure of the subendothelium would be expected to result in increased adherence of platelets. Indeed, platelets sequester in the lungs of animals treated with MCTP (White and Roth, 1988) but not until several days after treatment. Enhanced retention of platelets in the lung occurs about the time of the development of major lung injury, which is delayed in onset. These findings *in vivo* are consistent with our observation *in vitro* that gaps in the endothelium do not appear until several days after endothelial cell injury is initiated.

Cell types differ in sensitivity to MCTP. Concentrations that enhanced cell detachment and caused release of LDH from monolayers of BECs produced little change in monolayer morphology or LDH release from BSMCs. The resistance of BSMCs to the cytotoxic effects of MCTP may be due to inherent differences in MCTP-cell interaction and/or in cellular protective mechanisms. For example, a cell's ability to produce a pericellular matrix might influence toxicity of highly reactive molecules like MCTP. BSMCs produce a basement membrane-like extracellular matrix which surrounds cells. Endothelial cells, by contrast, have no protective matrix on the luminal surface. MCTP might bind to nucleophilic regions of the BSMC matrix, limiting the toxicant's access to the cell membrane. The stacking of cells that occurs in confluent BSMC monolayers could also protect lower layers of cells from exposure to MCTP. However, such an

explanation does not account for the equal sensitivity of BSMCs to the cytostatic effects of MCTP.

Endothelial cells produce a variety of factors that influence smooth muscle cell growth or vasomotor activity. PGI_2 is one such factor. The accumulation of 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, was elevated in media above monolayers of BECs after exposure to MCTP. The enhanced production of PGI_2 was dependent on the dose of MCTP. There does, however, appear to be an optimal dose for maximal release of this product, since 50 μg/ml MCTP consistently resulted in less accumulation of 6-keto-$PGF_{1\alpha}$ in the media than did 25 μg/ml. A similar, optimal PGI_2 response to increasing concentrations of endotoxin has also been reported (Meyrick, 1986). As with the other markers of endothelial injury, the release of PGI_2 was delayed and progressed with time. The enhanced release of PGI_2 paralleled the enhanced cell detachment from monolayers. The release of PGI_2 from endothelial cell monolayers exposed to other toxicants has also been associated with enhanced cell detachment and lysis (Nawroth *et al.*, 1984).

The damage to cultured endothelial cell monolayers after MCTP exposure appears to develop much more slowly than damage caused by many other endothelial cell toxicants. The earliest morphologic evidence of endothelial cell injury in this study was a mild increase in cell detachment detected 20 or more hours after exposure to the higher concentrations of MCTP. Endothelial cell detachment became more pronounced over the subsequent 2 to 3 days. In contrast, bacterial endotoxin, homocysteine and cyclosporin A cause significant endothelial cell detachment at 6 hours or earlier and a pronounced release of LDH

activity or ^{51}Cr into the incubation medium at 24 hours or earlier (Harlan *et al.*, 1983; Meyrick, 1986; Zoja *et al.*, 1986; Wall *et al.*, 1980). These toxicants caused marked disruption of endothelial cell monolayers within 24 hours of exposure as endothelial cells underwent contraction, pyknosis and eventual detachment; however, cell hypertrophy following exposure to these toxicants has not been described. The difference in time course of endothelial cell damage does not appear to be merely a function of toxicant concentration. Higher concentrations of MCTP (100 μg/ml) did not produce injury to monolayers at substantially earlier times (unpublished observations). *In vivo*, pulmonary edema induced by endotoxin or α-naphthylthiourea occurs much more rapidly than that induced by MCTP (Meyrick and Brigham, 1983; Fantone *et al.*, 1984). Moreover, lung edema resolves relatively rapidly after a single injection of either of these two toxicants at sublethal doses. Thus, the slowly developing nature of endothelial cell injury resulting from a single dose of MCTP is not common to other pneumotoxicants.

In vitro, endothelial monolayer damage induced by hyperoxia (Phillips *et al.*, 1988) or gamma irradiation (Rosen *et al.*, 1989) has several similarities to the damage induced by MCTP. For instance, hyperoxia can induce injury to endothelial monolayers that is delayed in development and progressive. In addition, hyperoxia or irradiation can induce endothelial cell hypertrophy and inhibit the ability of cells to proliferate. Interestingly, *in vivo* both hyperoxia and lung irradiation can cause chronic interstitial lung damage, vascular remodeling and chronic PH which is similar to that induced by MCTP (Jones *et al.*, 1981; Coflesky *et al.*, 1988; Perkett *et al.*, 1986; Meyrick *et al.*, 1987). The

fact that these insults produce similar responses in cultured cells and also result in persistent PH *in vivo* suggests that endothelial cellular changes may be important in the development of PH.

Ultrastructural studies of rat lungs have indicated that endothelial cell destruction occurs but is not widespread after administration of MCTP (Butler, 1970; Plestina and Stoner, 1972; Hurley and Jago, 1975). Similarly, electron microscopic examination of cultured endothelial cells 5 days or more after exposure to MCTP did not reveal marked degenerative changes in the vast majority of enlarged cells. Indeed, other than thickened endothelial cell profiles and changes in the nucleus and nucleolus, consistent changes were difficult to identify. After MCTP treatment *in vivo*, leakage of intravenously administered thorotrast from the pulmonary vasculature actually occurred in vessels with relatively normal-appearing endothelial cell profiles (Plestina and Stoner, 1972; Hurley and Jago, 1975). This is in contrast to the more pronounced endothelial cell injury associated with other pneumotoxicants like α-naphthylthiourea or endotoxin (Fantone *et al.*, 1984; Cunningham and Hurley, 1972; Meyrick, 1986). Whether subtle injury to endothelial cells can be detected with classic morphologic techniques has been questioned (Reidy, 1985; Reidy and Schwartz, 1984). Less severe, morphologic changes or subtle alterations in functional capacity of the endothelium could be overlooked, but such changes may nevertheless have important consequences regarding lung function.

Morphologic evaluation suggested the occurrence of endothelial cell hyperplasia (Turner and Lalich, 1965), and increased tritiated thymidine incorporation has been reported in the pulmonary vasculature after feeding

rats *Crotalaria spectabilis* (Meyrick and Reid, 1982). Our findings of impaired proliferative capacity indicate that it is unlikely that MCTP produces a hyperplastic response directly. Rather, if such a response occurs *in vivo*, it may represent a reparative response of vascular cells that were not exposed to the toxicant.

In summary, MCTP causes direct, dose-dependent injury to BECs. The injury is slow to develop but results in progressive deterioration of endothelial cells. Cell proliferation is also markedly inhibited by MCTP and may limit effective repair of defects in endothelial cell monolayers. Similar responses *in vivo*, might result in the delayed and progressive vascular leak and pulmonary edema that characterize MCTP and MCT pneumotoxicity.

Figure 4-1. Photomicrographs of bovine pulmonary arterial endothelial cells 14 days after a single exposure to MCTP. A, 0 μg/ml (DMF vehicle). B, 0.5 μg/ml. C, 5 μg/ml. D, 50 μg/ml. Cells exposed to 5 and 50 μg/ml MCTP were markedly enlarged compared to those exposed to 0 or 0.5 μg/ml. Photomicrographs were taken at comparable magnifications using a phase-contrast microscope.

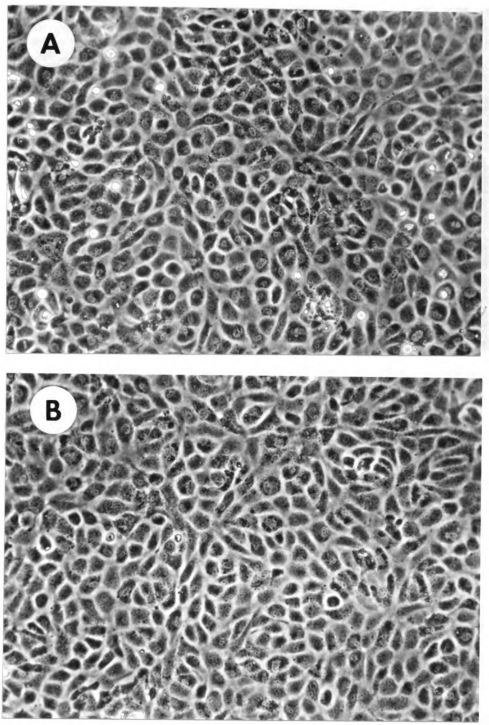


Figure 4-1.

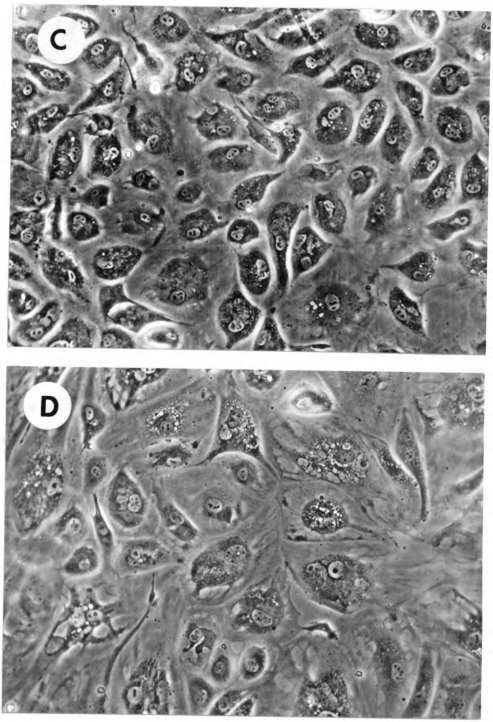


Figure 4-1. (cont.)

Figure 4-2. Photomicrographs of bovine smooth muscle cells 7 days after a single exposure to MCTP. Cells were exposed to A, 0 μg/ml (DMF vehicle); B, 0.5 μg/ml; C, 5 μg/ml; D, 50 μg/ml. Photomicrographs were taken at comparable magnifications using phase-contrast microscopy on hematoxylin-stained cell monolayers.

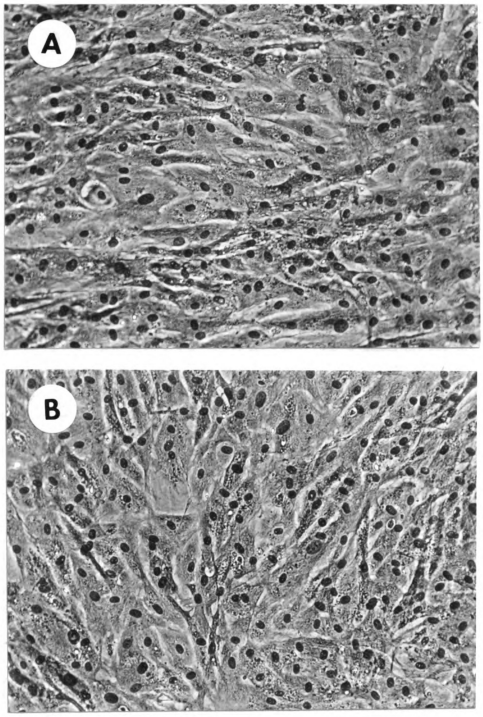


Figure 4-2.

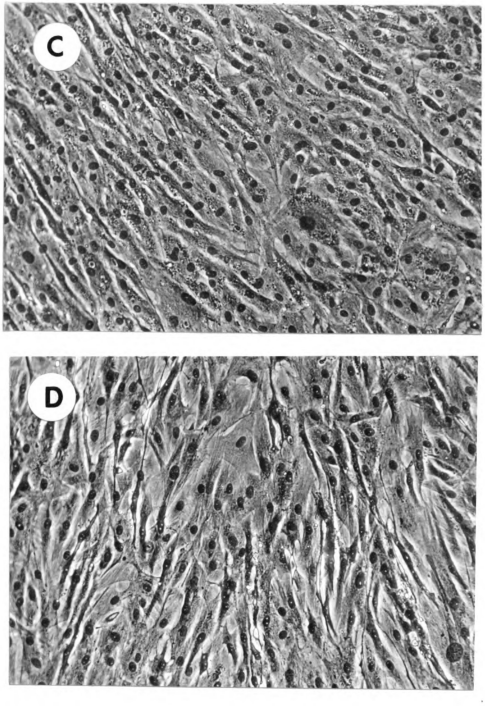


Figure 4-2. (cont.)

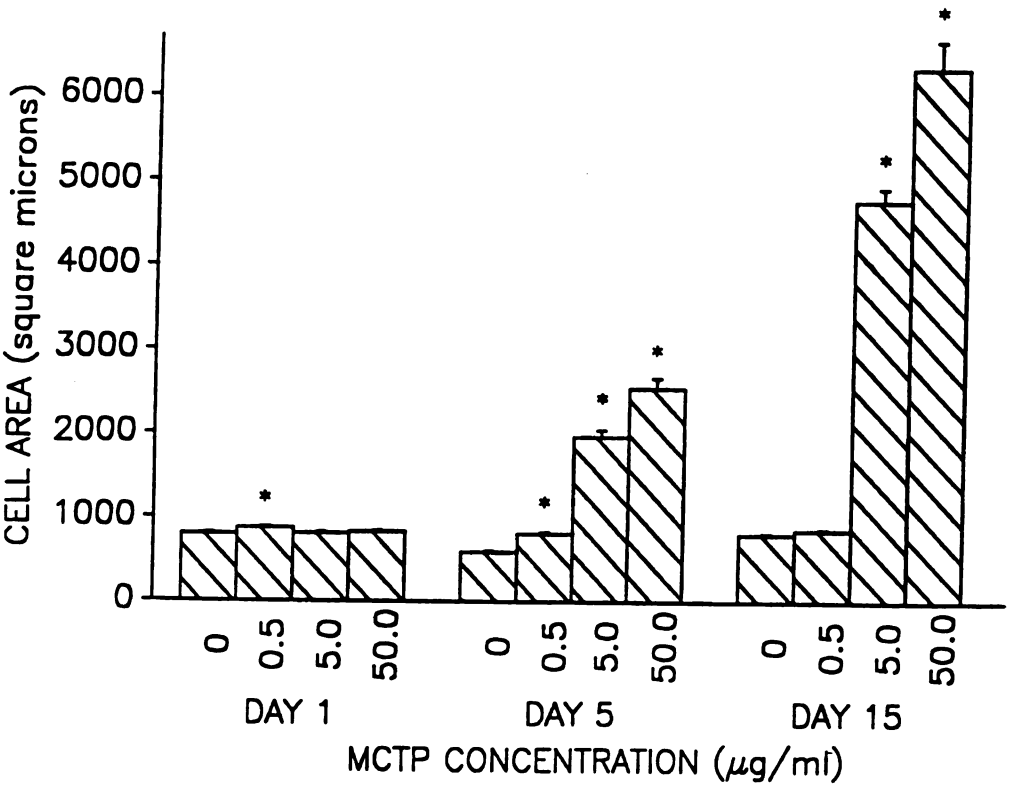


Figure 4-3. Effect of MCTP on two-dimensional surface area of bovine pulmonary arterial endothelial cells at 1, 5 and 15 days after a single treatment with MCTP. Results are expressed as mean ± SEM for 150 to 200 cells per treatment group. Asterisks indicate significant differences from cells exposed to 0 μg/ml MCTP, $p<0.05$.

Figure 4-4. Electron micrographs of bovine pulmonary arterial endothelial cells 5 days after exposure to 0 (A) or 50 μg/ml (B) MCTP. The endothelial cell exposed to MCTP has an enlarged cell profile and a nucleus with prominent, multiple nucleoli.

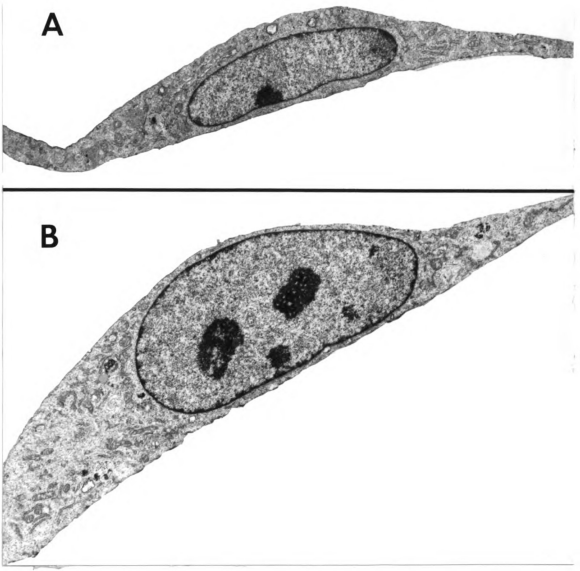


Figure 4-4.

Figure 4-5. Lactate dehydrogenase activity release (% maximal release) from monolayers of bovine pulmonary arterial endothelial cells (A) and bovine smooth muscle cells (B) after a single exposure to MCTP on day 0. Three replicates were used to determine the percentage release for each run. Results are expressed as a mean of run values $\pm$ SEM. (n=5 for BECs, n=3 for BSMCs) Asterisks indicate difference from 0 μg/ml MCTP dose groups, $p<0.05$.

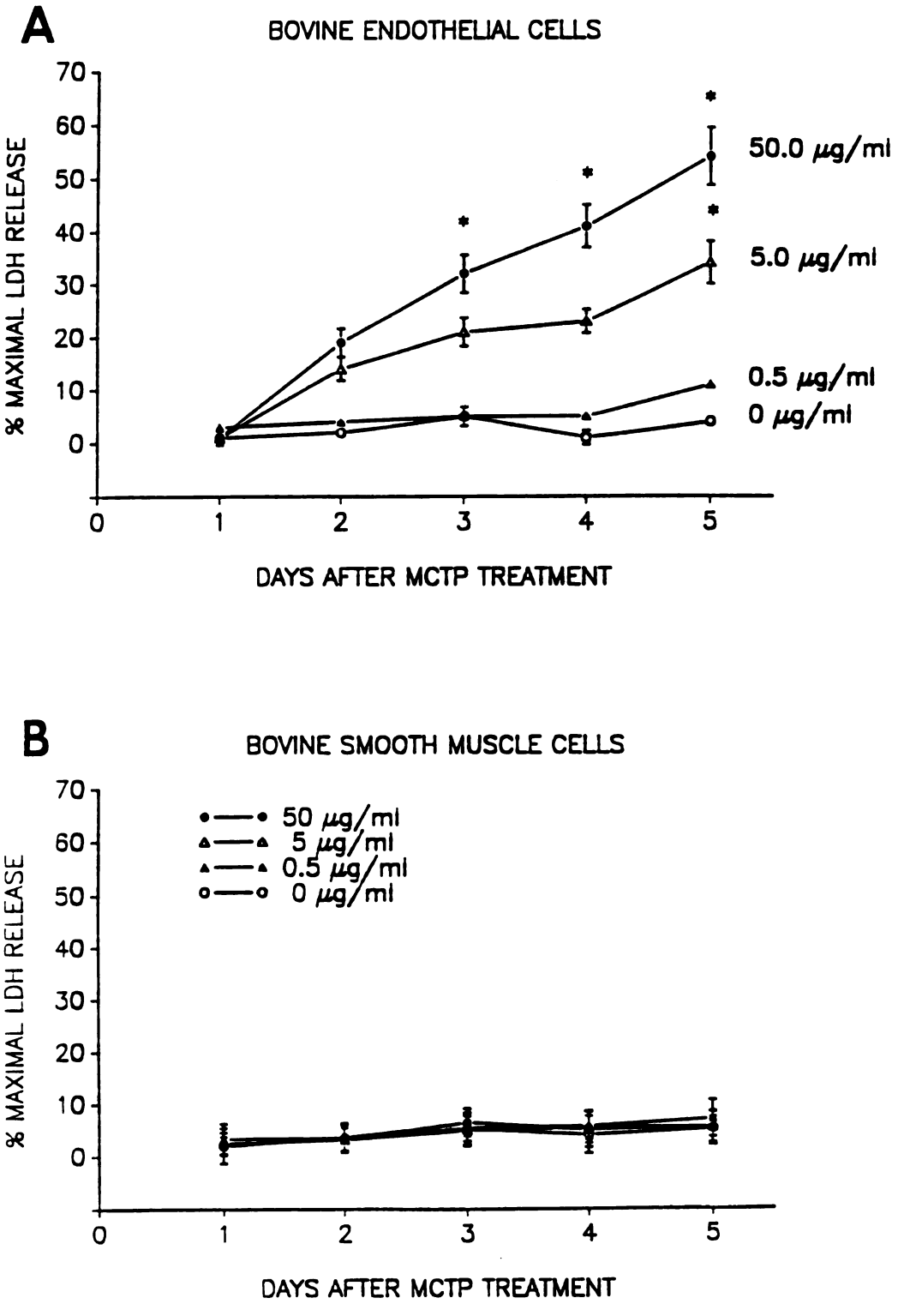

A
BOVINE ENDOTHELIAL CELLS
% MAXIMAL LDH RELEASE
70
60
50
40
30
20
10
0
0
1
2
3
4
5
DAYS AFTER MCTP TREATMENT
50.0 µg/ml
5.0 µg/ml
0.5 µg/ml
0 µg/ml
B
BOVINE SMOOTH MUSCLE CELLS
50 µg/ml
5 µg/ml
0.5 µg/ml
0 µg/ml
% MAXIMAL LDH RELEASE
DAYS AFTER MCTP TREATMENT

Figure 4-5.

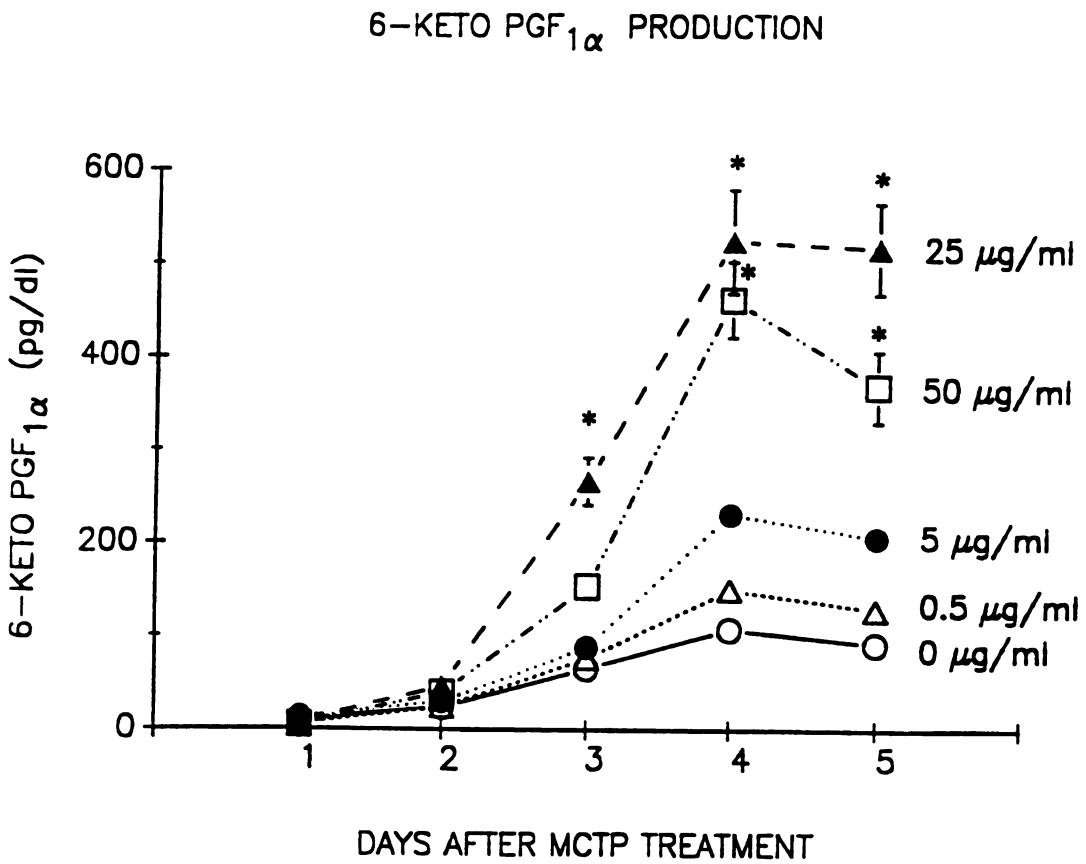


Figure 4-6. 6-Keto-$PGF_{1\alpha}$ (prostacyclin) concentration in medium above monolayers of bovine pulmonary arterial endothelial cells exposed to MCTP on day 0. Values represent mean ± SEM. Asterisks indicate significant differences from concentrations of 6-keto-$PGF_{1\alpha}$ in medium from above monolayers exposed to 0 μg/ml (DMF vehicle), $p<0.05$.

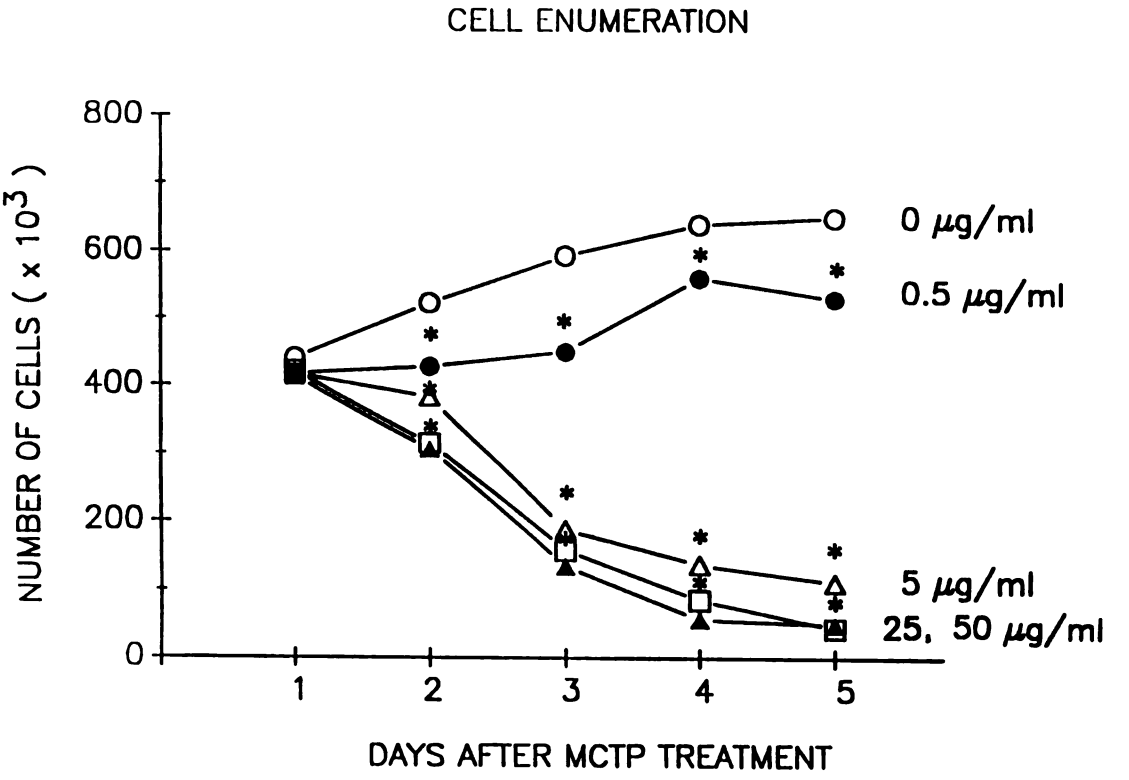
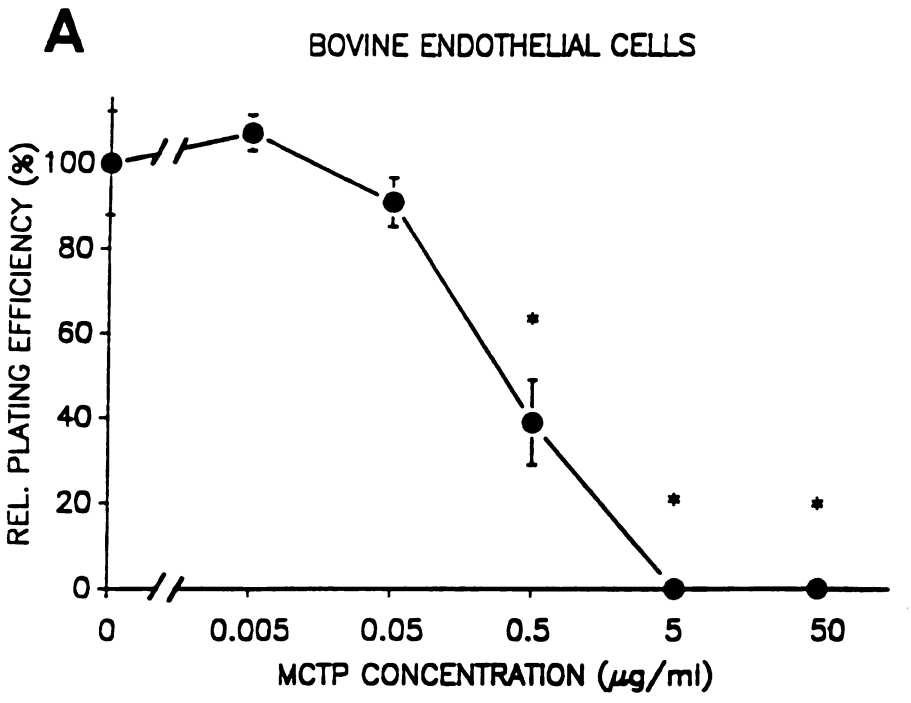


Figure 4-7. Effects of MCTP on numbers of bovine pulmonary arterial endothelial cells in cell monolayers after a single exposure to MCTP on day 0. Values represent group means (n=4). Standard error for individual comparisons was 10.24 by Tukey's omega test. Asterisks indicate significant differences from monolayers to 0 μg/ml. $p<.05$.

Figure 4-8. Colony forming efficiency of bovine pulmonary arterial endothelial cells (A) and smooth muscle cells (B) exposed to MCTP. Results are expressed as a percentage of the mean number of colonies observed in plates receiving 0 μg/ml (DMF vehicle). Value represent the mean $\pm$ SEM (n=4). Asterisks indicate significant differences from controls (0 μg/ml), $p<0.05$.


A
BOVINE ENDOTHELIAL CELLS
REL. PLATING EFFICIENCY (%)
100
80
60
40
20
0
0
0.005
0.05
0.5
5
50
MCTP CONCENTRATION (μg/ml)

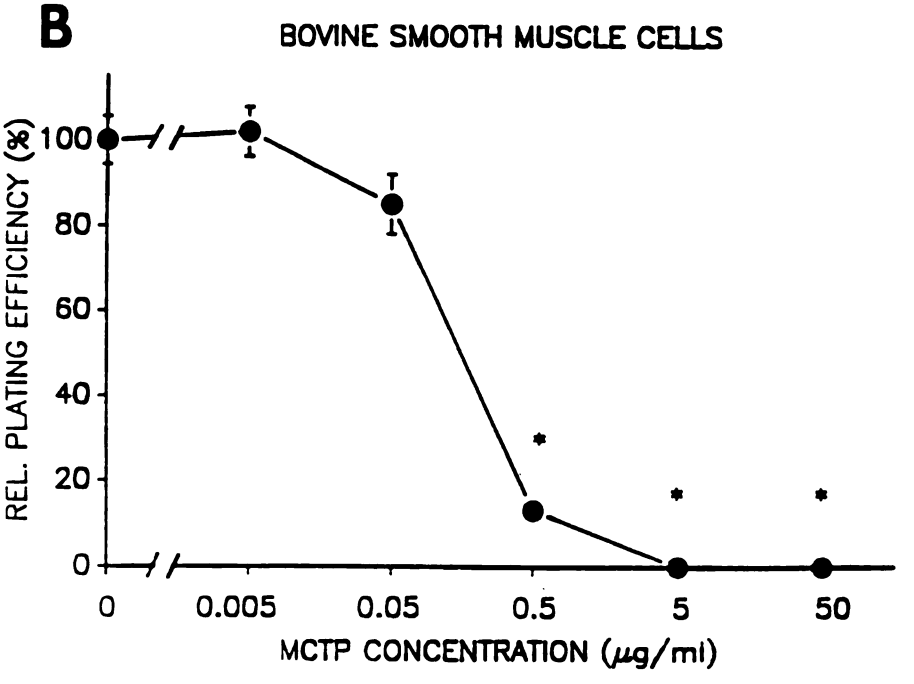

B
BOVINE SMOOTH MUSCLE CELLS
REL. PLATING EFFICIENCY (%)
100
80
60
40
20
0
0
0.005
0.05
0.5
5
50
MCTP CONCENTRATION (μg/ml)

Figure 4-8.

CHAPTER V

COMPARISON OF THE RESPONSE OF BOVINE AND PORCINE PULMONARY ARTERIAL ENDOTHELIAL CELLS TO MONOCROTALINE PYRROLE

SUMMARY:

In many species, monocrotaline (MCT) intoxication is associated with vascular injury in liver and lung. This injury is thought to be caused by bioactivation products of MCT metabolism such as monocrotaline pyrrole (MCTP). The responses of pulmonary arterial endothelial cells of bovine and porcine origin to a single administration MCTP were compared. MCTP caused delayed and progressive cytolytic injury to bovine endothelial cells (BEC) resulting in a delayed and progressive, dose-dependent release of lactate dehydrogenase activity (LDH) from monolayers and a gradual and pronounced decrease in monolayer cellularity. Surviving cells underwent progressive and marked cell hypertrophy. Porcine endothelial cells (PEC) were much less sensitive to the cytolytic effects of MCTP. A minimal increase in release of LDH activity from monolayers was not apparent until 5 days after exposure to 50 μg MCTP/ ml. This correlated with minimal cell detachment from monolayers observed morphologically. Hypertrophy of surviving PECs was not as pronounced as in BECs. Monolayer cellularity, assessed by PEC enumeration, decreased in a dose-dependent manner, possibly due to enhanced cell lysis during enzymic dissociation. MCTP caused enhanced release of prostacyclin (6-keto-$PGF_{1\alpha}$) from monolayers of BECs and PECs exposed to 10 μg MCTP/ml, and concentrations of 0.5 μg/ml or greater caused equivalent reduction in colony forming efficiency in both cell types. In summary, whereas BECs appear to be more susceptible to the cytolytic and hypertrophic effects of MCTP, BECs and PECs respond

similarly with regard to comparable prostacyclin release and had equal sensitivity to the cytostatic effects of this compound.

INTRODUCTION:

Pyrrolizidine alkaloids (PAs) are structurally related chemical compounds found in hundreds of plant species distributed throughout the world (Bull et al., 1968). Many are harmless, but others are toxic to man and animals. Consumption of toxic PAs causes hepatotoxic and pneumotoxic effects which are responsible for chronic disease in humans and substantial economic losses to the animal industry (McLean, 1970; Sippel 1964; Huxtable, 1979).

Animal species vary widely in sensitivity to the toxic effects of PAs (Cheeke and Pierson-Goeger, 1983; Gardiner et al., 1965; Lalich and Ehrhart, 1962). In addition, species differ in clinical signs associated with PA intoxication, the major target organ damaged by PAs and the histological pattern of changes in organs following exposure (Huxtable 1979; Schoental 1968; Kelly 1985). Variability in sensitivity to PAs has been attributed to differences in hepatic microsomal metabolism of PAs and the proportion of toxic versus inactive metabolites produced (Cheeke and Pierson-Goeger, 1983; Shull et al., 1976a; Chesney and Allen, 1973b). In addition, interspecies variation might arise from different sensitivities or responses of target cells to reactive metabolites of PAs.

Pyrrolic PA derivatives, produced by hepatic microsomal metabolism, are thought to be the proximate toxicants responsible for PA-induced hepatic and pulmonary injury (Mattocks, 1968a). These pyrroles bind

locally to hepatocellular constituents and produce toxic effects. A portion may also escape binding locally in hepatic parenchymal cells and pass into the blood stream where metabolites can interact with cells of the hepatic venous system and pulmonary vascular bed (Barnes et al., 1964; Mattocks, 1968a). Here these reactive electrophiles can bind to endothelial cells and cause injury by mechanisms that are largely unknown. The ultimate consequence of the interaction of toxic pyrroles with cells *in vivo* is vascular damage and structural remodeling that results in hepatic veno-occlusive disease in some animal species (cattle, horses, nonhuman primates, human)(Sanders et al., 1936; Hill and Martin, 1951; Cox et al., 1958; Rose et al., 1959; Allen and Carstens, 1968b; Allen et al., 1965,1969; Bras et al., 1954) or delayed and progressive pulmonary vascular remodeling and chronic pulmonary interstitial inflammation in others (rats, pigs)(Emmel et al., 1935; Harding et al., 1964; Allen and Carstens, 1970; Turner and Lalich, 1965; Kay and Heath, 1969; Peckham et al., 1974; Ghodsi and Will, 1981; Meyrick and Reid, 1980).

Monocrotaline pyrrole (MCTP) is a putative toxic metabolite of the PA, monocrotaline. Low doses of MCTP (<10 mg/kg) administered intravenously into rats cause delayed and progressive pulmonary vascular disease, pulmonary interstitial inflammation and pulmonary hypertension virtually identical to that induced by systemic administration of MCT (Butler et al., 1970, Barnes et al., 1964; Chesney et al., 1974a; Mattocks and Driver, 1983; Bruner et al., 1983a). A single exposure to this short-lived electrophile also causes delayed and progressive cytolytic injury to bovine pulmonary arterial endothelial cell monolayers in culture (Reindel and Roth, 1989; Roth and Reindel, 1988). Bovine endothelial

cells (BECs) which survive the cytolytic effects of MCTP undergo progressive and marked cell hypertrophy. Concentrations of MCTP less than those which caused cytolytic injury inhibit proliferation of BECs. The progressive cytolytic injury and block in cell proliferation prevented effective monolayer repair. Bovine pulmonary arterial smooth muscle cells (BSMCs) differed from BECs in their response to MCTP, in that they were resistant to the cytolytic effects of this compound but had comparable sensitivity to the cytostatic effects of MCTP. The reason for this difference in sensitivity to the cytolytic effects is not clear, but it suggests that quantitative and perhaps qualitative differences in responses occur among different cell types.

The purpose of this study was to compare responses of endothelial cells from two animal species to cytotoxic concentrations of MCTP to determine if there are species differences in responses or sensitivity of endothelial cells to this toxicant. Toward this end, porcine pulmonary artery endothelial cells (PECs) and BECs were exposed to MCTP, and responses of cells were assessed by morphologic and biochemical methods.

We found that PECs and BECs differ qualitatively and quantitatively in their responses to MCTP. Such differences in target cell sensitivity in vivo may affect animal sensitivity to the PAs and the pathologic changes observed during PA intoxication.

MATERIALS AND METHODS:

Preparation of Endothelial Cells

Lines of BECs and PECs were derived from segments of pulmonary artery by modifications of the method of Jaffe (1973). Freshly isolated

segments of pulmonary artery from young calves and pigs were washed with Hanks balanced salt solution (HBSS), and the adventitia and external elastic laminae carefully removed by dissection. The vessels were incised and placed into petri dishes containing sufficient collagenase (Type IA, 0.1%, Sigma Chemical Co.) to immerse only the luminal surface. After incubation for 3 to 5 minutes at 30°C, the luminal surface of the vessels were gently brushed with a single stroke of a rubber policeman. The brushings were resuspended in fresh HBSS. Individual, small clusters of cells were collected from this suspension with the aid of a 200 μl Pipetman^R pipetter and an inverted microscope (TMS, Nikon). These were placed in wells of 12 or 24 well tissue culture clusters (Costar) and allowed to attach for 3 to 4 hours. The wells were washed vigorously with HBSS, and fresh medium was added. Plates were incubated for 3 to 7 days during which time cell colonies developed in many wells. Colonies from individual wells which were free of spindle cells (smooth muscle cells or fibroblasts) were selectively removed with a 200 μl Pipetman^R pipetter and mechanically dispersed into larger tissue culture plates for expansion of the cell clones. Selected, expanded clones of endothelial cells which had the characteristic cobblestone, epithelial-type pattern and were free of contaminating spindle cells were passaged using either enzymic (0.025% trypsin- 0.27 mM EDTA) or mechanical dissociation.

Endothelial cells were maintained in Dulbecco's Modified Eagles Medium (DMEM, Gibco) or M199 Medium (Gibco) containing 2 mM glutamine, 1% penicillin (100 units/ml)-streptomycin (100 μg/ml)-fungizone (0.25 μg/ml) (Antibiotic-Antimycotic; Gibco). Cells were used between passages 2 and

12. They were passed by enzymic (0.025% trypsin - 0.27mM EDTA; Gibco) or mechanical dissociation and split at 1:3 to 1:6 ratio.

Monolayers from lines of BECs had a characteristic cobblestone pattern, and cells stained positive for factor VIII-related antigen and had characteristic ultrastructural appearance of endothelial cells. PEC monolayers also had a characteristic cobblestone pattern and had ultrastructural features of endothelial cells. PECs were not examined for factor VIII-related antigen activity since cultured endothelial cells from this species do not stain for the antigen.

Preparation of MCTP

MCTP was prepared from MCT (Transworld Chemical, Washington D.C.) via an N-oxide intermediate by the method of Mattocks (1969). MCTP isolated from this synthesis procedure has Ehrlich activity (Mattocks and White, 1971) and a structure compatible with MCTP as determined by mass spectrometry and nuclear magnetic resonance (Bruner et al., 1986). MCTP was dissolved in N,N-dimethylformamide (DMF; Sigma Chemical Co.) at a concentration of 20 mg/ml or 40 mg/ml and all dilutions of MCTP were made with DMF. A 2.5 μl volume of MCTP solutions or DMF vehicle (0 μg/ml MCTP) per ml of medium was used in all studies to achieve the nominal concentrations of MCTP (0.005-100 μg/ml) used in the study.

Morphology of Endothelial Cells and Cell Monolayers

The morphology of endothelial monolayers was monitored on a daily basis for up to two weeks after a single exposure to MCTP. At 5 and 14 days posttreatment, monolayers of cells exposed to 50, 5, 0.5 or 0 μg MCTP/ml of medium were fixed with 2% glutaraldehyde. Two dimensional

surface area of approximately 150 cells per group was assessed morphometrically with the aid of a Joyce Loebl Magiscan Image Analysis System (Nikon) attached to an TMS inverted microscope (Nikon).

Cellular Release of Lactate Dehydrogenase Activity

Bovine and porcine endothelial cells were seeded into 12 well tissue culture clusters and allowed to proliferate until monolayers reached confluency. At this time (day 0), monolayers were exposed to 50 or 0 μg/ml MCTP. On each of the subsequent 5 days, one 12 well plate for each species was used for determination of LDH activity. Medium from above monolayers was removed for LDH analysis and monolayers were washed twice with HBSS. Two ml of fresh medium were replaced in all wells and cells were lysed with 15 μl of a 10% solution of Triton X 100 (Sigma). Percent LDH release was determined by the following formula:

$$\% \text{ release} = \frac{\text{LDH in medium above monolayers}}{\text{LDH from lysed cells + LDH in medium above monolayer}} \times 100.$$

In separate studies, the dose-response relationship for the accumulation of LDH activity in the incubation medium above cell monolayers was examined. BECs and PECs were exposed to 100, 10, 1, or 0 μg MCTP/ml. Percent maximal release of LDH activity was determined 3 days after exposure.

Colony Forming Efficiency

BECs and PECs were plated at low density (500 and 1000 cells per plate, respectively) in 100 mm^2 tissue culture plates. Cells were incubated for 3 to 4 hours to allow for cell attachment, and then were

exposed to 50, 5, 0.5, 0.05, 0.005 or 0 μg MCTP/ml. Medium was changed on day 7 postexposure, and on day 14 cell colonies on plates were fixed with 10% neutral buffered formalin and stained with crystal violet (0.5%). Colonies of more than 50 cells were counted. Results are expressed as a percent of the number of colonies in plates exposed to 0.0 μg/ml of MCTP.

Effects of MCTP on Monolayer Cellularity

After exposure of confluent cell monolayers in 24-well cell culture clusters to MCTP on day 0, cells remaining in the monolayers of BECs and PECs were counted on sequential days. Medium from above monolayers of cells was removed, and cell monolayers were rinsed twice with 0.5 ml of HBSS without Ca^{++} or Mg^{++} to remove nonadherent cells. Adherent cells were enzymically removed from the plate surface with 0.025% trypsin - 0.27 mM EDTA solution (Gibco) and enumerated using a hemacytometer or Coulter Counter.

6-keto-prostaglandin $F_{1\alpha}$ Analysis

BEC and PEC monolayers in 24 well tissue culture clusters were exposed to 100, 10, 0.1 or 0 μg/ml MCTP in a total volume of 2.0 ml of medium. On day 3 after exposure, medium from above monolayers was collected in polystyrene tubes containing 15 μg of indomethacin. Tubes were centrifuged at -20°C, and supernatant fluids were collected and stored at -70°C until they were analyzed.

Unextracted samples of medium were analyzed for 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), the stable breakdown product of prostacyclin (PGI_2), by radioimmunoassay (RIA). Standard curves were constructed using DMEM as a diluent. Specific antibodies and antigen were purchased from Seragen

(Boston, MA). The crossreactivity at 50% maximal binding of antigen (B/Bo) for 6-keto-$PGF_{1\alpha}$ antibody was 7.8% for $PGF_{1\alpha}$, 6.8% for 6-keto-PGE_1, 2.2% for $PGF_{2\alpha}$, 0.7% for PGE_1, 0.6% for PGE_2 and <0.1% for PGD_2, PGA_2, PGB_2, TxB_2, 15-keto-PGF_2, 15-keto-$PGF_{2\alpha}$, $DHKE_2$, or $DHKF_{2\alpha}$ (Seragen).

Statistical Analysis

Data are presented as means ± standard error of the means (SEM). Percentage data were transformed by the arc $\sin^{-1}$ transformation prior to statistical analysis. The data from the time course assessment of endothelial injury was analyzed by a completely blocked analysis of variance (ANOVA), and individual comparisons were made with the Tukey's omega-test. Morphometric data were analyzed using Bonferroni's correction for multiple comparisons. Data for the remaining studies were analyzed using a completely random ANOVA, and individual comparisons were made using the Tukey's omega-test. The criterion for significance was $p<0.05$.

RESULTS:

Morphology of Cells and Cell Monolayers

Monolayers of BECs exposed to concentrations of MCTP greater than 1 μg/ml showed dose-dependent injury first apparent as a subtle increase in the appearance of retractile floating cells above intact monolayers at approximately 24 hours or more following treatment. Thereafter, cell detachment became more pronounced with time. In addition, surviving BECs in monolayers exposed to concentrations of MCTP greater than 0.5 μg/ml underwent a dose-dependent cell hypertrophy and features of cellular atypia appeared as the posttreatment interval increased. Cytoplasmic

vacuoles developed in scattered cells of the monolayer with time and cytoplasmic stress fibers were evident in some enlarged cells. The nuclei of cells became enlarged, and prominent, multiple nucleoli were evident in most cells. Cell hypertrophy compensated to a large degree for loss of BECs from the monolayer; thus, monolayer integrity was maintained throughout the study in wells exposed to concentrations of MCTP less than 50 μg/ml. Only small gaps were apparent between cells in monolayers exposed to 50 and 100 μg MCTP/ml of medium by days 5 and 14. Photomicrographs of BEC monolayers at 14 days after exposure to doses of MCTP are shown in figure 5-1.

PECs exposed to concentrations of MCTP greater than 5 μg/ml had a subtle increase in cell detachment first evident 3 or more days posttreatment. This cell detachment was much less pronounced than observed with BECs, and monolayer integrity was maintained throughout the duration of the study at all concentrations of MCTP tested. Photomicrographs of PEC monolayers 14 days after a single exposure to doses of MCTP are shown in figure 5-2. PECs exposed to 50 and 100 μg MCTP/ml became more spindle shaped with time. Their cytoplasm became granular in appearance and the nuclei of the cells enlarged. The cell enlargement of PECs was much less pronounced than that observed in BECs. The cell density of the PEC monolayers exposed to concentrations of MCTP greater than 5 μg/ml appeared to decrease with time relative to monolayers exposed to lesser concentrations of MCTP. This relative decrease in cellularity appeared to be due to continued proliferation of PECs in monolayers of cells exposed to concentrations of MCTP less than 0.5 μg/ml

rather than to a distinct increase in cell detachment from the monolayers exposed to higher concentrations of MCTP.

Figure 5-3 depicts data from morphometric assessment of two dimensional surface area of MCTP exposed cells at 5 and 14 days postexposure. BECs exposed to 5 and 50 μg MCTP/ml of medium had an increase in surface area (2.5 and 6.0 x, respectively) relative to vehicle treated cells by day 5 posttreatment. The surface area of these cells increased further by day 14 to 5.0 and 11.0 x control values, respectively (Figure 5-3). For PECs, surface area of cells had increased only slightly (1.08 x) by day 5 in monolayers exposed to 50 μg of MCTP/ml of medium. By day 14, cells exposed to 50 μg MCTP/ml of medium had increased further in size (2.15 x) and cells exposed to 5 μg MCTP/ml of medium were larger than controls by 1.90 x control values. Thus, BECs exposed to 5 or 50 μg MCTP/ml underwent a gradual marked increase in cell size whereas enlargement of PECs occurred more slowly and was less pronounced.

Cellular Release of Lactate Dehydrogenase Activity

Monolayers of BECs exposed to 50 μg MCTP/ml of medium had an increased release of LDH activity that was first apparent in wells exposed to 50 μg MCTP/ml at 48 hours posttreatment, and this increase became more pronounced with time (Figure 5-4). In contrast, an increase in accumulation of LDH activity in medium above monolayers of PECs was not evident until 5 days after treatment (Figure 5-4). Even at this time the magnitude of the increase was quite small compared to that released by BECs.

A concentration/response study in BECs revealed that, at 3 days posttreatment, greater than 1 μg MCTP/ml caused enhanced accumulation of LDH activity in medium above monolayers (Figure 5-5). In PEC monolayers, MCTP did not cause a significant enhancement in the release LDH activity at this time; however, a trend toward enhanced release was apparent.

Monolayer Cell Density

Effects of MCTP on cell density of monolayers treated at confluency are shown in figure 5-6. The numbers of cells in monolayers of BECs decreased in a dose-dependent manner after exposure to MCTP at concentrations greater than 0.5 μg/ml. This decrease was apparent at 2 days posttreatment and became more pronounced with time. The cellularity of vehicle-treated BEC monolayers increased slightly with time.

The numbers of cells in PEC monolayers exposed to concentrations of 50 μg MCTP/ml decreased with time, although cellularity did not diminish as rapidly as in BEC monolayers, and it was less pronounced in magnitude. The cellularity of monolayers exposed to 5.0 μg MCTP/ml did not decrease significantly with time, and that of monolayers exposed to lesser concentrations increased slightly with time.

Colony Forming Efficiency

MCTP inhibited proliferation of cells in a dose-dependent manner for both cell types (fig. 7). Concentrations of MCTP equal to or greater than 5 μg/ml caused complete inhibition of colony formation, and 0.5 μg MCTP/ml caused greater than 50 percent reduction in colony forming efficiency for both cell types. In wells exposed to doses of 5 μg MCTP/ml or greater,

individual atypical cells were apparent attached to the plate surface, but there was no evidence of colony formation.

6-keto-prostaglandin $F_{1\alpha}$ Accumulation

MCTP, at concentrations of 10 μg/ml or greater enhanced the release of 6-keto-$PGF_{1\alpha}$ from monolayers of BECs and PECs (fig. 8). A dose of 1 μg/ml did not enhance accumulation of 6-keto-$PGF_{1\alpha}$ from monolayers of either cell type.

DISCUSSION:

Many alterations in the liver and lung caused by PAs may arise from the vasotoxic properties of PA metabolites. The endothelium of the hepatic venous system and pulmonary vascular bed is a proposed target for a portion of toxic PA metabolites generated in the liver (Barnes et al., 1964; Butler et al., 1970; Mattocks, 1968a). Differing sensitivity or responses of these target cells has not been considered as a factor which could influence organ toxicity. Indeed, the differences in sensitivity or responses of endothelial cells may in part account for distinctions among animal species in the pattern of pathologic changes associated with PA intoxication.

Endothelial cells derived from pulmonary arteries of bovine and porcine species differed quantitatively and qualitatively in certain responses to MCTP. BECs were sensitive to the cytolytic effects of MCTP as indicated by delayed and progressive detachment of cells from confluent cell monolayers and the accumulation of LDH activity in medium above monolayers. The cytolytic effects in PECs were much less pronounced. In

addition, the damage to PEC monolayers progressed much more slowly when compared to responses of BEC monolayers.

Species differences were also apparent in the morphologic response to MCTP. BECs underwent dramatic alterations in cell size and appearance. These cells gradually enlarged to cover 11 times the surface area of vehicle-treated controls. Some cells in the monolayers became vacuolated with time, and many developed cytoplasmic stress fibers that radiated from the perinuclear region. Despite the gradual detachment of many BECs, monolayer integrity seemed to be maintained temporarily by the enlargement and spreading of the remaining adherent cells. With time, however, small gaps developed between altered cells, indicating a loss of integrity of the endothelial monolayer. By contrast, PECs exposed to comparable concentrations of MCTP underwent relatively minor enlargement, and cell monolayers did not develop gaps in monolayer integrity during the 14 day study. Thus, the hypertrophic response of cells in PEC monolayers was far less pronounced than in BEC monolayers.

Although pronounced cell hypertrophy was not a feature of MCTP-treated PECs, they did undergo other morphologic changes after exposure to high concentrations of the toxicant. These alterations included becoming spindle-shaped in appearance and an apparent attenuation of cytoplasm at cell margins. The perinuclear cytoplasm of cells became granular in appearance, but distinct vacuolation of cells was not evident. Binucleate PECS appeared more common in MCTP-treated monolayers. With time, monolayers of PECs exposed to higher MCTP concentrations had reduced cellularity.

Species differences in morphologic effects of PAs or pyrrolic derivatives on vascular endothelium or other cell types *in vivo* have not been reported. However, there are some differences among animal species in the response of hepatocytes to these compounds. For instance, people who have ingested PAs do not have megalocytotic hepatocytes, a hallmark of hepatocellular response in many other species (McLean and Mattocks, 1980). Centrilobular necrosis and occlusion of central veins do occur in human livers as seen in many other species. The reason for the species difference in the megalocytotic response has puzzled investigators. Our findings suggest that this may be related to a specific difference in pathophysiologic response to pyrrolic metabolites rather than just to differences in the amount of toxic pyrroles that cells produce or encounter.

Not all of the markers of cell injury we employed revealed species differences. For example, enhanced PGI_2 release by endothelial cells, which may signal perturbation of endothelial cell membranes, occurred at similar MCTP doses in BECs and PECs. Similarly, the cytostatic effects of MCTP, assessed as colony forming efficiency, was comparable for both cell types.

For BECs exposed to concentrations of MCTP of 5 μg/ml or higher, both cytostatic and cytolytic changes were evident. For PECs, however, these higher concentrations of MCTP did not induce a pronounced cytolytic response. Concentrations of 0.5 μg MCTP/ml inhibited the proliferation of both cell types by greater than 50% without causing increased release of LDH activity from monolayers or distinct morphologic alterations in cells or cell monolayers. Cytostatic effects of MCTP can therefore be

separated from the cytolytic effects of the toxicant, particularly in PECs.

Results of studies in our laboratory have indicate that MCTP is cytostatic to other primary and transformed cells (Reindel and Roth, 1989; Roth and Reindel, 1988; Reindel *et al.*, 1988; unpublished observations). In fact, the effects of MCTP on colony forming efficiency has occurred at virtually identical concentrations for all cell types we have tested to date. In Madin-Darby canine kidney (MDCK) cells, Crandel-Rous feline kidney (CRFK) cells, undifferentiated human keratinocytes and bovine pulmonary arterial smooth muscle cells, a concentration of 5 μg MCTP/ml caused complete cessation of cell proliferation and a concentration of 0.5 μg MCTP/ml caused nearly a 50 percent reduction in the colony forming efficiency, just as in BECs and PECs. Thus, the ability of MCTP to inhibit cell proliferation is similar across a wide range of cell types.

Although there was little variation among different cell types in the cytostatic effects of MCTP, there was variation in the dose of MCTP which caused cytolytic effects in these cells. For undifferentiated human keratinocytes or bovine smooth muscle cells, concentrations of 50 μg MCTP/ml did not cause substantial cell detachment or release of LDH activity from cell monolayers. With MDCK cells and CRFK cells, concentrations of 50 but not 5 μg MCTP/ml caused enhanced cell detachment and lysis of cells in the monolayers. Although an enhanced cytolytic response was not apparent in cells exposed to 5 μg/ml, they were distinctly altered by treatment. Many showed morphologic features of cellular atypia as the posttreatment interval increased, including cell enlargement and nuclear abnormalities (Reindel et al., 1988).

The results of the LDH release assay for PEC monolayers and the effects of MCTP on PEC monolayer cellularity as determined by cell enumeration are disparate. The results of the LDH assay suggest that MCTP does not cause an appreciable cytolytic response in PECs. In addition, morphologic observations confirmed that there was little increase in cell detachment from monolayers exposed to as much as 100 μg MCTP/ml. Yet enumeration of cells comprising treated monolayers indicated an appreciable decrease in cell numbers. The reason for this apparent discrepancy is unknown. It is possible that PECs exposed to MCTP may be more prone to lysis during the process of enzymic removal from the plate prior to enumeration. Why PECs exposed to MCTP may not detach as readily as BECs from the monolayer but were prone to enzymic lysis and why this effect takes time to develop needs further investigation.

The findings in this study suggest that differences in the response of animal species to MCTP *in vivo* could reflect differences in cell sensitivity to toxic insult. For instance, BECs were relatively sensitive to the cytolytic effects of MCTP. Such sensitivity may predispose cattle to develop veno-occlusive lesions in the liver. The relatively rapid deterioration of the endothelium in this species could result in a rapid increase in vessel leak, insudation of the vascular intima with large serum proteins such as fibrinogen, intimal thickening, vascular thrombosis and consequent veno-occlusion. This sequence of vascular damage indeed occurs in central veins of the liver and small hepatic veins of veno-occlusion-prone nonhuman primates treated with MCT (Allen and Carstens, 1968; Allen et al., 1969). Like non-human primates, cattle develop

pronounced veno-occlusive lesions in the liver in addition to pulmonary edema.

PECs were less sensitive to the cytolytic effects of MCTP and alterations in the PEC cell monolayers occur relatively slowly compared to BEC monolayers. If a comparably slow deterioration of endothelium occurs *in vivo* in swine and other animal species with endothelial cells which are resistant to the cytolytic damage, this might preclude the development of veno-occlusive lesions liver (a process that may require relatively rapid endothelial destruction) but allow other pathologic changes to develop in the vasculature, such vascular structural remodeling (Peckham et al., 1974; Harding et al., 1964). Well developed, veno-occlusive lesions of the liver have not been described in swine, but pulmonary interstitial inflammation and vascular lesions are prominent (Peckham et al., 1974; Harding et al., 1964).

In summary, MCTP causes injury to endothelial cells, but the manifestations of this injury are dependent upon the species from which the cells are derived. The species differences in sensitivity of endothelial cells to reactive pyrroles such as MCTP may influence the pattern of organ pathology observed in animals during PA intoxication.

Figure 5-1. Photomicrographs of BEC monolayers exposed to 0 (A), 0.5 (B), 5 (C) and 50 (D) μg MCTP/ml of medium 14 days after a single administration. Phase contrast. 180x Mag.

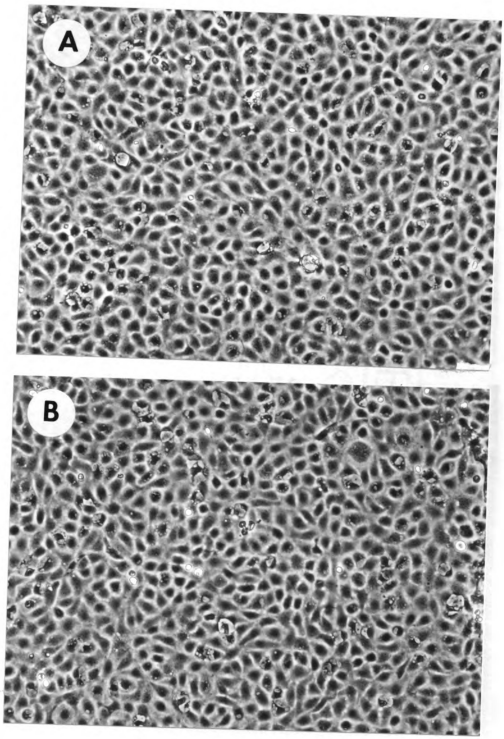


Figure 5-1.

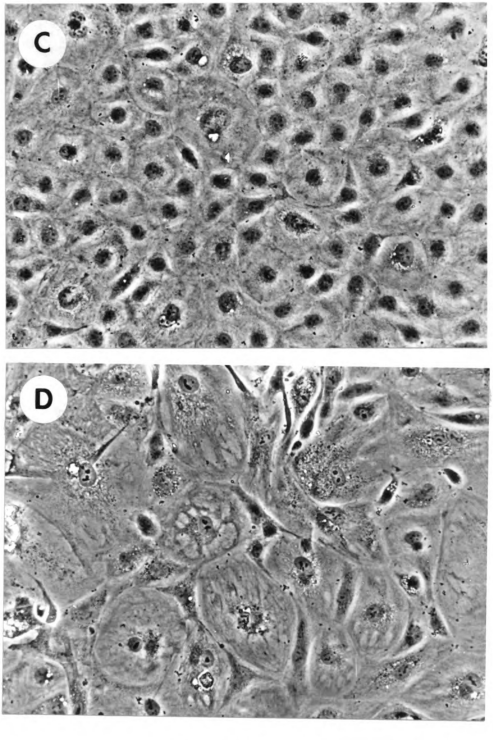


Figure 5-1. (cont.)

Figure 5-2. Photomicrographs of PEC monolayers exposed to 0 (A), 0.5 (B), 5 (C) and 50 (D) μg MCTP/ml of medium 14 days after a single administration. Phase contrast. 180x Mag.

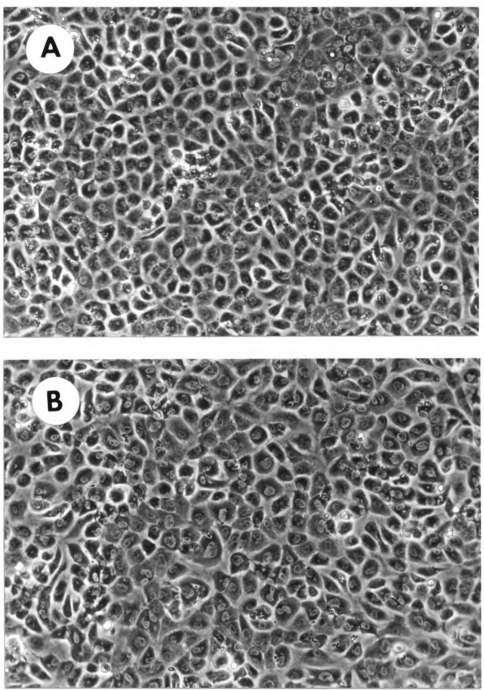


Figure 5-2.

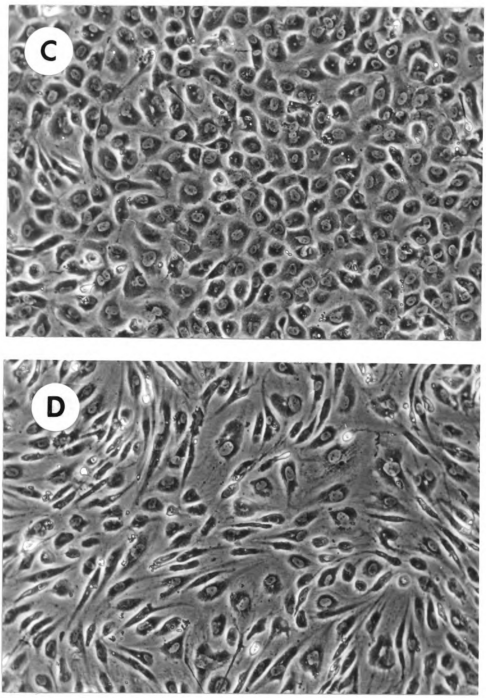


Figure 5-2. (cont.)

Figure 5-3. Morphometric data depicting two-dimensional surface area of BECs (A) and PECs (B) 5 and 14 days after a single exposure to MCTP. Data are presented as mean ± SEM for approximately 150 cells per treatment group. Asterisks indicate significant difference from corresponding control (0 μg/ml) at $p<0.05$.

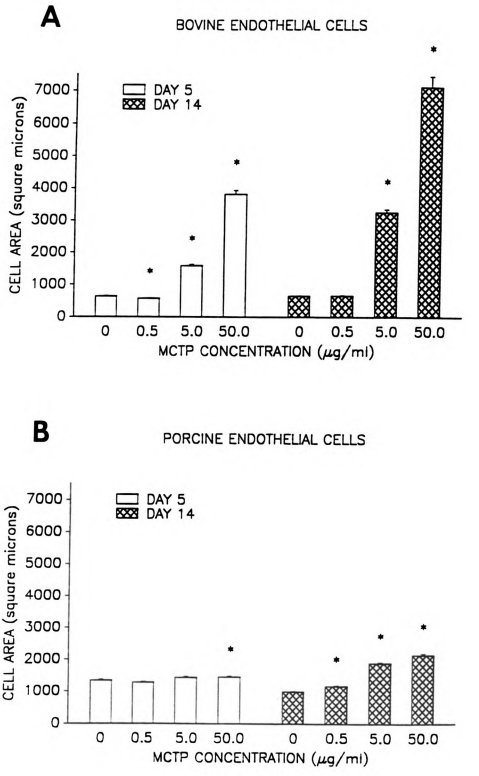

A
BOVINE ENDOTHELIAL CELLS
DAY 5
DAY 14
CELL AREA (square microns)
7000
6000
5000
4000
3000
2000
1000
0
0 0.5 5.0 50.0 0 0.5 5.0 50.0
MCTP CONCENTRATION (μg/ml)
B
PORCINE ENDOTHELIAL CELLS
DAY 5
DAY 14
CELL AREA (square microns)
7000
6000
5000
4000
3000
2000
1000
0
0 0.5 5.0 50.0 0 0.5 5.0 50.0
MCTP CONCENTRATION (μg/ml)

Figure 5-3.

Figure 5-4. Time course of lactate dehydrogenase activity release from monolayers of BECs (A) and PECs (B) exposed to 0 or 50 μg MCTP/ml of medium on day 0. Asterisks indicate significant difference from control of the same time at $p<0.05$. n=6.

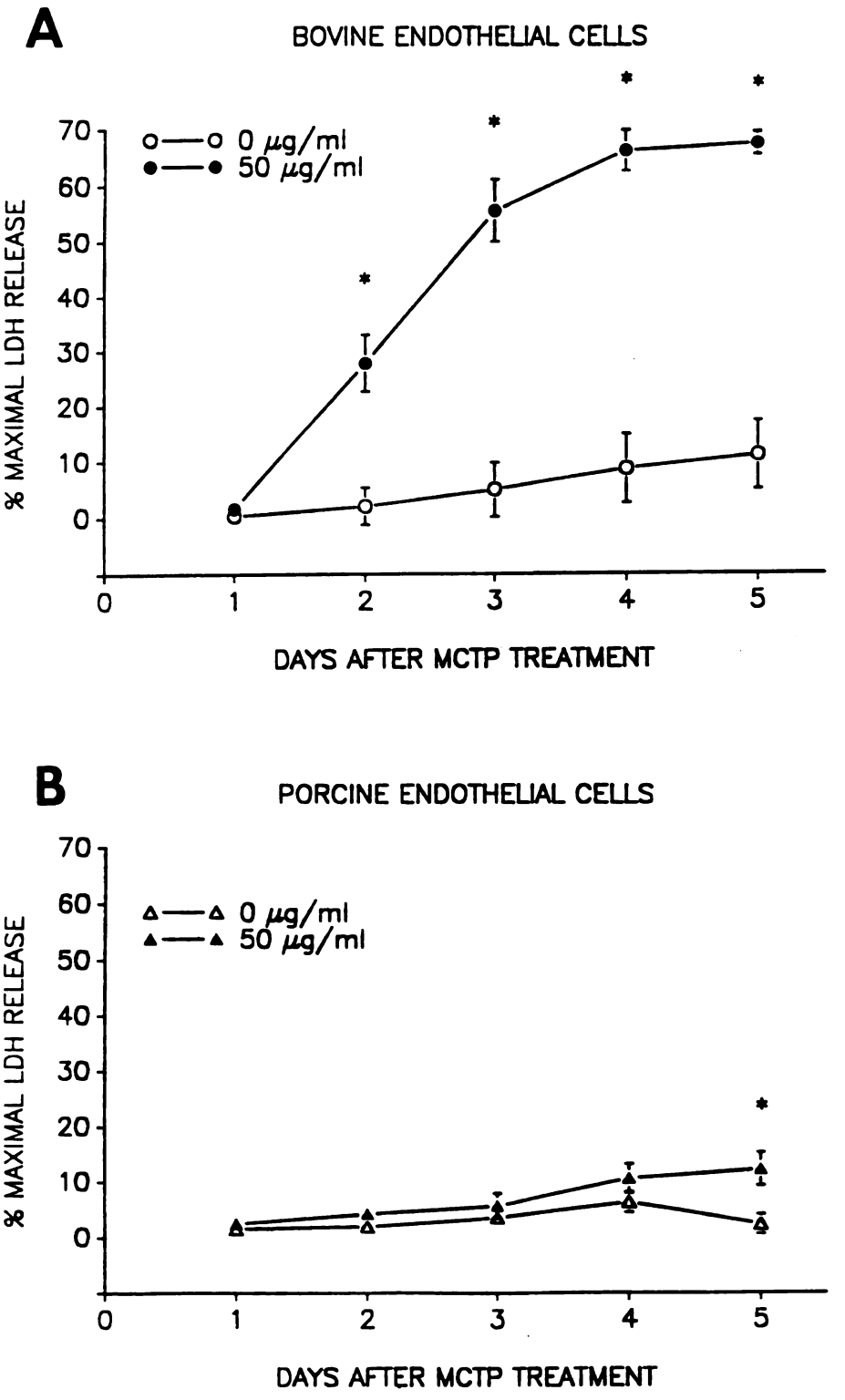

A
BOVINE ENDOTHELIAL CELLS
o—o 0 μg/ml
●—● 50 μg/ml
% MAXIMAL LDH RELEASE
70
60
50
40
30
20
10
0
0
1
2
3
4
5
DAYS AFTER MCTP TREATMENT
B
PORCINE ENDOTHELIAL CELLS
△—△ 0 μg/ml
▲—▲ 50 μg/ml
% MAXIMAL LDH RELEASE
70
60
50
40
30
20
10
0
0
1
2
3
4
5
DAYS AFTER MCTP TREATMENT

Figure 5-4.

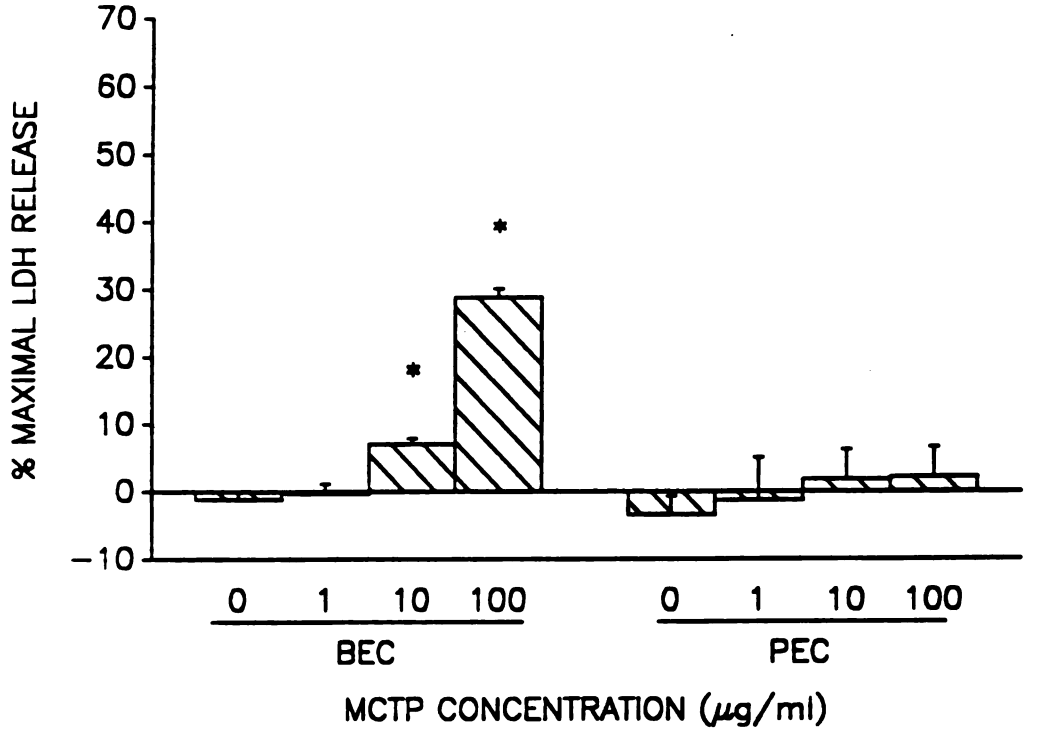


Figure 5-5. Dose-response relationship for LDH activity release from monolayers of BECs and PECs exposed to 100, 10, 1, and 0 μg MCTP/ml of medium. Asterisks indicate significant difference from respective control at $p<0.05$. n=6.

Figure 5-6. Effects of MCTP on cellularity of BEC (A) and PEC (B) monolayers exposed to a single administration on day 0. Values represent means of 5 replicate studies. Standard error for individual comparisons was 45.18 for BECs and 15.64 for PECs. Asterisks indicate significant differences from control at the same time at $p < 0.05$. n=5.

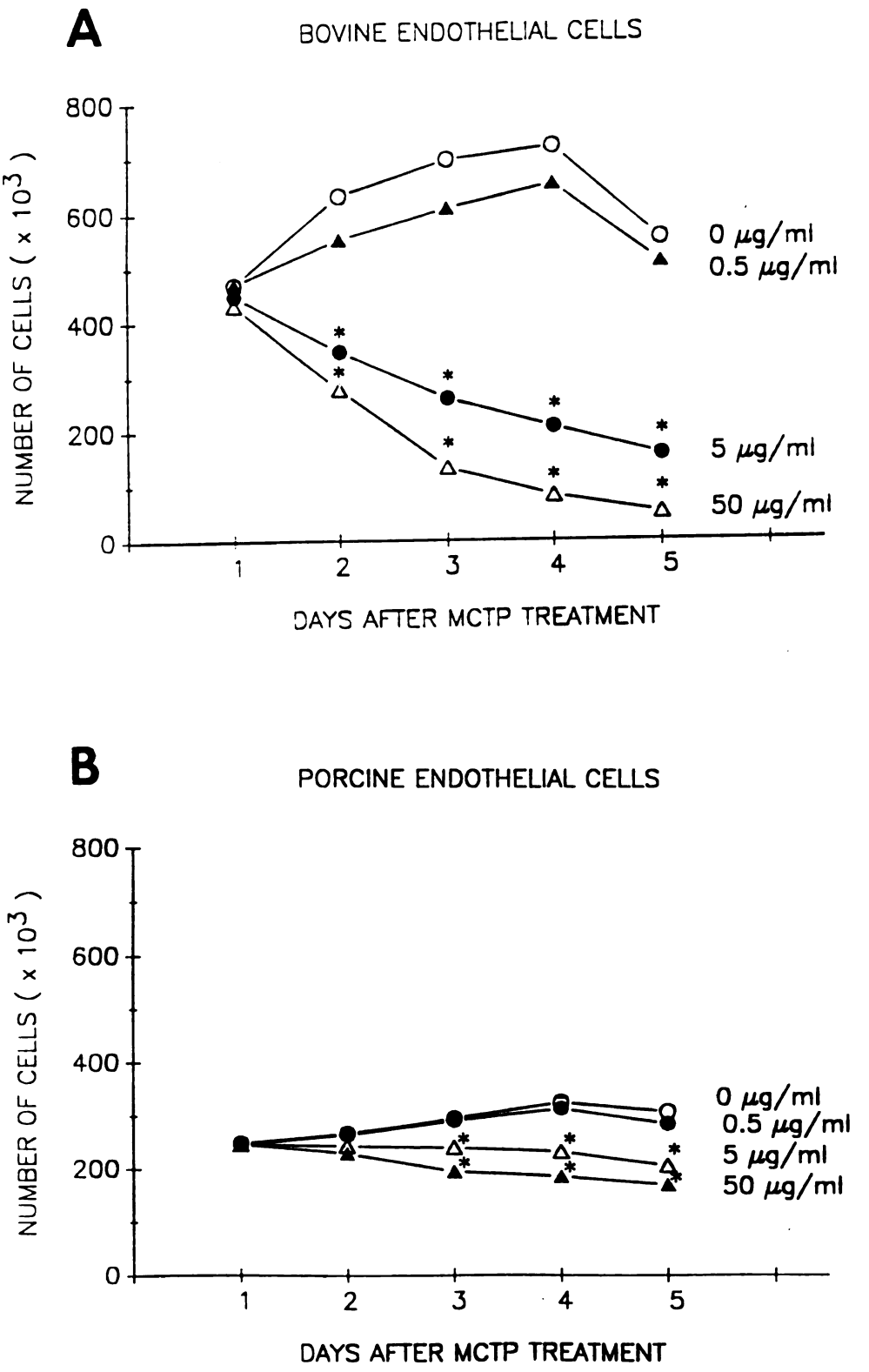

A
BOVINE ENDOTHELIAL CELLS
NUMBER OF CELLS (x 10^3)
800
600
400
200
0
1
2
3
4
5
DAYS AFTER MCTP TREATMENT
0 μg/ml
0.5 μg/ml
5 μg/ml
50 μg/ml
B
PORCINE ENDOTHELIAL CELLS
NUMBER OF CELLS (x 10^3)
800
600
400
200
0
1
2
3
4
5
DAYS AFTER MCTP TREATMENT
0 μg/ml
0.5 μg/ml
5 μg/ml
50 μg/ml

Figure 5-6.

Figure 5-7. Effects of MCTP on relative colony forming efficiency of BEC (A) and PEC (B) cells. Asterisks indicate significant differences from control at $p < 0.05$. n=4.

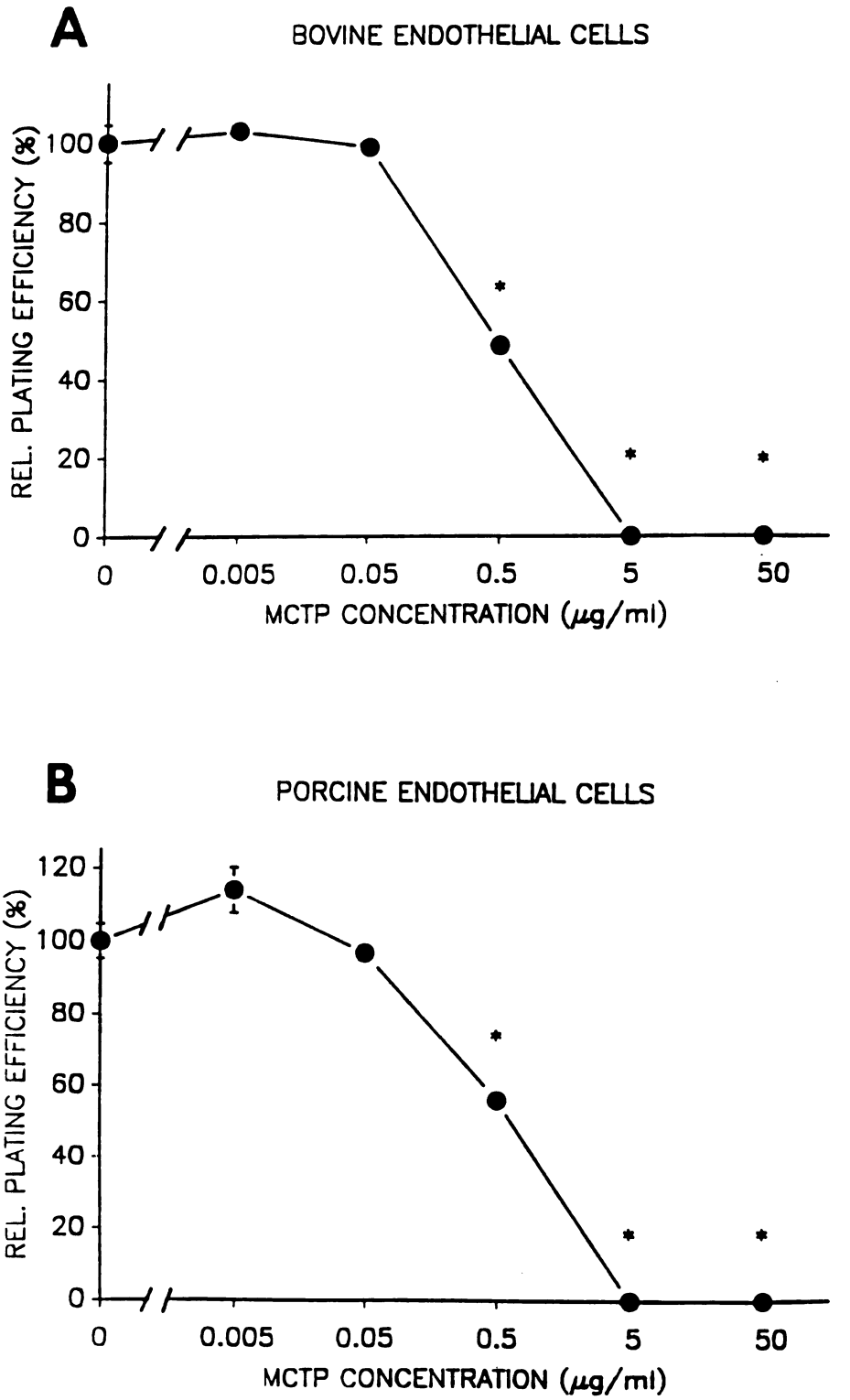

A
BOVINE ENDOTHELIAL CELLS
REL. PLATING EFFICIENCY (%)
100
80
60
40
20
0
0
0.005
0.05
0.5
5
50
MCTP CONCENTRATION (μg/ml)
B
PORCINE ENDOTHELIAL CELLS
REL. PLATING EFFICIENCY (%)
120
100
80
60
40
20
0
0
0.005
0.05
0.5
5
50
MCTP CONCENTRATION (μg/ml)

Figure 5-7.

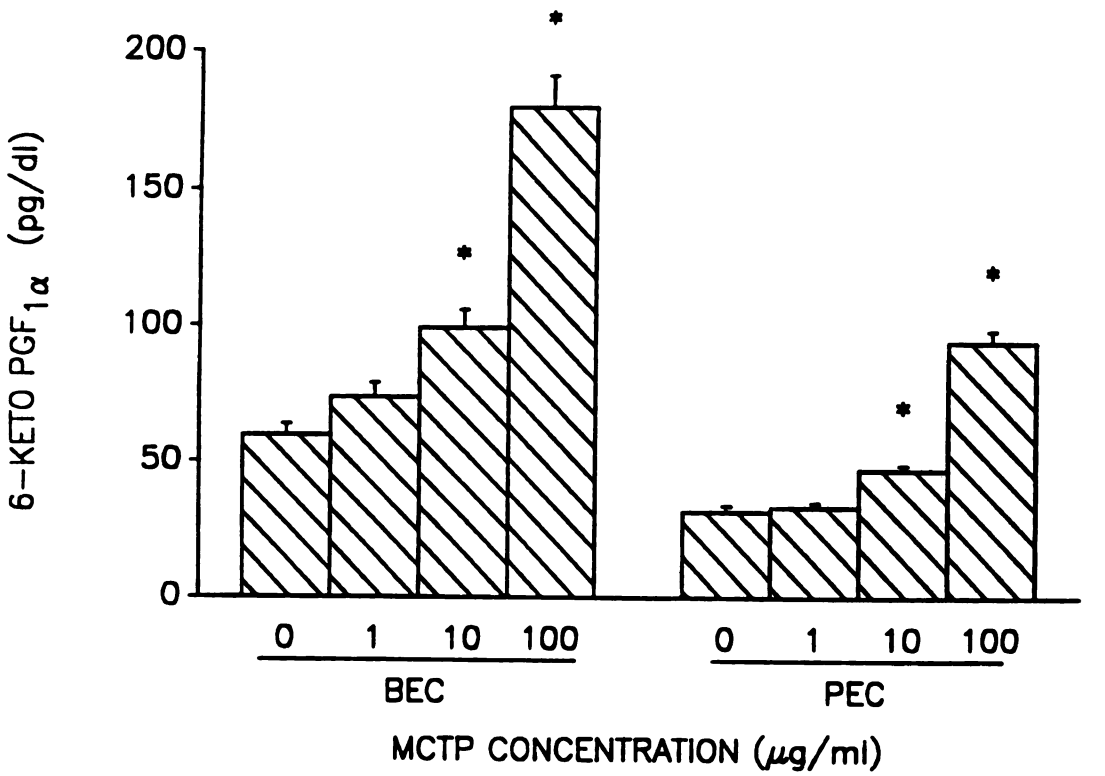


Figure 5-8. Effects of MCTP on 6-keto-$PGF_{1\alpha}$ accumulation in the medium above monolayers of BECs and PECs three days after a single exposure to concentrations of MCTP. Asterisks indicate significant differences from respective controls. $p<0.05$. n=6.

SUMMARY

The results of these studies support the premise that MCTP is the proximate toxicant of MCT. MCTP caused lung injury that was delayed in development and relatively subtle early in the course of the disease. This injury later progressed to widespread and severe injury, much like that induced by MCT. The injury was first evident as a subtle pulmonary vascular leak and interstitial edema (Figure S-1). These became more pronounced and widespread with time and were followed by progressively more severe pulmonary inflammation which was predominantly mononuclear in character. Pulmonary vascular remodeling, including thickening of medial layers of pulmonary arteries, was evident at 5 days after MCTP treatment and became more pronounced thereafter. The increase in medial thickness of arterial walls preceded the increase in pulmonary arterial pressure and the right ventricular enlargement, indicating that increased pulmonary arterial pressure is not solely responsible for this vascular remodeling. The development of the MCTP-induced lung injury and vascular remodeling parallels the course of lung injury and vascular remodeling induced by administration of MCT.

Results of studies *in vitro* indicate that MCTP can cause direct cell injury, and the injury is dependent on the dose of MCTP to which cells are exposed, on cell type and on species from which a cell type is derived. The injury in BEC, MDCK and CRFK cells and cell monolayers was delayed

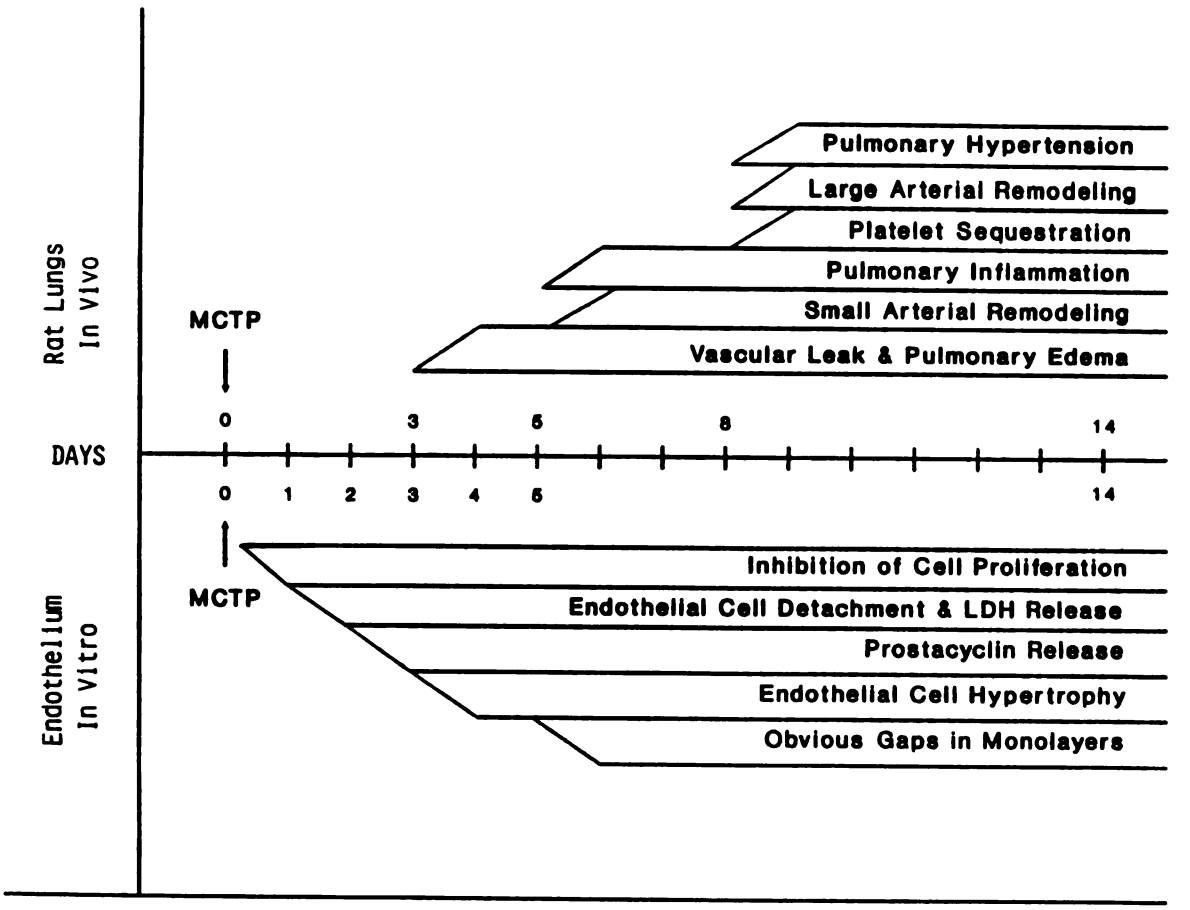


Figure S-1. Diagrammatic summary of the time course of alterations in rat lungs *in vivo* and those which occurred in BEC monolayers (endothelium) *in vitro* after a single exposure to MCTP. Numbers refer to days after MCTP administration when effects generally became obvious. Most effects were minimal when first apparent and became pronounced with time. Responses of PEC monolayers were similar to those observed with BEC monolayers although changes were slower to develop.

in development, inasmuch as there were few or no changes observed until at least 24 hours after exposure (Figure S-1). Injury was progressive in that there was a slowly developing increase in cell detachment from the monolayers and a gradual increase in the accumulation of LDH in the culture medium above monolayers with time. Monolayer integrity was temporarily maintained, in spite of cell loss, by spreading and hypertrophy of surviving cells. This delayed the appearance of obvious gaps in the monolayer until days to weeks after damage was initiated. The cell hypertrophy and appearance of atypical cellular features suggested that surviving cells had altered metabolism, may have had altered gene expression and may have been producing altered amounts or types of cell products.

Responses of PECs differed quantitatively and qualitatively from responses of CRFK and MDCK cells and of BECs. PECs did not release significant LDH within the first few days following exposure to high concentrations of MCTP. PECs and PEC monolayers were altered by MCTP; however, the injury developed much more slowly than with other cell types. Although not lysed by MCTP, PECs released enhanced amounts of PGI_2, and they appeared to be more prone to lysis by mechanical trauma. These cells also underwent shape changes after treatment but did not undergo marked cell hypertrophy within the duration of the study. That endothelial cells derived from the same tissue source from different animal species responded differently to MCTP suggests that some species differences in response to PAs may be due to differing responses of target cells.

As with PECs, BSMCs were not sensitive to the cytolytic effects of MCTP. There did not appear to be disruption of BSMC monolayers or marked

hypertrophy of these cells following exposure. That BSMCs did not undergo marked hypertrophy or proliferate following exposure to MCTP indicates that the smooth muscle cell hypertrophy and hyperplasia that occur *in vivo* are not likely direct responses to MCTP exposure. They may be a response to factors which leak from the vasculature, factors produced by MCTP-altered endothelial cells, or factors produced by platelets or by the perivascular inflammatory cells that accumulate in the lung.

MCTP inhibited cell proliferation, assessed as colony forming efficiency, of all cell types examined in these studies. The effects of MCTP concentration on colony forming efficiency of cells were in fact virtually identical for all cell types tested. For those cell types in which MCTP caused a cytolytic response, antiproliferative doses of MCTP were lower then those which caused release of LDH activity or enhanced cell detachment. The consequence of the antiproliferative effects of MCTP is that such cells of the monolayers are not viable reproductively in spite of being viable metabolically. These cells are ultimately destined to die over days to weeks but can temporarily maintain many cell functions and a largely intact monolayer. The slow attrition of cells from these monolayers without cell replacement contributes to the progressive deterioration of monolayer.

The delayed but progressive pulmonary edema and vascular leak observed in studies *in vivo* in light of the findings of studies *in vitro* suggest that there may be a close similarity between responses of the endothelium of the pulmonary vascular bed and cell monolayers *in vitro*. If the responses of endothelial cells in culture to MCTP reflect responses of cells composing the endothelium, then the pathogenesis of the slowly

developing lung injury can be explained. Cells of the vascular endothelium may be exposed to a range of MCTP concentrations, depending on their location in the vasculature, the rate of local blood flow and the dose of MCTP administered. MCTP might induce a mosaic effect on cells comprising the endothelium of the pulmonary vascular bed. Cells exposed to relatively high concentrations of MCTP would undergo cell degeneration and death over a period of time. The consequence would be progressive vascular leak from these areas of the vasculature and progressive pulmonary edema. Cell hypertrophy and spreading of surviving cells could compensate temporarily for cell loss, slowing the disruption of the endothelial barrier and the development of vascular leak.

Normally, the loss of cells from damaged endothelium would result in proliferation of adjacent cells and restoration of the endothelium. Because cell replication is inhibited in many of the surviving MCTP-exposed endothelial cells, effective repair of the damaged endothelial barrier would be inhibited, and this would contribute indirectly to the development and progression of pulmonary edema. The MCTP-exposed cells which are not reproductively viable may temporarily maintain many of the metabolic functions of normal endothelial cells but ultimately these cells will perish and metabolic functions of the lung endothelium will gradually decrease. As greater numbers of the MCTP-exposed endothelial cells are lost, larger plasma constituents would leak into the vessel wall and the pulmonary interstitium. Such plasma constituents can themselves instigate an inflammatory reaction in the pulmonary interstitium that would gradually increase as leak progresses. In addition, adhesion of platelets to gaps that develop in the endothelial barrier would occur. Platelets

and\or the interstitial perivascular inflammatory cells could release factors that contribute to the vascular remodeling and cause vasoconstriction. Platelet adhesion and aggregation may also cause microvascular occlusion in later stages of the disease. These factors may contribute to the increase in pulmonary vascular resistance and the increase in PAP that occur following MCTP administration.

The responses of cells to MCTP *in vitro* shed light on the possible mechanisms of the delayed and progressive lung injury induced by this compound. Additional studies are necessary to uncover other alterations in cell function which may contribute to the vascular cell injury and vessel remodeling that appear to be integral components of MCT- and MCTP-induced chronic pulmonary hypertension.

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