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THE ROLE OF THE HEMOSTATIC SYSTEM IN MONOCROTALINE PYRROLE-INDUCED PNEUMOTOXICITY AND PULMONARY HYPERTENSION

presented by

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has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Pathology-Environmental Toxicology

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Date 8/28/92

THE ROLE OF THE HEMOSTATIC SYSTEM IN MONOCROTALINE PYRROLE-INDUCED PNEUMOTOXICITY AND PULMONARY HYPERTENSION

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Pathology

1992

ABSTRACT

THE ROLE OF THE HEMOSTATIC SYSTEM
IN MONOCROTALINE PYRROLE-INDUCED PNEUMOTOXICITY AND
PULMONARY HYPERTENSION

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The administration of monocrotaline pyrrole (MCTP), a putative, toxic metabolite of the pyrrolizidine alkaloid, monocrotaline, causes pulmonary vascular thrombi that are associated with lung injury, vascular remodeling and pulmonary hypertension in rats. It is possible that the thrombi contribute to lung injury and pulmonary hypertension in this model. The mechanism(s) of the thrombosis and relationship of the thrombi to the pneumotoxicity and pulmonary hypertension are unknown.

Early changes in biochemical and cytologic markers of lung injury in rats treated with MCTP were examined. The results suggest that mild lung injury begins quite early after MCTP administration. However, major lung injury is delayed for 3-5 days after exposure to the toxicant. The subtle onset and progression of pneumotoxicity are consistent with the possibility that pulmonary microvascular thrombosis may be involved in the pathogenesis of the lung injury and pulmonary hypertension.

The hypothesis that changes in procoagulant or fibrinolytic activity in the systemic blood were responsible for the thrombosis in this model was examined. Changes in systemic procoagulant activity of blood from rats treated with MCTP were transient and mild and therefore probably not the cause of pulmonary

thrombosis. Significant changes in fibrinolysis in blood were restricted to an increase in the activity of plasminogen activator inhibitor (PAI) observed 8-14 days after MCTP intoxication. To address an alternate hypothesis that rats treated with MCTP may develop local alterations in fibrinolysis within the lung that may not be detected systemically, the fibrinolytic activity of lung tissue was measured. Three days after treatment with the toxicant, rats had decreased fibrinolytic activity of lung tissue that progressed with time.

To evaluate mechanisms(s) of hemostasis that may be altered in the lung microvasculature, the effect of MCTP was examined in cultured bovine endothelial cells (BECs). Five days after administration of MCTP, BECs had no change in production of the tissue factor, and only a mild increase in activity of Factor V/cell in culture medium. MCTP treatment caused a time-dependent increase in plasminogen activator activity and a decrease in PAI activity in the culture medium. Thus, BECs exposed to MCTP *in vitro* do not produce changes in procoagulant or fibrinolytic properties that would explain the thrombosis observed *in vivo*.

From the information gathered, alterations in fibrinolytic activity appear to be the most probable cause of the pulmonary microvascular thrombosis in the MCTP-treated rat model of lung injury and pulmonary hypertension.

ACKNOWLEDGEMENTS

I am a fortunate man, for there are many who have helped me to achieve my goals. The decision of a veterinarian to return to the university for specialty training and graduate education is a monumental one. I am grateful for the support of my family and friends during this challenging yet rewarding period of my life. Upon completion of my residency in pathology at Michigan State University, my entry into the Pathology/Toxicology training program of the Institute for Environmental Toxicology was encouraged by Dr. Robert Leader. I am thankful for his gentle persistence that I consider this educational opportunity.

I am particularly grateful to Dr. Robert A. Roth, Jr., who has been my thesis advisor, mentor and friend. He has shown me how to meld basic science research and diagnostic training in pathology into a productive and enjoyable career. His dedication to toxicology, meticulous standards for scientific writing and ability to work with investigators of diverse backgrounds in collaborative research are impressive and his excellent communication skills and talent to recognize and foster the better qualities in each individual are admirable. I hope to reflect his influence in my future work. I am also indebted to the other members of my thesis guidance committee: Drs. Stuart Sleight (academic advisor), Thomas Bell and Kenneth Schwartz. Many thanks for your patience, wise direction and helpful advice.

I acknowledge the valuable assistance of Katie Donnelly in preparation of my dissertation. Thanks are also extended to my colleagues in Dr. Roth's laboratory for

their kind support and assistance in completion of my research. Dr. Susan White, Dr. Paul Jean, Dr. Lawrence Dahm, Dr. James Hewett, Dr. Cindy Hoorn, Dr. Marc Bailie, James Wagner, and Maria Colligan, I appreciate your help!.

I would like to express my gratitude to the members of the Department of Pathology and the Department of Pharmacology and Toxicology at Michigan State University for their support and consultation in completion of my research. I will always feel fortunate to have been associated with these people.

Finally, I would like to thank those who provided financial support for my graduate work. My stipend was derived from NIH Training Grant #ES07146. My research and final two years of stipend were obtained from NIH Grant #ES02581.

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LIST OF ABBREVIATIONS

α ..... alpha
ACE ..... angiotensin converting enzyme
ADP ..... adenine diphosphate
alv. ..... alveolar
AMP ..... adenine monophosphate
ANP ..... atrial naturetic peptide
APC ..... activated Protein C
ARDS ..... adult respiratory distress syndrome
ATP ..... adenine triphosphate
ATIII ..... antithrombin III
β ..... beta
BALF ..... bronchoalveolar lavage fluid
BEC ..... bovine endothelial cell
Ca^{+2} ..... calcium
cap. ..... capillary
C. spectabilis ..... *Crotalaria spectabilis*
DEC ..... diethylcarbamazine
DFMO ..... α-defluromethylornithine
DMF ..... N,N-dimethylformamide
DNA ..... deoxyribonucleic acid
EGF ..... epidermal growth factor
FDP ..... fibrin degradation product
g ..... gram
Hep Sul PG ..... heparan sulfate proteoglycans
HMWK ..... high molecular weight kininogen
iv ..... intravenous
ip ..... intraperitoneal
5HT ..... serotonin
LDH ..... lactate dehydrogenase
LT ..... leukotriene
LW/BW ..... wet lung-to-body weight ratio
MCT ..... monocrotaline
MCTP ..... monocrotaline pyrrole
MCT(P) ..... monocrotaline and monocrotaline pyrrole
O_2^- ..... superoxide
ODC ..... L-ornithine decarboxylase
PA ..... pyrrolizidine alkaloid
PAF ..... platelet activating factor
PAI ..... plasminogen activator inhibitor
PC ..... Protein C
PDGF ..... platelet derived growth factor

PF_3 ..... platelet factor 3
plt. ..... platelet
PPH ..... primary pulmonary hypertension
PGI_2 ..... prostacyclin
pulm. ..... pulmonary
PRP ..... platelet rich plasma
RNA ..... ribonucleic acid
sc ..... subcutaneous
SE ..... standard error of the mean
TF ..... tissue factor
TM ..... thrombomodulin
TNCC ..... total nucleated cell count
tPa ..... tissue plasminogen activator
TxA_2 ..... thromboxane A_2
TxB ..... thromboxane B_2
uPa ..... urokinase-like plasminogen activator
vasc. ..... vascular
vWF ..... von Willebrand Factor
wk ..... week

PREFACE

Some of the data contained in this dissertation have been published previously. Chapter Two appeared as Schultze *et al.* (1991a) and Chapter Three as Schultze *et al.* (1991b).

CHAPTER ONE

LITERATURE REVIEW

I. Pyrrolizidine Alkaloids

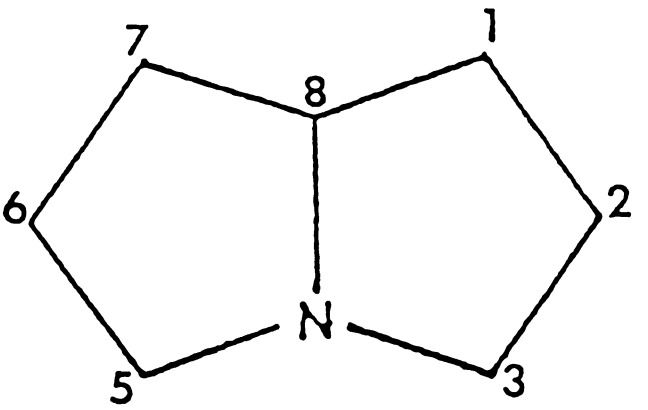
A. General Information

The term, pyrrolizidine alkaloid (PA), describes a group of structurally related compounds of plant origin that are characterized by a pyrrolizidine nucleus to which is attached a variable number of functional groups (McLean, 1970; Mattocks, 1986). The pyrrolizidine nucleus (Figure 1) consists of two fused five-membered rings that share a common nitrogen atom at the center or bridgehead of the molecule (Schoental, 1968; Huxtable, 1979). Side chains, attached at positions 1 and/or 7, may vary greatly in length, structure and composition and may be cyclic or acyclic (Huxtable, 1979; Mattocks, 1986).

Approximately 200 different PAs have been isolated and identified by various chromatographic and spectroscopic techniques. Plants that contain PAs grow on every continent and are a diverse group botanically, including 13 families (Figure 2), more than 60 genera and approximately 300 species (Bull *et al.*, 1968; Smith and Culvenor, 1981; Robbins, 1982; Mattocks, 1986; Huxtable, 1989, 1990).

PA-containing plants grow readily in recently disturbed soil and under specific conditions may spread rapidly. They evolved naturally in many locations and in others were introduced intentionally as ornamental plants, ground covers and nitrogen accumulators to improve fallow fields. They occasionally spread as weeds when seeds were scattered from the baggage of early settlers and travelers (Bull *et al.*, 1968; Cheeke, 1989). Some investigators suggest that the actual number of plant

Figure 1: Chemical structure of a pyrrolizidine nucleus



(Bull *et al.*, 1968; Huxtable, 1979, 1990)

Figure 2: 13 plant families that contain PAs

Apocyanaceae	*Orchidaceae*
Boraginaceae	*Ranunculaceae*
Celastraceae	*Rhizophoraceae*
Compositae	*Santalaceae*
Euphorbiaceae	*Sapotaceae*
Graminae	*Scrophulariaceae*
Leguminosae	

(Bull *et al.*, 1968; Smith and Culvenor, 1981; Mattocks, 1986; Huxtable, 1989, 1990)

species containing potentially toxic PAs is approximately 6,000, or 3% of the world's flowering plants (Smith and Culvenor, 1981; Huxtable, 1989). Overall, the PAs are a group of unpalatable, natural insecticides that represent an understudied yet extremely important source of mammalian exposure to plant toxicants and carcinogens (Huxtable, 1989).

B. Toxicity to Human Beings

Fortunately, not all PAs have deleterious effects on people. In most cases, PAs with major toxicological impact are restricted to the genera *Senecio*, *Heliotropium*, *Symphytum* and *Crotalaria* (Huxtable, 1990).

Most toxic PAs exist as esters of the necine bases retronecine or heliotridine (Winter and Segall, 1989). The chemical structural requirement for toxicity of PAs is the presence of a 1,2 double bond in the pyrrolizidine nucleus. Branching of ester side chains may amplify toxicity (Huxtable, 1979). PAs with cyclic diester linkages are more toxic than those with acyclic ester moieties, and acyclic diesters are generally more toxic than monoesters (Smith and Culvenor, 1981; Huxtable, 1989).

Human exposures to toxic PAs occur primarily via consumption of food or folk medicines. Exposures via food are further categorized as to whether the PA-containing plant was intentionally or unintentionally eaten. Episodes of intentional ingestion of PA-containing plants as food are few and are generally restricted to consumption of the green vegetables, *Symphytum* or *Petasites* (Huxtable, 1989). Most human exposures to toxic PAs can be classified as unintended in that the plant

material containing the toxicant was present as a contaminant and was not purposefully added to the product. Epidemic poisonings from consumption of PAs have occurred worldwide but are more common in less industrialized countries. Ingestion of cereal grain products harvested from fields contaminated with plants that produce toxic PAs have resulted in the majority of fatalities. The seeds and foliage of *Heliotropium* in Afghanistan (Mohabbat *et al.*, 1976), *Senecio* in South Africa (Willmot and Robertson, 1920) and *Crotalaria* in India (Tandon *et al.*, 1976) were processed into flour or cooking oils. When ingested as baked goods, these PA-containing plants resulted in massive outbreaks of fatal liver disease.

Another mode of exposure to PAs is the ingestion of specific medicinal herbs. "Bush teas" made from plants gathered from the bush or scrub of the land and selected for their reputed beneficial medical effects provide a vehicle for exposure to toxic PAs. Endemic hepatic veno-occlusive disease in the West Indies (Hill *et al.*, 1951; Bras *et al.*, 1954, 1957; Stuart and Bras, 1957) has been traced to consumption of home-brewed or "bush teas" prepared from *Senecio* or *Crotalaria* species. In the United States, infant mortalities have been attributed to consumption of gordoloba yerba, a commercially available medicinal tea that was contaminated with *Senecio longilobus* (Stillman *et al.*, 1977; Fox *et al.*, 1978).

Of increasing interest is the potential human exposure to alkaloids via foods that would not be immediately associated with plants. Milk, meat and honey are other sources for exposure to potentially toxic alkaloids (Huxtable, 1989). Milk from cows and goats that grazed on pastures contaminated with plants which produce

toxic PAs has been shown to contain concentrations of PAs that cause liver injury in rats (Dickinson *et al.*, 1976; Goeger *et al.*, 1982; Deinzer *et al.*, 1982; Miranda *et al.*, 1981). Rat dams fed *Senecio* alkaloids in quantities insufficient to cause hepatotoxicity in the adult females, produced concentrations of PAs in milk capable of causing liver disease in their suckling offspring (Schoental, 1959). In addition, mutagenic effects, measured by the Ames test, have been detected in milk from goats fed *Senecio jacobaea* (White *et al.*, 1984). While there are no proven cases of human morbidity or mortality from exposure to contaminated milk, speculation is growing that babies may be exposed to toxic PAs via breast milk from mothers who drink large quantities of "bush teas" (Huxtable, 1989).

It has been suggested that meat may serve as a source of human exposure to toxic PAs. This seems an unlikely scenario due to the rapid metabolism of PAs, the quick excretion of unmetabolized PAs (Huxtable, 1989) and the intensive, highly controlled rearing of most meat animals in industrialized nations. Even in countries that lack highly regulated meat production industries, animals would need to ingest large doses of toxic PAs and then be slaughtered within hours to present a potential hazard. There is currently no data to incriminate meat as a source of PA-induced morbidity or mortality.

Low concentrations of toxic PAs have been detected in honey produced by bees in the United States (Deinzer *et al.*, 1977). Fortunately, the bitter taste imparted by the alkaloids and need for blending of the domestic honey prior to marketing, coupled with the low consumption of honey by most Americans, makes

honey a minimal risk for exposure to toxic PAs in the United States (Mattocks, 1986; Huxtable, 1989). The risk is greater in Australia, where 10-15% of honey production is affected by PA contamination, and the level of contamination is two to three times that found in the United States (Culvenor *et al.*, 1981; Culvenor, 1985; Huxtable, 1989).

Most clinical cases of human poisoning by PAs result in hepatotoxicity (Kasturi *et al.*, 1979; Huxtable, 1989; Mattocks, 1986). Acute exposures to many PAs result in fatal centrilobular hepatic necrosis and hemorrhage. More often people ingest low levels of PAs over an extended period of time, resulting in development of hepatic veno-occlusive disease. Clinical signs are somewhat variable but may include a combination of vomition, jaundice, hepatomegaly, ascites and edema of lower limbs (Hill *et al.*, 1951; Bras *et al.*, 1954; Stuart and Bras, 1957; Huxtable, 1989). Biochemical evidence of liver dysfunction, such as increased activity of liver-specific enzymes in blood and decreased ability to remove the dye, bromsulfophthalein, from the circulation may also be detected. The pathognomonic lesions are characterized by loss of hepatic parenchymal cells, centrilobular fibrosis, megalocytosis and collagen deposition. Concurrently, small hepatic veins become occluded due to endothelial cell proliferation and medial hypertrophy. The final sequelae are cirrhosis and liver failure.

Rarely, people exposed to toxic PAs develop pulmonary hypertension and *cor pulmonale* (Heath *et al.*, 1975). More often, pulmonary lesions are limited

to pleural effusions that occur concurrently with hepatic veno-occlusive disease (Hill *et al.*, 1951; Stuart and Bras, 1957; Stillman *et al.*, 1977; Fox *et al.*, 1978).

The mutagenic and carcinogenic effects of PAs *in vitro* are well known (Mattocks, 1986; Bull *et al.*, 1968; IARC, 1976; McLean, 1970). Some investigators suggest that PAs may be responsible for naturally occurring malignancies in human beings (Schoental, 1968). No strong evidence yet exists to support this speculation.

The diagnosis of PA poisoning in people is a difficult and arduous process. Assessment of patient history, results of physical examination, serum biochemical evidence of hepatotoxicity and tests of decreased liver function provide supportive data. Definitive diagnosis, however, is restricted to cases in which a triad of criteria are fulfilled. Observation of pathognomonic lesions by biopsy or autopsy, demonstration of a responsible PA-containing plant in food, medicament or gastrointestinal contents and calculation of alkaloid quantity ingested sufficient to cause toxicity are necessary to confirm the diagnosis (Huxtable, 1989).

C. Toxicity to Animals

Consumption of toxic PAs causes considerable animal morbidity and mortality as well as substantial economic losses for the livestock industry and for owners of companion animals. Plants from the families *Boraginaceae*, *Compositae* and *Leguminosae* are responsible for most animal poisonings due to ingestion of PAs. In North America, *Senecio* and *Crotalaria* species cause the greatest number of fatalities (Cheeke, 1989). In addition, animal poisonings due to *Cynoglossum officinale* are a problem of increasing frequency (Knight *et al.*, 1984).

Herbivorous animals, primarily horses and cattle, that graze on pastures containing plants that produce toxic PAs may develop one of several fatal hepatic, gastrointestinal, neurological or pulmonary diseases (Huxtable, 1979; Cheeke, 1989). Although plants containing toxic PAs are not particularly palatable, they may be consumed readily when animals are forced to graze poor pastures or open rangeland. Exposure may also occur when animals are fed commercially manufactured forage or grain products obtained from fields contaminated with toxic alkaloid-producing plants. In addition, some avian species may be poisoned when they are maintained on litter that is contaminated with the seeds of PA-containing plants (Sippel, 1964).

The exact mammalian response to toxic PAs is dependent upon several factors including: specific PA ingested, amount or dose of PA, chronicity of exposure, species, age, sex and diet (Mattocks, 1986; Huxtable, 1989; Cheeke, 1989). Numerous organs may be affected by toxic PAs, and the selective lesions that occur after exposure are believed to be due to pharmacokinetic differences among PAs rather than variability in inherent tissue susceptibility (Huxtable, 1989).

As with people, the most common sequela of naturally occurring PA toxicosis in animals is hepatotoxicity. Swine and dogs develop chronic liver lesions similar to those seen in human beings (Huxtable, 1989). Proliferative interstitial pneumonia in pigs has been reported late in the disease progression after chronic ingestion of *Crotalaria* species (Peckham *et al.*, 1974). In addition, capillary and arterial vascular lesions in the lungs and kidneys are observed in pigs that consume

various *Crotalaria* species (McGrath *et al.*, 1975; Hooper and Scanlan, 1977; Ross, 1977). Cattle develop liver lesions but also manifest a gastrointestinal syndrome (Mattocks, 1986; Huxtable, 1989). A recent clinical report describes pulmonary vascular and cardiac lesions in dairy calves suspected of having *Crotalaria* intoxication (Pringle *et al.*, 1991). However, investigators were unable to confirm the presence of *Crotalaria* in the feed of the affected animals. The main target of PAs in horses is the liver but the most common clinical presentation is a neurotoxicity believed to occur secondary to hyperammonemia (Cheeke, 1989). Sheep exposed to PAs develop a hemolytic disease (Huxtable, 1979) and may develop spongy degeneration of the central nervous system secondary to severe hepatic lesions and hyperammonemia (Hooper *et al.*, 1974). In Australia, sheep that graze upon *Crotalaria* species develop acute, fatal pulmonary congestion and edema (Laws, 1968). Poultry may develop lesions in liver and lungs (Allen *et al.*, 1960, 1963; Huxtable, 1989).

Diagnosis or detection of toxicity in animals is often complicated by the delayed and progressive nature of the vague clinical signs that may ensue (Huxtable, 1989). Differences in PA metabolism, variation in responses to toxic levels of PAs, variation in serum enzyme specificity between species, lack of closely supervised environment and inconsistent monitoring of food intake prohibit definitive diagnosis in many cases. As with human beings, demonstration of pathognomonic lesions, identification of offending plant and calculation of dose necessary to cause toxicity are the criteria necessary to confirm the diagnosis.

II. Monocrotaline

A. General Information

Monocrotaline (MCT) or (12β,13β-dehydroxy-12α,13α,14α-trimethyl-crotal-1-enine) is a bitter tasting PA that is well known for its hepatic and cardiopulmonary toxicity in animals. Numerous plants of the *Crotalaria* genus produce MCT in their seeds and leaves (Figure 3) (IARC, 1976; Mattocks, 1986). *Crotalaria spectabilis*, *Crotalaria retusa* and *Crotalaria sericea* produce the greatest concentrations of MCT and may be used for industrial isolation of the compound (IARC, 1976).

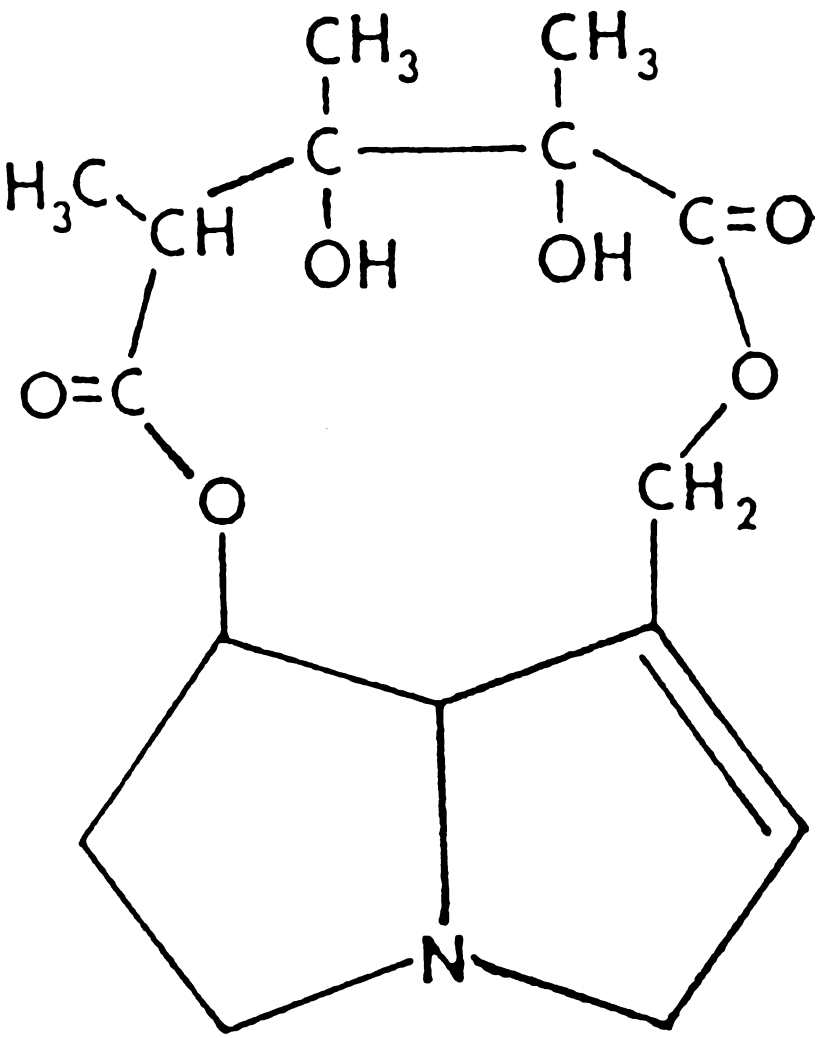
In the early 1920s, *Crotalaria spectabilis*, also called "rattle box" or "rattle pod", was introduced in the United States by the Florida Agricultural Experiment Station as a leguminous cover crop to prevent erosion and improve nitrogen content of poor soil (Bull *et al.*, 1968). The plant continues to thrive in the southeastern United States and is the primary source for isolation of the compound for most scientific studies (Kay and Heath, 1969; Cheeke, 1989).

MCT is a macrocyclic diester of the necine base, retronecine. More specifically, the molecule is characterized by the typical pyrrolizidine nucleus to which is attached, via ester linkages at positions 1 and 7, the dicarboxylic acid, monocrotalic acid. The chemical structure of MCT (Figure 4) was first identified by Adams and Rogers (1939) and has been confirmed by nuclear magnetic resonance, infrared and mass spectroscopic techniques (Culvenor and Dal Bon, 1964; Bull *et al.*, 1968). When

Figure 3: Plants that contain monocrotaline

Family	**Genus**	**Species**
Leguminosae	*Crotalaria*	*aegyptiaca*
		assamica
		burhia
		cephalotes
		crassipes
		crispata
		cunninghamii
		grahamiana
		leschenaultii
		leiloba
		mitchellii
		mysorensis
		nitens
		novae-hollandae
		paulina
		quinquefolia
		recta
		retusa
		sagittalis
		sessiloflora
		sericea
		spectabilis
		stipularia
Boraginaceae	*Lindelofia*	*spectabilis*

(Bull *et al.*, 1968; Smith and Culvenor, 1981; Mattocks, 1986; Huxtable, 1989, 1990)



MONOCROTALINE

Figure 4: Chemical structure of monocrotaline

purified, MCT is a colorless, crystalline powder of molecular weight of 325.3 g/mole. It has a melting point of 202-203°C. Additional chemical and physical data are available elsewhere (IARC, 1976).

B. Metabolism and Bioactivation

MCT is stable and inert. Under physiologic conditions, it reacts little with living tissue while in its native form (Mattocks, 1986). If applied topically or injected, it does not cause a localized toxicity (Winter and Segall, 1989). MCT will, however, cause lesions in several organs after adsorption and hepatic bioactivation.

Within the liver, MCT may undergo one of several chemical reactions including N-oxidation, dehydrogenation, ester hydrolysis and hydroxylation. Ester hydrolysis, hydroxylation and N-oxide derivatives are not toxic (Lalich and Ehrhart, 1962; Bull *et al.*, 1968; McLean 1970; Mattocks and White, 1971; Mattocks, 1986). Pyrrolic derivatives, formed by dehydrogenation within liver microsomes, cause extensive hepatotoxicity and pneumotoxicity (Mattocks, 1986). Monocrotaline pyrrole (MCTP), also called dehydromonocrotaline, is a putative toxic pyrrolic derivative of MCT formed by the cytochrome P450 mixed function oxidase system in liver microsomes (Figure 5) (Mattocks and White, 1971). MCTP is a reactive and unstable electrophile that is capable of causing extensive tissue injury. Intravenous administration of chemically synthesized MCTP to rats results in lung (Butler, 1970; Butler *et al.*, 1970; Bruner *et al.*, 1983a, 1986) and liver lesions (Newberne *et al.*, 1971; Hsu *et al.*, 1973) similar to those caused by the parent compound, MCT.

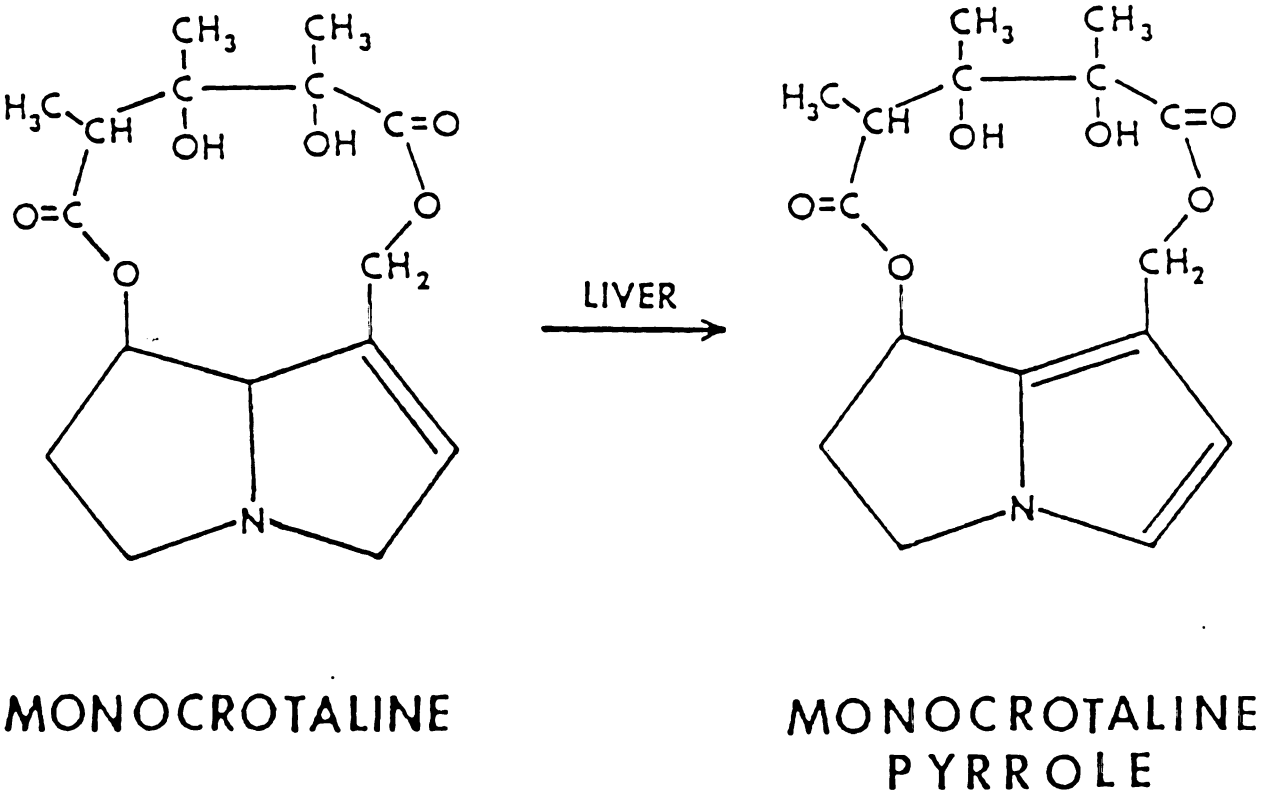


Figure 5: Bioactivation of monocrotaline within the liver.

Chloramphenicol and SKF-525A, chemicals that inhibit cytochrome P450 monooxygenase activity, decrease the MCT-induced organ toxicity (Mattocks and White, 1971; Chesney *et al.*, 1974ab; Allen *et al.*, 1972; Tuchweber *et al.*, 1974). Treatment with phenobarbital, a chemical that induces P450-containing monooxygenases, increases pyrrole production (Mattocks and White, 1971; Lafranconi *et al.*, 1985) and augments the toxicity of MCT (Mattocks, 1972; Allen *et al.*, 1972; Tuchweber *et al.*, 1974). Although cytochrome P450 enzymes have been identified in lung Clara cells, pulmonary P450 monooxygenase activity is not induced by phenobarbital treatment (Conney, 1967; Sunderman and Leibman, 1970). Therefore, increased pulmonary toxicity that follows phenobarbital pretreatment must result from an increased quantity of pyrrolic derivatives from other organs, presumably the liver.

There has been debate about whether MCTP, the putative product of hepatic bioactivation, could survive long enough in circulation to cause pneumotoxicity (Huxtable, 1990). The metabolism of ^{14}C-MCT was studied *in vitro* using an isolated perfused rat liver in a recirculating system (Lame' *et al.*, 1991). The major metabolite in the perfusate was identified as monocrotalic acid. ^{14}C-MCT and few water soluble metabolites were also present in trace amounts. The investigators concluded that MCTP, the putative metabolite of MCT, could react with nucleophiles, thus producing the measured monocrotalic acid. Mattocks *et al.* (1990) and Mattocks and Jukes (1990) demonstrated thiol sepharose trapping and covalent binding of pyrrolic metabolites that escaped isolated rat livers perfused with MCT.

Survival of pyrrolic conjugates in blood was substantiated by the identification of glutathione conjugated pyrrole in blood and urine of rats treated with ^{14}C-MCT (Estep *et al.*, 1990; Mattocks and Jukes, 1990; Lame' *et al.*, 1990). In addition, Pan *et al.* (1992) compared the pneumotoxicity of MCT, MCTP, the glutathione conjugate of pyrrolic MCT and the N-acetylcysteine conjugate of pyrrolic MCT. Although MCT and MCTP caused comparable degrees of pneumotoxicity and *cor pulmonale*, neither the glutathione adduct nor cysteinyl conjugate of the MCT metabolites caused significant lung or cardiac alterations. Results of these studies suggest that thiol conjugation of pyrrolic metabolites attenuates or prevents lung injury, and only the administration of free MCTP or its precursor, MCT, will result in significant pneumotoxicity.

C. Pharmacokinetics

Early pharmacokinetic studies of PAs were limited due to inability to detect accurately tissue levels of metabolites using radiolabeled PAs that lacked high specific activity. Original studies using ^{3}H-MCT administered in a dose of 60 mg/kg to rats revealed that approximately 70% of the MCT was recovered unmetabolized from the urine at 3 hours (Hayashi, 1966). The remaining 30% of labeled MCT was detected in the bile as metabolites. Measurements of pyrrolic activity using the Ehrlich assay (Mattocks and White, 1970) indicate that pyrrole derivatives accumulate in the lungs, liver and kidneys within minutes after administration of MCT. Peak activity occurs between 25-90 minutes and then decreases to low levels by 48 hours (Allen *et al.*, 1972; Mattocks, 1972).

Recently, Estep and colleagues (1991) treated rats with 60 mg ^{14}C-MCT/kg and found that 90% of the radioactivity was present in the urine and bile at 7 hours. While the concentration of radioactivity in plasma decreased dramatically at 7 hours, the content of radioactivity in erythrocytes decreased much less, signifying MCT equivalents were retained in erythrocytes. These data suggest that erythrocytes may serve as carriers of MCT or its metabolites and may be involved in the pulmonary toxicity that occurs after its administration. Using tandem liver and lung perfusion preparations, Pan and associates (1991) demonstrated that the transport of ^{14}C-MCT metabolites from liver to lung was increased by erythrocytes. They concluded that erythrocytes stabilize hepatic metabolites of ^{14}C-MCT and that these metabolites may covalently bind to pulmonary tissue. While interesting, the results of these studies are not definitive and the potential involvement of blood cells in transport of pyrrolic conjugates certainly warrants more attention. The binding of MCTP or other toxic metabolites to erythrocytes could explain one vehicle for movement of toxicants of liver origin to the lung. In the future, it will be necessary to determine which metabolites of MCT are carried by erythrocytes and, in particular, where in the erythrocytes metabolites may bind in a reversible manner for release in the lung.

D. Biologic Effects of MCT and MCTP

1. Species affected

MCT causes toxicity in numerous domesticated animals, including cattle (Sanders *et al.*, 1936; Becker *et al.*, 1935), horses (Gibbons *et al.*,

1953; Rose *et al.*, 1957), goats (Dickinson, 1980), swine (Emmel *et al.*, 1935), poultry (Thomas, 1934; Allen *et al.*, 1960, 1963; Simpson *et al.*, 1963), dogs (Miller *et al.*, 1981), rabbits (Gardiner *et al.*, 1965), mice (Harris *et al.*, 1942; Goldenthal *et al.*, 1964) and rats (Rose *et al.*, 1945; Schoental and Head, 1955). MCT intoxication has been documented in higher species, including nonhuman primates (Allen *et al.*, 1965, Allen and Carstens, 1971; Allen and Chesney, 1972; Raczniak *et al.*, 1978) and human beings (McFarlane and Branday, 1945; Kasturi *et al.*, 1979). A few species, including guinea pigs (Chesney and Allen, 1973a), gerbils and hamsters (Cheeke and Pierson-Goeger, 1983), are relatively resistant to the deleterious effects of MCT intoxication.

2. Routes of exposure

The natural route of exposure of animals and people to MCT is by ingestion of plants that contain the toxicant. In addition, toxicity or carcinogenesis in laboratory animals results from topical, iv, ip or sc administration of MCT or its putative metabolite, MCTP (Schoental and Head, 1955; Roth *et al.*, 1981; Bruner *et al.*, 1983a; Mattocks, 1986).

3. Carcinogenesis

No reports of naturally occurring malignancies in people or animals attributed to poisoning with MCT and MCTP (MCT(P))* have been identified (McLean, 1970; Svoboda and Reddy, 1972; IARC, 1976). There is, however, an increasing amount of experimental data to suggest MCT(P) may induce

* *The abbreviation MCT(P) will be used to designate situations that apply to both MCT and MCTP.*

neoplasia in some animals. Approximately 25% of rats administered MCT by gastric intubation weekly for 42 weeks developed hepatocellular carcinomas (Newberne and Rogers, 1973). Rhabdomyosarcomas, hepatocellular carcinomas, pulmonary adenomas and myelogenous leukemia occurred in rats given sc injections of MCT or dehydroretronecine biweekly for 52 weeks (Allen *et al.*, 1975). Various carcinomas and sarcomas of skin, lymphomas and pulmonary adenomas were observed after chronic dermal application of dehydromonocrotaline (Mattocks and Cabral, 1982). In addition, pulmonary adenocarcinomas and insulinomas were observed in rats given MCT by sc administration (Shumaker *et al.*, 1976; Hayashi *et al.*, 1977).

4. Hepatic toxicity

Hepatotoxicity is the most common sequela of exposure of animals and people to MCT. The onset and character of lesions are dependent upon the dose of toxicant and its route of administration.

After administration of large doses of MCT, mice and rats experience acute, hemorrhagic hepatic necrosis and death within hours. Ascites, hepatomegaly, congestion and a granular appearance of the liver are common macroscopic lesions (Harris *et al.*, 1942; Schoental and Head, 1955). Histologically, the lesions are characterized by centrilobular necrosis, dilatation of sinusoids and the presence of large blood-filled lagoons associated with necrotic cords (McLean, 1970).

More common is the chronic oral exposure of people and animals to low doses of MCT via food or drink and development of classic, hepatic venoocclusive disease (McFarlane and Branday, 1945; Hill and Rhodes, 1953; Bras

et al., 1954; McLean *et al.*, 1964). Macroscopic lesions and histologic lesions are similar in animals and man and have already been described in this manuscript. Structural lesions may be accompanied by slightly to moderately increased concentrations of serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and bilirubin (Kasturi *et al.*, 1979). Serum globulin concentration and bromosulfophthalein retention increase while serum albumin concentration and production of specific clotting factors may decrease (Kasturi *et al.*, 1979; Rose *et al.*, 1945).

5. Renal toxicity

Studies describing renal alterations that occur after exposure to PAs are limited (Mattocks, 1986). Macroscopic lesions induced by administration of *C. spectabilis* or MCT included changes in kidney color to green or dark brown (Schoental and Head, 1955; Hayashi and Lalich, 1967; Carstens and Allen, 1970) and textural irregularities of the capsular surface (Hayashi and Lalich, 1967; Carstens and Allen, 1970). Petechial hemorrhages within the renal cortex have also been reported (Hayashi and Lalich, 1967).

Masugi *et al.* (1965) observed glomerular congestion, thickening of basement membranes and mesangium, and glomerular fibrin thrombosis in rats fed *C. spectabilis*. Swelling and granular changes of proximal tubular epithelial cells and protein casts were also seen. Vascular lesions included thickening and necrosis of the vasa afferentia and dilation and thickening of arterioles. Similar glomerular and vascular lesions were reported by Carstens and Allen (1970), who also observed

markedly decreased or obliterated vascular lumina due to deposition of an amorphous, fine, fibrillar material. Iron pigment in the proximal tubules of rats was identified by several investigators after MCT administration and has been associated with intravascular hemolysis resulting from extensive vascular lesions (Schoental and Head, 1955; Hayashi and Lalich, 1967).

The similarity of the vascular lesions in the kidney to those seen in the lungs from rats treated with MCT was reported by Hayashi and Lalich (1967). Swelling of capillary endothelial cells and thrombosis are prominent lesions common to both organs following administration of PAs.

Rats exposed to MCT develop azotemia and alterations in tubular function. Accumulation of p-aminohippuric acid was decreased in kidney slices, but accumulation of tetraethylammonium was increased in the same preparations from rats killed 28 days after administration of the toxicant (Roth *et al.*, 1981).

Feeder pigs given *C. spectabilis* in their food were studied by McGrath *et al.* (1975). They found dilation, reduced cellularity and hemorrhages in glomeruli. Necrosis of tubular epithelial cells was also observed.

In summary, rats and pigs exposed to *C. spectabilis* develop numerous morphologic lesions and physiologic changes in renal function. Most studies involved chronic administration or relatively high doses of the PAs and revealed changes that develop late in disease progression. The vascular lesions had

distinct similarities to pulmonary lesions that occur following administration of MCT(P). No reports describing renal lesions due to MCTP were identified.

6. Cardiac toxicity

Rats chronically exposed to MCT in drinking water or food (Lafranconi *et al.*, 1984; Huxtable *et al.*, 1977; Turner and Lalich, 1965) or to *C. spectabilis* seeds in food (Turner and Lalich, 1965; Hislop and Reid, 1974; Meyrick *et al.*, 1980) develop right cardioventricular hypertrophy. Similarly, rats given a single sc administration of MCT (Hayashi and Lalich, 1967; Ghodsi and Will, 1981; Werchan *et al.*, 1986, 1989; Oehlenschlager *et al.*, 1989) or a single iv injection of MCTP (Chesney *et al.*, 1974a; Bruner *et al.*, 1983a) also develop significant right heart enlargement. There is general agreement among investigators that the cardiac lesions occur as a physiologic response to an increased workload that occurs as a sequela of pulmonary vascular remodeling and chronic elevations in pulmonary artery pressure. Generally, lesions are limited to the right ventricle and interventricular septum. No major lesions in the left ventricle or atria have been reported.

The macroscopic evidence of right heart enlargement is accompanied by an increased rate of right ventricular protein synthesis (Huxtable *et al.*, 1977), an increase in total collagen content of the right ventricle (Lafranconi *et al.*, 1984), a marked decrease in ratio of right ventricle DNA:RNA but no change in ventricular lipid content (Lafranconi *et al.*, 1984).

Werchan and colleagues (1989) observed marked increases in both cross-sectional area and cell length in myocytes from the right ventricle.

Further investigation, in which several parameters of cardiac pump function were measured, led them to conclude that MCT-treated rats do not experience a decrease in myocardial function *in vivo*. Pump failure may occur, however, late in the disease development, after extensive pulmonary vascular remodeling and sustained increases in pulmonary vascular resistance that result in excessive increases in cardiac afterload.

Physiologic changes in electrical potential of the cardiac muscle occur concurrently with cardiac hypertrophy due to MCTP. A right shift in the mean electrical axis of the electrocardiogram was observed at 14 days after treatment of rats with pneumotoxic* doses of MCTP by Bruner *et al.* (1983a).

Detailed studies of the histologic changes in the myocardium that occur after exposure to MCT(P) are few. Rats chronically exposed to MCT in diet (Turner and Lalich, 1965) or by single sc injection (Hayashi and Lalich, 1967) developed histologic evidence of myocardial hypertrophy that was restricted to the right ventricular wall and interventricular septum. Few rats developed evidence of mild myocarditis of the right ventricular wall and interventricular septum. At 150 days survival, several rats had small foci of mild perivascular myocardial fibrosis (Turner and Lalich, 1965). When Hayashi and Lalich (1967) treated rats with a single sc injection of MCT, few rats developed evidence of myocarditis characterized by small foci of myocardial degeneration and of mild lymphocytic infiltration within the right ventricular wall and interventricular septum. Chesney *et al.* (1974a) noted

** For the purpose of this thesis, the term pneumotoxic doses of MCTP will be used to designate doses of 3-5 mg MCTP/kg unless stated otherwise.*

foci of hemorrhage and similar mononuclear infiltrates in the right ventricle and interventricular septum from rats given a single iv injection of MCTP.

Infant stumptail monkeys given several sc injections of MCT developed right ventricular hypertrophy with marked endocardial fibrosis characterized by subendothelial increases in collagen and elastic fibers within the right ventricle (Chesney and Allen, 1973b). Small foci of endocardial edema and hemorrhage and multifocal areas of right ventricular hemorrhage and myocytolysis were also observed. Some of the coronary arteries had prominent endothelial and medial lesions similar to those described in pulmonary vessels of rats treated with MCT(P).

The cardiac atria produce atrial naturetic peptide (ANP), a hormone that is believed to be important in blood pressure regulation. ANP inhibits synthesis of aldosterone (Atarashi *et al.*, 1984), is a direct vasodilator (Currie *et al.*, 1983), causes natriuresis and diuresis (Debold *et al.*, 1981) and inhibits vasopressin secretion (Samson, 1985). Oehlenschlager and associates (1989) treated rats with a single sc injection of MCT and noted a time-dependent cardioventricular hypertrophy. At 21 and 45 days, MCT-treated rats had significant increases in the level of ventricular immunoreactive ANP. Analysis of cardiac mRNA indicated significant increases in both atrial and ventricular ANP mRNA had occurred, suggesting that the cardiopulmonary lesions induced by MCT caused a compensatory extension in cardiac endocrine activity from the atria into the ventricles. Although the exact mechanism for ventricular production of ANP during pulmonary

hypertension is unknown, the investigators postulated prolonged stretching of cardiac myocytes may have caused induction of the gene responsible for ANP synthesis.

Generally, remission of right ventricular hypertrophy does not occur without amelioration of the inciting cause. In one study of *C. spectabilis* (Hislop and Reid, 1974), the investigators described significant reduction of right ventricular hypertrophy in rats allowed to recover for 31 and 54 days after feeding of the toxic alkaloid. The results must be viewed with caution for three reasons. Although a decrease in muscularity occurred in the pulmonary vessels of rats allowed to recover, arterial wall thickness and extension of smooth muscle to normally nonmuscular pulmonary arteries were still significant compared to controls. Experimental design allowed for unintentional selection of nonresponding rats to be grouped for recovery, thus biasing the data. Finally, the number of rats, at 2-3 per recovery group, was unacceptably low for accurate statistical analysis and data interpretation.

7. Pulmonary toxicity

a. Clinical signs

Clinical signs of illness are usually not evident immediately after a single exposure of rats to pneumotoxic doses of MCT(P). Within 3-7 days, rats may show signs of ill thrift, including anorexia, listlessness, failure to gain weight, tachypnea and ruffled hair coats. As lung injury and vascular remodeling progress, rats may develop variable degrees of dyspnea, weakness, diarrhea and peripheral cyanosis (Schoental and Head, 1955; Turner and Lalich, 1965; Merkow and

Kleinerman, 1966; Hayashi and Lalich, 1967; Hislop and Reid, 1974; Bruner *et al.*, 1983a; Bruner, 1986).

Intravenous administration of high doses of MCTP (15-30 mg/kg) to rats causes rapid onset of dyspnea and tachypnea due to massive acute pulmonary injury. Animals develop severe pulmonary edema and die within hours (Plestina and Stoner, 1972). In contrast, when rats are given a single, iv injection of a low dose of MCTP (3-5 mg/kg), they develop lung injury that is characterized as delayed and progressive in nature (Bruner *et al.*, 1983a). Vascular remodeling and sustained increases in pulmonary artery pressure follow the period of lung injury.

b. Macroscopic lesions

The pulmonary lesions induced by exposure of rats to MCT(P) are somewhat variable and tend to progress with time (Hayashi *et al.*, 1984). At necropsy, rats may have varying degrees of pleural effusion (Chesney *et al.*, 1974a; Hislop and Reid, 1974). The lungs are edematous and congested and may have multifocal petechial hemorrhages (Schoental and Head, 1955; Merkow and Kleinerman, 1966). Most prominent are multifocal, irregularly shaped, red-brown foci of induration, consolidation and atelectasis (Schoental and Head, 1955; Turner and Lalich, 1965; Merkow and Kleinerman, 1966). Reindel *et al.* (1990) determined the distribution of pulmonary lesions in rats treated with a low dose of MCTP (5 mg/kg). The dorsal anterior regions of the right caudal lung lobe were affected most often; the hilar regions of the right middle lobe and left lobe were also commonly affected.

c. Microscopic and ultrastructural lesions

1) Vascular lesions

MCT(P) cause marked alterations in the pulmonary vasculature, and these lesions are the main focus of many histologic investigations. The complexity and diversity of lesions require that the descriptions be subdivided by the layer or tunic of the vessel affected. For clarity, the discussion that follows will be divided into lesions of the lumina, intima, media and externa or adventitia.

a) Lesions of the lumina

The lumina of pulmonary vessels, particularly those of alveolar capillaries, may change in size or shape in animals treated with MCT(P). Swollen endothelial cells protrude into the vascular lumen, decreasing its size and altering blood flow (Allen and Carstens, 1970). Lucent cytoplasmic projections or blebs from endothelial cells may partially occlude many lumina. In addition, thrombi composed of fibrin, platelets, erythrocytes, leukocytes or cellular debris individually or in admixture may partially or completely obliterate the lumina of significant numbers of small vessels (Merkow and Kleinerman, 1966; Allen and Carstens, 1970; Hislop and Reid, 1974; Chesney *et al.*, 1974a; Lalich *et al.*, 1977). Recently, Schraufnagel and Schmid (1989), using corrosion casts and scanning electron microscopy, demonstrated a decrease in pulmonary capillary density in rats given MCT. A review of histologic sections revealed fibrin thrombosis of small capillaries as the reason for the decreased capillary filling.

In addition to alterations in lumina of alveolar capillaries, changes in lumen size and shape occur in other pulmonary vessels but are noticed less frequently due to the larger size of these vessels (Allen and Carstens, 1970).

b) Intimal lesions

The vascular intima is composed of endothelium, basement membrane and, in larger vessels, some connective tissue and smooth muscle. Treatment of rats with MCT(P) causes alterations in endothelial cells within 9-24 hours (Valdivia *et al.*, 1967ab; Butler, 1970; Plestina and Stoner, 1972). The lesions are characterized by vesiculation, thickening and thinning of cytoplasm. In some areas, endothelial cytoplasmic protrusions partially obstruct the capillary lumen (Valdivia *et al.*, 1967ab). Platelet and fibrin thromboemboli are first detected at these early times (Valdivia *et al.*, 1967ab; Plestina and Stoner, 1972). Rosenberg and Rabinovitch (1988) observed endothelial cell pallor and swelling at 4 days post-MCT treatment. Ultrastructurally, these endothelial cells had swollen mitochondria, a decreased number of microfilaments and significantly more ground substance than controls. At 7 days, endothelial cell nuclei were convoluted and protruded into the lumen causing partial obstruction. Retraction of endothelial cells with exposure of basement membranes was not a prominent feature of the lesions (Vincic *et al.*, 1989). Endothelial cell lesions progress with time and eventually extend to all levels of the pulmonary vasculature but are generally more severe in arterioles and capillaries (Turner and Lalich, 1965; Merkow and Kleinerman, 1966;

Valdivia *et al.*, 1967ab; Allen and Carstens, 1970). Hypertrophy and hyperplasia of endothelial cells occur within 7-14 days after MCT(P) treatment, and vascular lumina are compromised more frequently by enlarged cells and thrombi (Turner and Lalich, 1965; Hislop and Reid, 1974; Butler, 1970; Meyrick and Reid, 1982; Vincic *et al.*, 1989). In some foci, endothelial cytolysis occurs, and fibrin thrombi are incorporated into the vessel wall. Several investigators hypothesized that capillary luminal occlusion may increase vascular resistance and result in pulmonary hypertension and vascular remodeling after MCT(P) treatment (Valdivia *et al.*, 1967ab; Raczniak *et al.*, 1979). Overall, the potential contribution of capillary alterations and, in particular, the thrombotic component of the lesions have been largely overlooked in investigations of the pathophysiology of MCT- and MCTP-induced pneumotoxicity and pulmonary hypertension.

c) Medial lesions

The vascular media of arteries is composed of layers of smooth muscle and elastic connective tissue arranged in concentric layers. Changes in the medial layer due to *C. spectabilis* or MCT(P) administration occur after intimal changes and are characterized by hypertrophy and hyperplasia of smooth muscle and extension of smooth muscle to normally nonmuscular pulmonary arteries (Turner and Lalich, 1965; Hayashi and Lalich, 1967; Ghodsi and Will, 1981; Kay *et al.*, 1982ab; Hayashi *et al.*, 1984; Molteni *et al.*, 1984; Reindel *et al.*, 1990). Rats treated with MCT or fed seeds of *C. spectabilis* had increased thickness of the vascular media at 7-8 days or 10 days, respectively (Turner

and Lalich, 1965; Hayashi and Lalich, 1967; Meyrick and Reid, 1979a; Ghodsi and Will, 1981). Thickening of the media may occur due to a combination of increased collagen content (Kameji *et al.*, 1980), an increase in number of elastic laminae and proliferation of smooth muscle between the laminae (Kay and Heath, 1966). Remodeling of small pulmonary arteries precedes that of larger vessels and is usually the result of smooth muscle hypertrophy (Reindel *et al.*, 1990). Hyperplasia of smooth muscle between elastic laminae may also contribute to medial thickening (Meyrick and Reid, 1979a, 1982; Ghodsi and Will, 1981). The medial thickening of small arteries is of a proportionally greater degree than that of larger arteries (Turner and Lalich, 1965; Hayashi and Lalich, 1967; Meyrick and Reid, 1979a).

Extension of smooth muscle to previously nonmuscular small pulmonary arteries occurs after MCT(P) and *C. spectabilis* administration (Hislop and Reid, 1974, Kay and Heath, 1966; Langleben and Reid, 1985; Reindel *et al.*, 1990). Meyrick and Reid (1979a) observed this lesion 3 days after feeding *C. spectabilis* to rats.

Acute pulmonary arteritis is a localized and variable finding after MCT or *C. spectabilis* administration (Lalich and Merkow, 1961; Lalich and Ehrhart, 1962; Turner and Lalich, 1965; Kay and Heath, 1966). The lesions occur at sites of arterial branching and are characterized by necrosis of the external elastic lamina and medial layer and infiltration of inflammatory cells in the media and adventitia (Turner and Lalich, 1965; Kay and Heath, 1969). The lesions may progress, and the chronic phases are characterized by

replacement of the adventitia by granulation tissue and large numbers of inflammatory cells (Kay and Heath, 1969). Although the term necrotizing arteritis has been used to describe lesions after MCT and *C. spectabilis* treatment (Lalich and Merkow, 1961; Kay and Heath, 1966; Lalich and Ehrhart, 1962), more detailed studies of arteries revealed this term to be inappropriate since necrosis was not present. Ultrastructurally, PAS-positive material was deposited in the subendothelial intima, and the smooth myocytes had the appearance of increased cellular activity (Merkow and Kleinerman, 1966). Reindel (1989) suggested that acute pulmonary arteritis and necrotizing arteritis were the same lesion.

Histologic changes in pulmonary veins are reported infrequently. Rats fed *C. spectabilis* had evagination of smooth muscle cells and subsequent protrusion of endothelium into the vascular lumen (Smith and Heath, 1978). Reindel *et al.* (1990) observed thickening of muscular pads in some muscular pulmonary veins.

d) Adventitial lesions

The adventitia is composed of a fine mesh of connective tissue fibers that forms a continuum with the surrounding parenchyma. Generally, lesions of the adventitia caused by administration of MCT(P) or *C. spectabilis* to rats are mild and are characterized by edema and the infiltration of few inflammatory cells (Lalich and Merkow, 1961; Allen and Carstens, 1970; Reindel *et al.*, 1990). With time, the perivascular edema and degree of inflammation become more severe. Late in disease progression, fibroblast prolifera-

tion, collagen deposition, hemorrhage, copious proteinaceous edema, abundant fibrin and marked infiltration of various leukocytes are observed (Kay and Heath, 1966; Merkow and Kleinerman, 1966; Reindel *et al.*, 1990).

2) Lesions of airways and alveoli

Thickening of alveolar walls due to capillary congestion and mild interstitial edema occurs within a few hours to several days after treatment with MCT(P) (Valdivia *et al.*, 1967ab, Hayashi and Lalich, 1967; Heath, 1969; Reindel *et al.*, 1990). Shortly thereafter, mild mononuclear hypercellularity characterized by infiltration of small lymphocytes and macrophages is observed in the pulmonary interstitium. A few segmented neutrophils, eosinophils and mast cells may accompany the primarily mononuclear infiltrates (Butler, 1970; Hayashi and Lalich, 1967; Valdivia *et al.*, 1967ab). Interstitial edema progresses with time and eventually the alveolar sacs fill with serous or serofibrinous exudate (Valdivia *et al.*, 1967ab; Hayashi *et al.*, 1984; Reindel *et al.*, 1990). Cuboidal to columnar metaplasia of alveolar epithelial cells and hypertrophy of type II alveolar epithelial cells occur late in disease progression (Turner and Lalich, 1965; Masugi *et al.*, 1965; Kay and Heath, 1966; Kay *et al.*, 1969; Reindel *et al.*, 1990). Thinning of elastic membranes in alveolar walls and replacement by collagen bundles thickens the oxygen exchange barriers (Meyrick *et al.*, 1980). In the later stages of pneumotoxicity, alveolar sacs may contain fibrin tangles, hemorrhage and cellular debris (Kay *et al.*, 1969; Butler *et al.*, 1970; Smith and Heath, 1978; Reindel *et al.*, 1990). Alveolar macrophages

increase in number, and many become large and foamy (Sugita *et al.*, 1983; Kay and Heath, 1966).

Lesions of the bronchi and bronchioles in animals treated with MCT(P) are emphasized less frequently than vascular lesions. Hyperplasia and hypertrophy of bronchiolar epithelial cells late in disease progression is the most frequently observed lesion (Lalich and Merkow, 1961; Lalich and Ehrhart, 1962; Turner and Lalich, 1965; Kay and Heath, 1969; Merkow and Kleinerman, 1966; Reindel *et al.*, 1990). Extension of airway epithelium into alveoli, peribronchiolar lymphatic dilatation and lymphatic hemorrhage occur in rats treated with MCT or fed *C. spectabilis* (Hayashi and Lalich, 1967; Kay and Heath, 1969; Kay *et al.*, 1969). Reindel and colleagues (1990) observed mild peribronchial and peribronchiolar edema in rats at 3 days post-injection of MCTP. In addition to hyperplasia and hypertrophy of airway epithelium, they detected marked goblet cell hyperplasia and degenerative changes in Clara cells in more severely affected large bronchioles at 8-14 days after MCTP intoxication.

3) Summary of the anatomic lesions

MCT and MCTP cause pulmonary lesions that are numerous and complex. Both the vasculature and parenchyma are injured in a delayed and progressive manner. Endothelial cells, particularly those of alveolar capillaries and small arterioles, are among the first cells to develop lesions. With time, vascular injury progresses in severity and extends to all levels of vessels. Vascular remodeling, characterized by smooth muscle hypertrophy and hyperplasia,

occurs at several levels of vessels. In addition, there is extension of muscle to normally nonmuscular vessels. Significant parenchymal lesions include alveolar epithelial metaplasia, edema and infiltration of numerous inflammatory cells.

d. Pulmonary mechanics and airway function

There are few studies of respiratory mechanics following exposure to MCT. Gillespie and colleagues (1985b) treated rats with a single, sc injection of MCT and, at 20 days, measured numerous markers of pulmonary function. Total lung capacity and residual volume were reduced in MCT-treated rats. Volume of relaxation, however, was increased. In addition, MCT caused a decrease in respiratory frequency, tidal volume and dynamic lung compliance. The gas exchange function, or coefficient of diffusion, was decreased and resistance to air flow was elevated. In summary, the mechanical, ventilatory and gas exchange alterations signified a decreased capacity for gas exchange and lung elasticity and were consistent with those of humans with some forms of pulmonary hypertension (Gillespie *et al.*, 1985b).

e. Hemodynamic alterations

Hemodynamic alterations that occur after exposure to MCT or its metabolites include changes in blood gas composition and arterial blood pressure, histologic evidence of vascular remodeling, hemorrhage and thrombosis and variations in blood cell numbers (Meyrick *et al.*, 1980; Ghodsi and Will, 1981; McNabb and Baldwin, 1984; Reindel *et al.*, 1990).

Rats fed *C. spectabilis* seeds had only mild changes in blood gas measurements (Meyrick *et al.*, 1980). Carbon dioxide tension (PCO_2) was slightly decreased at day 7, and bicarbonate (HCO_3-) concentration was slightly decreased at day 21. Only moribund rats had decreased oxygen tension (PO_2) at days 28 and 33. Arterial oxygen saturation was decreased significantly only at 33 days, but the venous oxygen saturation was decreased at 7 and 33 days in rats fed the toxic seeds.

The changes in blood gases were accompanied by vascular remodeling and alterations in pulmonary artery pressure (Meyrick *et al.*, 1980). Extension of smooth muscle to normally nonmuscular pulmonary arteries was first observed at 7 days. At 14 days, sustained increases in pulmonary artery pressure and increased thickness of the medial layer in arteries <200 μm diameter were detected. Pulmonary hypertension and vascular remodeling progressed, and right ventricular hypertrophy was observed at 28 days.

Rats treated with a single, sc injection of MCT (60 mg/kg) had significant thickening of the medial layer of small pulmonary arteries at 1 week post-injection (Ghodsi and Will, 1981). A significant increase in pulmonary artery pressure and mild right ventricular hypertrophy were observed at 14 days. Thickening of the pulmonary vascular walls, elevations in pulmonary artery pressure and severity of right ventricular hypertrophy increased in a linear fashion over the 3-4 weeks after MCT intoxication.

A correlative study of the hemodynamic and morphologic lesions that occur with a single iv administration of a low dose of MCTP (3.5 mg/kg) has only recently been completed (Reindel *et al.*, 1990). On day 5, rats developed mild thickening of the vascular wall in arteries <60 μm diameter. Vascular remodeling progressed, and at day 8 both small and large pulmonary vessels had mildly to moderately thickened walls. An increase in pulmonary arterial blood pressure, right cardioventricular hypertrophy and thickened arterial walls, characterized by both intercellular edema and hypertrophy of the smooth myocytes in the vascular media, were present at day 14. Previous studies of pulmonary hypertension after exposure to MCTP indicated that sustained increases in pulmonary artery pressure may be present as early as day 7 or 8 post-injection (Bruner *et al.*, 1983a; White *et al.*, 1989).

On day 7 after a single iv administration of 5 mg MCTP/kg, rats developed a mild leukocytosis that progressed slowly until 14 days. The leukocytosis was characterized by a trend for increased numbers of band neutrophils, segmented neutrophils, lymphocytes and monocytes. On day 14, MCTP-treated rats had hemoconcentration. There were no significant differences in the platelet count of MCTP-treated rats throughout the 14 day time course (Bruner *et al.*, 1983a).

Rats given 105 mg MCT/kg sc developed a moderate thrombocytopenia at 2 and 5 days post-injection. At day 10 they had only a mild

thrombocytopenia that was followed by a rebound thrombocytosis at day 14 (Hilliker *et al.*, 1982).

Fibrin and platelet thromboembolic lesions and pulmonary microhemorrhages are important hemodynamic lesions that occur in rats exposed to MCT(P) (Merkow and Kleinerman, 1966; Valdivia *et al.*, 1967ab, Plestina and Stoner, 1972; Chesney *et al.*, 1974a; Lalich *et al.*, 1977; Meyrick *et al.*, 1980; Reindel *et al.*, 1990). These lesions will be discussed in greater detail later in the text.

In summary, many different hemodynamic alterations occur in rats fed *C. spectabilis* seeds or given injections of MCT(P). The earliest changes occur in small pulmonary vessels, can be detected within a few days following intoxication, and progress with time. Sustained increases in pulmonary arterial pressure are delayed until approximately 7-14 days after exposure and occur simultaneously with vascular remodeling of medium sized vessels. The severity of vascular lesions and elevations in pulmonary arterial pressure progress, and right ventricular hypertrophy develops late in disease progression at approximately 2-4 weeks.

f. Biochemical, metabolic and functional lesions

In addition to the morphologic lesions described previously, MCT(P) cause numerous biochemical, metabolic and functional changes in the lungs. A short synopsis of the major alterations in laboratory animals treated with these toxicants follows. The possible involvement or mechanism(s) by which

these changes may be involved in the pathophysiology of the pneumotoxicity and pulmonary hypertension will be discussed later in the text.

1) Angiotensin converting enzyme

Angiotensin converting enzyme (ACE) is an endothelial cell product believed important in regulation of blood pressure. Although present in several organs, the majority of ACE is localized to the caveolae of pulmonary endothelium (Ryan *et al.*, 1975; Ng and Vane, 1967). Functions associated with ACE include conversion of angiotensin I to angiotensin II, a vasoconstrictor and smooth muscle mitogen (Ryan and Ryan, 1984; Dzau *et al.*, 1991), and inactivation of bradykinin, a potent vasodilator (Ryan and Ryan, 1984).

Changes in the activity of ACE after exposure of rats to MCT(P) have been the subject of many investigations and numerous disagreements. Molteni and associates (1984) reported an increase in the ACE activity of lung homogenates from rats 1 week after MCT administration. Subsequently, lung ACE activity decreased until 6 weeks, when it reached a plateau of 55% normal activity. Lafranconi and Huxtable (1983) observed that rats given MCT in drinking water for 3 weeks had reduced "specific activity" of lung ACE, a value determined by normalization of ACE activity to lung protein content. However, if ACE activity was expressed as total lung ACE, there was no significant difference between MCT-treated and control animals. In this study, there was an increase in the protein content of lungs from rats treated with MCT, hence the decrease in "specific activity" of lung ACE was observed. Other investigators have reported decreases in specific

activity of lung ACE (Keane *et al.*, 1982; Hayashi *et al.*, 1984) and total lung ACE activity (Keane and Kay, 1984) after exposure to MCT. Morphometric and hemodynamic measurements indicated that pulmonary vascular changes preceded the decrease in specific activity of lung ACE. Since changes in lung ACE activity and pulmonary hypertension developed concurrently, Kay *et al.* (1982b) concluded that the decrease in ACE activity was a response to sustained pressure elevations and a potential mechanism for its control.

2) Polyamines

The polyamines, a group of structurally related intracellular chemical compounds that regulate cell growth and differentiation (Heby, 1981), stimulate DNA condensation prior to cell replication and promote protein biosynthesis at several different steps (Hougaard, *et al.*, 1987ab; Pohjanpelto and Knuutila, 1982; Rao and Johnson, 1971). Olson *et al.* (1984a) demonstrated increased activity of lung L-ornithine decarboxylase, the rate limiting enzyme in polyamine synthesis, and increased concentrations of the polyamines, putrescine, spermidine and spermine (Olson *et al.*, 1984b), in lung tissue homogenates from rats treated with MCT. These pulmonary biochemical changes occurred well before vascular remodeling and onset of pulmonary hypertension.

3) Biogenic Amines

Serotonin or 5-hydroxytryptamine (5HT) is a vasoactive amine secreted by numerous cell types and has been suggested as a possible mediator of pulmonary hypertension after exposure to MCT(P). Vascular

endothelial cells remove 5HT from the circulation by a carrier mediated transport system (Hughes *et al.*, 1969; Strum and Junrod, 1972; Iwasawa *et al.*, 1973). Intracellularly, 5HT is metabolized by monoamine oxidases to inactive molecules (Roth, 1985).

Administration of MCT in drinking water (Gillis, *et al.*, 1978; Huxtable *et al.*, 1978) or by sc injection (Hilliker *et al.*, 1982) or an iv administration of MCTP (Hilliker *et al.*, 1983ab) results in decreased removal of 5HT by isolated perfused rat lungs or lung slices. The impaired removal of 5HT following MCT (Hilliker *et al.*, 1982) has been characterized as delayed and progressive and is dose and time dependent.

Rats treated with MCTP have increased vascular pressor responses. At 7 days, pulmonary vascular responsiveness is only mildly increased but at 14 days after MCTP-intoxication a three fold increase in vascular pressor responses of isolated lungs to 5HT is observed (Hilliker and Roth, 1985a).

Vascular endothelial cells remove the biogenic amine norepinephrine from the circulation and metabolize it in a manner similar to 5HT. MCT intoxication also impairs removal of norepinephrine from rat lungs *in vitro* (Gillis, *et al.*, 1978; Hilliker *et al.*, 1984a).

4) Protein, DNA and RNA content in lungs

After chronic administration of MCT in drinking water, rats develop significant increases in lung mass and absolute lung protein content. Total pulmonary lipids and RNA content increase, but DNA content

remains unchanged. Concurrently, the rates of protein and RNA synthesis increase. The decrease in lung DNA:RNA ratio that occurs following MCT administration is consistent with cellular hypertrophy (Lafranconi *et al.*, 1984).

By using autoradiographic studies of ^{3}H-thymidine incorporation into lungs of rats fed *C. spectabilis*, Meyrick and Reid (1982) evaluated cellular proliferation in pulmonary arteries at two levels: the hilar and intra-acinar regions. A biphasic response in labeling was observed in the endothelial cells, smooth muscle cells and fibroblasts of the hilar region. ^{3}H-thymidine incorporation was increased at 3 days in medial smooth muscle cells and at 7 days in endothelial cells and fibroblasts. ^{3}H-thymidine labeling returned to baseline at 14 days and was markedly elevated again at 21 days in all 3 cell types. Labeling levels again returned to control values at 35 days.

At 14 days, the extension of smooth muscle to previously nonmuscular arteries in the intra-acinar regions was recognized. Intra-acinar pulmonary arterial endothelial cells had increased ^{3}H-thymidine labeling at 14 days followed by increased labeling of precursor smooth muscle cells by day 35. Intra-acinar pulmonary venular endothelial cells had a biphasic response similar to that of the hilar arteries. Results of this study, together with detailed histologic and ultrastructural studies, confirmed that *C. spectabilis* causes hypertrophy and hyperplasia at two levels of the pulmonary artery. The vascular response occurs in several phases after exposure to MCT. The first phase correlates well with initial injury and subsequent DNA incorporation. The second phase of ^{3}H-thymidine

labeling is both delayed and intense in nature and probably involves a cellular response to ongoing injury or is a compensatory response to pulmonary hypertension.

5) Lavage fluid lactate dehydrogenase activity

Lactate dehydrogenase (LDH) is a cytosolic enzyme of mammalian cells. Analysis of LDH activity in the bronchoalveolar lavage fluid provides a sensitive but nonspecific marker of lung injury. Following exposure of rats to MCT(P), LDH activity of bronchoalveolar lavage fluid is increased in a delayed and progressive manner (Roth, 1981; Roth *et al.*, 1981; Bruner *et al.*, 1983a).

6) Lavage fluid protein concentration

One of the more sensitive indicators of pulmonary inflammation is an increase in the protein content of bronchoalveolar lavage fluid (Henderson *et al.*, 1978ab, 1985). Rats treated with MCT(P) develop marked and sustained increases in the protein content of lavage fluid (Roth, 1981, Bruner *et al.*, 1983a).

III. Pulmonary Hypertension

A. Chronic Pulmonary Hypertension in Human Beings

The normal pulmonary arterial pressure for resting adult human beings measured at sea level is 14 $\pm$ 3 mm Hg (Reeves and Grover, 1985). Pulmonary hypertension has been defined as a pulmonary arterial pressure of $\geq$ 25 mm Hg (Marshall and Marshall, 1991). Chronic pulmonary hypertension in people has been

observed in disease states or conditions that either increase pulmonary blood flow, increase pulmonary outflow pressure or result in increased pulmonary vascular resistance (Marshall and Marshall, 1991). Chronic pulmonary hypertension occurs in people with cystic fibrosis (Ryland and Reid, 1975), thoracic radiation (Gross, 1977), adult respiratory distress syndrome (Zapol and Snider, 1977), chronic bronchitis and emphysema (Semmens and Reid, 1974) and urticarial vasculitis (Falk, 1984). In addition, ingestion of Aminorex (Wagenvoort and Wagenvoort, 1977), "toxic oil syndrome" (Fernandez-Segoviano *et al.*, 1983), sepsis, recurrent pulmonary thromboembolism, hypoxia and various cardiac abnormalities may also cause chronic pulmonary hypertension (Marshall and Marshall, 1991). Finally, there exists a group of people with pulmonary hypertension for which no known cause can be determined. People with this condition are diagnosed as having primary pulmonary hypertension.

B. Animal Models of Pulmonary Hypertension

Patients with pulmonary hypertension are a perplexing problem for physicians. The disease is uncommon and most medical treatments, short of a heart/lung transplant, are ineffective. Therefore, further research is necessary to understand the mechanisms that cause pulmonary hypertension and to develop effective prevention programs and treatment regimens. Development of an appropriate animal model for this mysterious human vascular disease could hasten these processes.

There are few animal models of chronic pulmonary hypertension. A single injection of MCT in rats or one prolonged administration of thoracic radiation

in sheep will produce chronic pulmonary hypertension (Kay and Heath, 1969; Perkett *et al.*, 1986). Three other ovine models of pulmonary hypertension include repeated sc injections of indomethacin (Meyrick *et al.*, 1985), repeated iv injections of *Escherichia coli* endotoxin (Meyrick and Brigham, 1986), and continuous air embolization (Perkett *et al.*, 1988). Chronic or repeated exposure of rats to hyperoxia (Coflesky *et al.*, 1988), hypoxia (Meyrick and Reid, 1979b; Rabinovitch *et al.*, 1976), carrageenan (Herget *et al.*, 1981) or α-naphthylthiourea (Hill *et al.*, 1984) also causes persistent pulmonary hypertension.

There are some distinct similarities in the pathophysiology of these animal models and that of humans with pulmonary hypertension. The similarities and differences have been discussed in detail elsewhere (Meyrick *et al.*, 1987; Reid and Davies, 1989; Marshall and Marshall, 1991). In addition, each animal model is also accompanied by certain economic, space, and welfare factors that influence its availability for use in research. Each model may potentially reveal specific aspects of mechanisms involved in development of pulmonary hypertension.

C. MCT(P) as a Model of Human Pulmonary Vascular Diseases

1. Primary pulmonary hypertension

Primary pulmonary hypertension (PPH) is an insidious, structural disease of human lungs that is known by several names including essential, idiopathic and cryptogenic pulmonary hypertension (Davies *et al.*, 1987; Reid and Davies, 1989). This uncommon disease affects people of both sexes and all ages, but there is a higher incidence in female infants and post-adolescent women (Voelkel and Reeves,

1979). The diagnosis of PPH is dependent upon fulfillment of 3 criteria: 1) a sustained increase in pulmonary arterial pressure with normal pulmonary wedge pressure, 2) right cardioventricular hypertrophy in the absence of other cardiac lesions and 3) exclusion, at autopsy, of all known causes of chronic pulmonary hypertension (Voelkel and Reeves, 1979). The histologic lesions in people with PPH are similar to those observed in the lungs of rats treated with MCT(P). In rats, the endothelium appears to be the site of initial injury (Reid *et al.*, 1986). Endothelial cell hypertrophy, platelet and fibrin thromboemboli, intimal proliferation and fibrosis, vasculitis and vascular occlusion have been observed (Walcott *et al.*, 1970; Watanabe and Ogata, 1976; Wagenvoort and Wagenvoort, 1977; Voelkel and Reeves, 1979; Palevsky and Fishman, 1985; Reid *et al.*, 1986; Voelkel and Weir, 1989; Reindel *et al.*, 1990). Pulmonary vascular remodeling, characterized by medial hypertrophy of pulmonary arterioles and extension of smooth muscle to previously nonmuscular pulmonary arterioles, also occurs. Although the plexiform vascular lesion is a common feature of PPH in people (Wagenvoort and Wagenvoort, 1977; Voelkel and Reeves, 1979), this lesion has been reported only rarely in rats with MCT-induced pulmonary hypertension (Watanabe and Ogata, 1976).

The mechanism of PPH has not been elucidated, but evidence exists for involvement of several etiologic factors in the pathogenesis, including congenital factors, familial occurrence, thromboembolism, autoimmune mechanisms, hepatic dysfunction, vasoconstriction, drugs, pregnancy, diet and infectious agents (Voelkel and Reeves, 1979; Voelkel and Weir, 1989). PPH may be the final

manifestation of several factors acting singly or concurrently (McLeod and Jewitt, 1986).

It is not clear whether thromboembolism of small lung vessels is a cause or a response to vascular lesions in PPH. Some investigators believe plexogenic and microthrombotic pulmonary arteropathies may be separate pathologic entities in this disease (Fuster *et al.*, 1984; Voelkel and Weir, 1989; Palevsky and Weiss, 1990). In most histologic investigations of PPH, both thromboembolic and plexiform lesions are observed in the lungs (Wagenvoort and Wagenvoort, 1977; Wagenvoort, 1980; Bjornsson and Edwards, 1985). In several investigations the incidence of thromboembolic lesions was twice that of plexiform lesions (Fuster *et al.*, 1984; Bjornsson and Edwards, 1985). Microthrombotic pulmonary lesions have been identified in the lungs of rats treated with MCT(P), but plexiform lesions are observed infrequently.

The prognosis for people with PPH must be considered poor. Symptoms usually do not occur until pulmonary vascular lesions are well developed. Survival after diagnosis is brief and averages 2-3 years (Voelkel and Reeves, 1979). Given the similarities in pathophysiology, rats treated with MCT(P) provide useful animal models for the study of this disease.

2. Adult respiratory distress syndrome

The adult respiratory distress syndrome (ARDS) is a morbid condition of human beings characterized by evidence of diffuse bilateral pulmonary infiltrates by roentgenographic examinations, hypoxemia, decreased pulmonary

compliance and severe dyspnea (Connors *et al.*, 1981; Murray *et al.*, 1988). The syndrome has been recognized since the early 1900s and has had many different medical designations or names (Connors *et al.*, 1981). ARDS may be precipitated by a plethora of pathologic conditions, including shock, aspiration, severe trauma and sepsis (Connors *et al.*, 1981; Murray *et al.*, 1988). The incidence of ARDS in the United States is estimated at approximately 150,000 cases annually, of which more than 50% are fatal (Connors *et al.*, 1981; Fowler *et al.*, 1985; Murray *et al.*, 1988; Repine, 1992). Although numerous treatments have been applied to patients with ARDS, none are entirely successful (Macnaughton and Evans, 1992).

The pulmonary response in ARDS may be divided into two phases: an initial exudative phase and a delayed proliferative phase. The acute exudative phase occurs within 24-96 hours after a pulmonary insult and is characterized by an increase in capillary membrane permeability. Histologic lesions observed at this time include congestion, marked alveolar and interstitial edema, necrosis of type I pneumocytes, mild endothelial cell lesions and hyaline membranes in alveoli and respiratory bronchioles. Alveolar capillary neutrophilia and fibrin and platelet thromboembolic lesions are observed frequently (Connors *et al.*, 1981; Snow *et al.*, 1982; Tomashefski *et al.*, 1983).

The proliferative phase occurs approximately 3-10 days after the initial insult and is associated with pulmonary hypertension. Histologic alterations observed at this time include resolving alveolar edema, marked endothelial cell lesions and, eventually, fibrosis of alveolar septae and hyaline membranes. In

addition, thickening of the medial layer of small pulmonary vessels and extension of smooth muscle to previously nonmuscular arteries are observed (Snow *et al.*, 1982; Zapol and Jones, 1987).

There are numerous similarities between the pathophysiologic alterations in ARDS and MCT(P)-induced pneumotoxicity and pulmonary hypertension. Both have two rather distinct phases of pulmonary response. The early exudative phase of ARDS and the period of pneumotoxicity due to MCT(P) are characterized by marked increases in vascular permeability and subsequent pulmonary edema (Plestina and Stoner, 1972; Hurley and Jago, 1975). Pulmonary platelet and fibrin thromboemboli are the most consistently observed lesion of ARDS (Tomashefski *et al.*, 1983; Heffner *et al.*, 1983, 1987) and are also observed frequently after MCT(P). People with ARDS and rats treated with MCT(P) have numerous endothelial cell lesions and decreased ability to remove biogenic amines from circulation (Morel *et al.*, 1985).

The proliferative phase of ARDS and the period of pulmonary hypertension that occurs after MCTP administration are characterized by sustained increases in pulmonary arterial pressure and vascular remodeling. Endothelial cell lesions progress with time in both pathologic conditions. Smooth muscle extends to small, nonmuscular arteries, and pre-existing muscular arteries develop increased thickness of the medial layer.

Although there are some differences, the similarities between ARDS and MCT(P)-induced pneumotoxicity and pulmonary hypertension are

striking. Numerous investigators recognize these similarities and support the use of the MCT(P)-treated rat as an experimental model for the proliferative phase of ARDS (Snow *et al.*, 1982).

3. Interest in the MCT(P)-treated rat

There are several distinct advantages in the use of the MCT(P)-treated rat as a research model. The loss of domestic animals due to PA-induced toxicoses is estimated in millions of dollars annually. Fatality due to ingestion of toxic PAs is also a well documented human public health problem. The MCT(P)-treated rat provides an effective animal model for the study of this naturally occurring environmental health problem of animals and people. In addition, the pathophysiologic changes induced in the lungs of rats treated with MCT(P) are similar to those observed in the lungs of human beings with certain chronic pulmonary vascular diseases. Therefore, the MCT(P)-treated rat is a useful experimental model for PPH and the late phases of ARDS.

The use of the putative active metabolite that presumably initiates tissue damage affords several advantages over the use of MCT itself. These include a shorter time required for lesion development, elimination of the necessity for bioactivation, a uniform dose of reactive metabolite administered to all animals, elimination of variability due to differences among animals in hepatic metabolism of MCT and the capacity to relate pathophysiologic changes to a well defined, bolus exposure to the proximate toxicant.

IV. Mechanisms of Pulmonary Action of MCT(P)

A. General Information

The complete pathogenesis of MCT(P)-induced pneumotoxicity and pulmonary hypertension has not been elucidated. Given the complexity of the histologic lesions and the numerous biochemical, metabolic and functional alterations that occur in the lungs after administration of these toxicants, it is highly probable that more than one mechanism operates simultaneously and may involve many mediators. Several lines of investigation have revealed portions of mechanisms that may be important in the disease progression. The following section will summarize several areas of mechanistic investigation of MCT(P) and will emphasize pharmacologic interventions used to confirm or rule out specific mediators in the pathogenesis of MCT(P)-induced lung injury and pulmonary hypertension.

B. Mechanisms

1. Angiotensin converting enzyme

Changes in lung activity of angiotensin converting enzyme (ACE) after exposure of rats to MCT(P) have been a subject of great interest among investigators. Molteni *et al.* (1984) reported an initial increase in ACE activity in lung homogenates from rats exposed to MCT in drinking water. Since ACE, an endothelial cell product, is believed to be involved in control of blood pressure, the ability to manipulate ACE activity in a model of pulmonary hypertension might be especially valuable.

Administration of Captopril, an ACE inhibitor, to rats treated with MCT in drinking water was studied by Molteni and colleagues (1985). Rats that received Captopril plus MCT had less right heart enlargement, vascular remodeling and pulmonary fibrosis than those that received only MCT. While initially exciting, results of these experiments must be interpreted with caution for the following reasons: rats treated with Captopril alone did not experience a decrease in lung ACE activity, but they did develop an increase in serum ACE activity. These data suggest that inhibition of lung ACE activity was not responsible for the amelioration of MCT-induced effects in rats treated with Captopril. An explanation for the increase in serum ACE activity was not apparent. In addition, interpretation of results from this study was complicated because rats treated with Captopril alone had a decrease in body weight, a nonspecific change that has been associated with protection from MCT-induced lesions.

The effect of ACE inhibitors on MCT-induced pneumotoxicity and pulmonary hypertension continues to provoke interest and controversy. Protection from right heart enlargement has been afforded to rats treated with MCT by concurrent administration of CL242817, a competitive sulfhydryl-containing inhibitor of ACE structurally similar to Captopril, as well as nonsulfhydryl-containing ACE inhibitors (Molteni *et al.*, 1986ab). All studies have been complicated by one or more factors, including decreased heart weight-to-body weight ratios, weight loss of test animals, or increased serum ACE activity in rats treated with the ACE inhibitor only (Molteni, *et al.*, 1986ab). For these reasons, the definitive involvement

of changes in ACE activity in the pathogenesis of MCT-induced pulmonary hypertension and right heart enlargement has not been established.

2. Collagen synthesis

The administration of MCT to rats causes numerous lung lesions, including interstitial fibrosis (Kay *et al.*, 1969; Butler, 1970, Meyrick and Reid, 1979a; Meyrick *et al.*, 1980). Pulmonary collagen deposition, evaluated by measurement of lung hydroxyproline concentration and electron microcopy, was increased by MCT treatment, and this was attenuated in rats treated with D-penicillamine (Molteni *et al.*, 1985). Other agents known to decrease lung hydroxyproline concentration in MCT-treated rats include the ACE inhibitors, Captopril and CL242817 (Molteni *et al.*, 1985, 1986b). D-penicillamine will chelate copper and is an inhibitor of collagen cross-linking and maturation (Nimni *et al.*, 1972). The exact mechanism(s) by which D-penicillamine decreases collagen deposition in lungs of rats treated with MCT is uncertain. The mechanism(s) by which the ACE inhibitors decrease pulmonary fibrosis after MCT treatment is unknown, but experimental data suggest their protective effects after MCT administration may be due to properties other than inhibition of ACE activity (Molteni *et al.*, 1985).

3. Immune system

The delayed and progressive nature of pneumotoxicity and the histologic lesions that follow MCTP administration in rats suggest that the immune system may mediate the lung injury (Bruner, 1986). Graft rejection responses and

MCT(P)-induced pneumotoxicity share common lesions suggestive of vasculitis and are characterized by "fibrinoid necrosis", fibrin and platelet thromboemboli, leukocyte infiltration, endothelial cytolysis and pronounced increases in vascular permeability (Carpenter *et al.*, 1976; Till *et al.*, 1982, 1983).

Accordingly, Bruner and associates (1983b, 1987a, 1988) investigated potential involvement of immune mechanisms in MCTP-induced pneumotoxicity. MCTP-treated rats cotreated with the immunosuppressants cyclosporin A or antilymphocyte serum were not protected from pulmonary injury. Adoptive transfer of sensitized lymphocytes to MCTP-treated rats did not change the time of onset or severity of lung lesions (Bruner *et al.*, 1987a). Complement activation was not detected in rats after administration of MCTP, and complement depletion with cobra venom factor did not alter significantly the character or severity of lesions. Lastly, addition of MCTP to serum *in vitro* failed to activate complement (Bruner *et al.*, 1988). From these series of experiments, Bruner and colleagues concluded that the immune system did not play a significant role in MCTP-induced pulmonary injury.

4. Phagocytic cells

Pulmonary inflammatory cell infiltrates after exposure to MCT(P) have been described in detail (Takeoka *et al.*, 1962; Sugita *et al.*, 1983; Stenmark *et al.*, 1985; Czer *et al.*, 1986), but studies of cellular function after MCTP intoxication are few. Large numbers of intraalveolar macrophages were observed histologically following treatment of rats with MCT (Sugita *et al.*, 1983). Macrophage

cell numbers correlated positively with severity of pulmonary hypertension and dose of MCT administered. Originally described as "abnormal macrophages" due to their large size and foamy appearance, these cells are more appropriately described as cytologically activated (DeNicola *et al.*, 1981).

Stenmark and colleagues (1985) treated rats with a sc injection of MCT and performed bronchoalveolar lavage at 1, 2 and 3 weeks thereafter. The total nucleated cell counts of lavage fluid from rats treated with MCT were significantly increased at 3 weeks. Cytologic preparations of concentrated lavage were characterized by increased numbers of segmented neutrophils at 3 weeks and of large, "abnormal macrophages" similar to those described by Sugita *et al.* (1983) at 2 and 3 weeks. Increased hexosaminidase activity in the bronchoalveolar lavage fluid obtained at 3 weeks suggested that the leukocytes were "activated" (Stenmark, *et al.*, 1985).

Dahm and associates (1986) treated rats with a single iv injection of MCTP and performed bronchoalveolar lavage at several times later. The lavage fluid of MCTP-treated rats was characterized by significantly decreased numbers of macrophages at 5 days and by increased numbers of segmented neutrophils at days 7, 10 and 14. In addition, the bronchoalveolar lavage fluid of MCTP-treated rats had increased numbers of eosinophils at day 10 and significantly more lymphocytes than controls at day 14. The ability of cells in the lavage to cause superoxide (O_2-) production was examined. At 7, 10 and 14 days after MCTP administration, the O_2- production of lavaged cells was significantly decreased

compared to control values. The investigators proposed that the lavage fluid cell types may have been down-regulated following exposure to MCTP (Dahm *et al.*, 1986). An alternative explanation would be that the cells obtained by bronchoalveolar lavage had released O_2- *in vivo* prior to or during the lavage procedure and were incapable of O_2- production at the time of examination (Ganey, 1986). Such release *in vivo* might cause lung injury in MCTP-treated rats.

5. Reactive oxygen metabolites

Reactive oxygen metabolites cause toxicity in several models of lung injury (Fantone and Ward, 1982; Till *et al.*, 1982, 1983). Bruner and associates (1987b) investigated the involvement of toxic oxygen species in MCTP-induced lung injury by cotreating rats with one of several agents known to prevent formation of oxygen metabolites or inactivate reactive oxygen species once they are generated. Administration of deferoxamine mesylate, polyethylene-coupled catalase or dimethyl sulfoxide failed to protect rats from MCTP-induced pneumotoxicity. Hence, Bruner and colleagues (1987b) concluded toxic oxygen radicals may not be major factors in the pathogenesis of MCTP-induced pneumotoxicity.

6. Leukotrienes

The metabolism of arachidonic acid via the lipoxygenase pathway results in the production of several leukotrienes (LTs) which are important mediators of biologic events. Some of the effects attributed to leukotriene mediators include leukocyte chemotaxis, increased vascular permeability and pulmonary vasoconstriction (Samuelsson, 1983; Yokochi *et al.*, 1982; Burghuber *et al.*, 1985;

Voelkel *et al.*, 1984). The similarities between the pneumotoxic response in rats treated with MCT and the biologic effects of LTs have moved several investigators to explore the involvement of LTs as mediators in models of lung injury and pulmonary hypertension.

Stenmark *et al.* (1985) identified increased concentrations of slow-reacting substance of anaphylaxis, LTB_4 and LTC_4 in the bronchoalveolar lavage fluid from rats treated with MCT. Administration of diethylcarbamazine (DEC), an inhibitor of LT synthesis (Mathews and Murphy, 1982), decreased pulmonary inflammation and attenuated right ventricular hypertrophy after MCT treatment (Stenmark *et al.*, 1985). In contrast, DEC cotreatment did not afford a similar degree of protection to rats treated with MCTP, the putative toxic metabolite of MCT (Bruner and Roth, 1984). Although lung injury was slightly attenuated at day 7, there was no protection from lung injury or right heart hypertrophy at day 14.

The results of these studies are in contrast, and the reason for the disparity is unclear. One explanation suggested is that the dosing regimen of DEC may be critical. Stenmark *et al.* (1985) gave DEC prior to MCT, but Bruner and Roth (1984) administered DEC 3 days after MCTP. Alternatively, DEC may interact with MCT to prevent its conversion to pyrrolic metabolites capable of causing toxicity. In addition, the administration of DEC in doses given by Stenmark and colleagues (1985) decreased weight gain in rats (Morganroth *et al.*, 1985) and may therefore have inhibited the development of MCT-induced lung injury and pulmonary hypertension. More studies of LTs and MCT(P) and use of specific LT

inhibitors are necessary to clarify the potential involvement of LTs in these models of pneumotoxicity and pulmonary hypertension.

7. Polyamines

A single injection of MCT has been shown to activate spermidine/spermine acetyl transferase, an enzyme that regulates spermidine acetylation, in a dose and time dependent manner (Orlinska *et al.*, 1989). The increased activity of this enzyme was accompanied by a significant increase in the lung content of acetylated polyamine intermediates, suggesting that a single administration of MCT causes not only *de novo* synthesis of polyamines but also increases polyamine synthesis by interconversion pathways.

Treatment of rats with α-difluromethylornithine (DFMO), an irreversible inhibitor of L-ornithine decarboxylase and polyamine synthesis, attenuated the vascular hyperresponsiveness of isolated lungs from rats treated with MCT (Gillespie *et al.*, 1985a). Similarly, DFMO administration decreased lung levels of putrescine and spermidine and prevented increases in lung wet-to-dry weight ratio, pulmonary vascular medial hypertrophy and compensatory right sided cardioventricular hypertrophy (Olson *et al.*, 1985). These results suggest that polyamines may be involved in pulmonary vascular hyperresponsiveness and lung injury in rats treated with MCT.

Supplementation of exogenous ornithine, a polyamine precursor, resulted in increased concentrations of the lung polyamines, putrescine and spermidine and attenuated the protective effect of DFMO in preventing pulmonary

hypertension and right ventricular hypertrophy, suggesting that polyamines are critical for the delayed and sustained increases in pulmonary arterial pressure that occur after administration of MCT (Olson *et al.*, 1989).

8. Biogenic amines

The interest in 5HT as a mediator of pulmonary hypertension in animals exposed to MCT(P) is based upon the following observations. 5HT causes pulmonary vasoconstriction (Rickaby *et al.*, 1980; Tucker and Rodeghero, 1981), and circulating 5HT may increase pulmonary artery pressure *in vivo* (Ozdemir *et al.*, 1972). Mast cells, capable of releasing large granular stores of 5HT, are observed in increased numbers in lungs of rats treated with MCT (Takeoka *et al.*, 1962; Turner and Lalich, 1965). Platelets store 5HT in dense granules (Holmsen *et al.*, 1969), and platelet thromboembolic lesions are observed histologically in lungs of rats treated with MCT(P) (Merkow and Kleinerman, 1966; Chesney *et al.*, 1974a, Hurley and Jago, 1975; Heath and Smith, 1978). Antibody-mediated thrombocytopenia attenuates MCTP-induced pulmonary hypertension in rats (Hilliker *et al.*, 1984b; Ganey *et al.*, 1988; White *et al.*, 1989). Endothelial cells remove 5HT from the circulation and metabolize it to inactive products. Pulmonary endothelial cells are biochemically and histologically altered after exposure to MCT(P) (Molteni *et al.*, 1985, 1986ab; Reindel *et al.*, 1990), and isolated perfused lungs from rats treated with MCT(P) have decreased ability to remove 5HT from circulation (Hilliker *et al.*, 1982, 1983a).

Although there are ample reasons to suggest that 5HT may cause pulmonary hypertension after MCT(P) treatment, the evidence does not confirm its involvement as a mediator in disease progression. The concentration of 5HT in serum and platelets from rats fed *C. spectabilis* was not significantly different from control values (Kay *et al.*, 1968). Both chemical sympathectomy with 6-hydroxydopamine and inhibition of 5HT synthesis with *p*-chlorophenylalanine decreased the magnitude of right ventricular hypertrophy after a single injection of MCT. However, these treatments did not reduce the pulmonary vascular changes associated with MCT (Tucker *et al.*, 1983), and the results were difficult to interpret due to weight loss in animals treated with *p*-chlorophenylalanine alone. In addition, Tucker and colleagues (1983) did not confirm the depletion of 5HT following treatment. Administration of the 5HT uptake inhibitor, imipramine, failed to alter vascular responsiveness of isolated rat lungs in MCTP-treated rats (Hilliker and Roth, 1985a). Cotreatment of MCTP-intoxicated rats with either metergoline or ketanserin, two 5HT receptor antagonists, also did not prevent pneumotoxicity or cardioventricular hypertrophy (Ganey *et al.*, 1986). Hence, it must follow that 5HT does not contribute significantly in the pathogenesis of MCT(P)-induced lung injury and pulmonary hypertension.

9. Prostanoids

Arachidonic acid metabolism via the cyclooxygenase pathway results in production of prostacyclin (PGI_2), an inhibitor of platelet aggregation and a vasodilator. Increased production of PGI_2 is an indication of endothelial cell injury.

Treatment of rats with MCT causes activation of the cyclooxygenase pathway (Stenmark *et al.*, 1985). Increased amounts of 6-keto prostaglandin $F_{1\alpha}$, a metabolite of PGI_2, were detected in the bronchoalveolar lavage fluid of rats at 1 and 3 weeks after treatment with MCT. Molteni *et al.* (1984) observed increased concentrations of 6-keto prostaglandin $F_{1\alpha}$ in lung homogenates from rats at 6 and 12 weeks after chronic administration of MCT in drinking water. Similarly, bovine endothelial cells released increased concentrations of 6-keto prostaglandin $F_{1\alpha}$ in culture media after treatment with MCTP (Reindel *et al.*, 1991). In contrast, Ganey and Roth (1987a) did not detect increased amounts of 6-keto prostaglandin $F_{1\alpha}$ in the perfusate of isolated lungs from rats treated with MCTP.

Thromboxane A_2 is a potent prostanoid vasoconstrictor and mediator of platelet aggregation and vascular permeability that is produced by platelets and endothelial cells. Like PGI_2, increased thromboxane production is also a marker of endothelial cell injury. At 3 weeks after treatment with MCT, the bronchoalveolar lavage fluid of rats contained increased thromboxane B_2 (TxB_2), a metabolite of thromboxane A_2. TxB_2 was measured in isolated perfused lungs from MCTP-treated rats (Ganey and Roth, 1987a, 1988). At 7 days after MCTP intoxication, there was no difference in release of thromboxane from the isolated lung preparations. However at 14 days, a time when pulmonary hypertension is well established, isolated lungs from MCTP-treated rats released significantly more TxB_2 than controls.

The administration of indomethacin, an inhibitor of cyclo-oxygenase metabolism, attenuated prostanoid synthesis but did not prevent pulmonary hypertension in rats treated with MCT (Stenmark *et al.*, 1985). These results suggest PGI_2 or TxA_2 are not major mediators of lung injury or pulmonary hypertension in this model of chronic injury. In addition, indomethacin treatment prevented the increase in TxB_2 in bronchoalveolar lavage fluid but did not attenuate or prevent the pulmonary hypertension due to MCTP treatment (Stenmark *et al.*, 1985). Administration of L640-035, a TXA_2 receptor antagonist, or Dazmegrel, an inhibitor of TXA_2 synthetase, to rats treated with MCTP, did not attenuate the vascular leak or right cardioventricular hypertrophy associated with the toxicant (Ganey and Roth, 1986). These results also suggest that TXA_2 is not required for the cardiopulmonary lesions that occur after MCTP-treatment in rats.

In experiments of acute pulmonary injury in dogs, administration of 30 mg MCT/kg iv caused thrombocytopenia, pulmonary platelet deposition and pulmonary hypertension. In this model, increases in pulmonary vascular resistance were biphasic and occurred in a parallel manner with increases in plasma thromboxane concentration (Czer *et al.*, 1986). The administration of exogenous PGI_2 attenuated the thrombocytopenia, pulmonary platelet sequestration, pulmonary hypertension and thromboxane production.

A more detailed description of the prostanoids and their involvement in MCT(P)-induced pneumotoxicity and pulmonary hypertension will follow in a review of the hemostatic system. Emphasis will be placed on PGI_2 and

endothelial cell function and thromboxane and platelet function as they relate to this model.

V. Summary of the Hemostatic System

A. Functions

The hemostatic system is a physiologic network that incorporates the vasculature, platelets and the coagulation and fibrinolytic systems in an intricate array of positive and negative feedback mechanisms (Thompson and Harker, 1983; Crutchley, 1987). The functions of the hemostatic system are to prevent exsanguination following injury and to maintain blood fluidity. (Hassouna, 1988).

B. Components and Their Response to Injury

1. Vessels

Endothelial cell disruption causes rapid vasoconstriction mediated by a combination of the α-adrenergic system and the secretion of serotonin and thromboxane A_2 from activated platelets (Jaffe, 1983; Bonner, 1988). In addition, vessels adjacent to those injured will constrict by reflex stimulation. The benefits of vasoconstriction include increased contact activation of platelets and coagulation proteins and reduced blood flow (Thompson and Harker, 1983).

2. Platelets

Circulating platelets are anucleate fragments of megakaryocytes that are required for normal hemostasis. They have a unique cytoskeletal structure composed of a dual membrane system used to mobilize the contents of their 3 types

of storage granules: α granules, dense granules and lysosomes (Thompson and Harker, 1983). This network of connecting channels consists of an open canalicular system and a dense tubular system. The α granules contain platelet factor 4, platelet derived growth factor, *B*-thrombomodulin, fibronectin, fibrinogen, albumin and coagulation Factors V and VIII (Stenberg and Bainton, 1986). ADP, calcium, serotonin and phosphates are contained in the dense granules (Huang and Detwiler, 1986). The lysosomes contain a variety of enzymes (Stenberg and Bainton, 1986).

Normally, platelets do not adhere to the vascular endothelium. However, after vascular injury, they perform four functions necessary for adequate hemostasis (Thompson and Harker, 1983). Platelets routinely seal minor defects in endothelium, thus maintaining vascular integrity. They adhere to sites of denuded endothelium and form the initial hemostatic plug. Thromboxane A_2 generated by platelet phospholipases and ADP released from adherent platelets cause more platelets to adhere and aggregate at the site of injury. Platelet factor 3, a phospholipid from platelet membranes, accelerates coagulation by serving as a cofactor for the intrinsic and common pathways. Fibrin, formed by the local generation of thrombin, stabilizes the platelet plug. Lastly, platelets promote vascular healing by releasing specific mitogens from α granules (Thompson and Harker, 1983).

3. The coagulation system of peripheral blood

Coagulation, or the conversion of the soluble plasma protein fibrinogen to an insoluble fibrin network, is the normal response of the hemostatic system to vascular injury (Hassouna, 1988). Coagulation depends upon a system of

soluble blood zymogens that, in the presence of cofactors and calcium may be sequentially activated to proteases. This autocatalytic system, in turn, converts prothrombin to thrombin, the only enzyme capable of converting fibrinogen to fibrin (Bloom, 1990). Coagulation may be initiated by either of two closely linked enzyme cascades called the extrinsic and intrinsic pathways. Both pathways join in a common pathway that culminates in fibrin formation (Jackson and Nemerson, 1980).

The extrinsic pathway is also called the tissue factor pathway (Figure 6). Tissue factor, an inducible cell surface protein, reacts with Factor VII, a zymogen, and converts it to Factor VIIa, a serine protease. Factor VIIa is then capable of activating Factor X, the first component of the common pathway. Tissue factor also acts to bridge the extrinsic and intrinsic pathways since, in addition to Factor VIIa-mediated activation of Factor X, the TF/Factor VIIa complex can activate Factor IX, a zymogen of the intrinsic pathway (Bloom, 1990; Benditt and Schwartz, 1988).

The intrinsic pathway of coagulation is initiated by contact activation of the zymogen, Factor XII (Figure 6). Contact activation occurs in the absence of calcium, involves a surface mediated conformational change and can be induced by exposure of Factor XII to high molecular weight kininogen, prekallikrein or other specific negatively charged biologic surfaces (Colman *et al.*, 1987; Bloom, 1990). Factor XIIa may then activate Factor XI to Factor XIa, beginning a cascade effect of zymogen activation that extends to and includes Factor IX.

Figure 6: Schematic representation of coagulation pathways within a blood vessel. In the intrinsic pathway, coagulation is initiated by exposure of circulating FXII to negatively charged subendothelial surfaces. In the extrinsic pathway, production of tissue factor by stimulated endothelial cells converts FVII to its active form. Both intrinsic and extrinsic pathways join in a common pathway that results in conversion of fibrinogen to fibrin. Thrombosis occurs when an insoluble fibrin network physically impedes blood flow. Tissue factor, TF; calcium, Ca^{+2}, platelet factor 3, PF_3.

Intrinsic Pathway
XII
XIIa
XI
XIa
IX
Ca+2
IXa
VIIIa
X
Ca+2
PF3
Xa
Va, PF3
Ca+2
Thrombin
Prothrombin
Fibrinogen
Fibrin (soluble)
XIIIa
Ca+2
Fibrin (insoluble)
Thrombosis
Common Pathway
Ca+2
VIIa
X
VII
TF
Extrinsic Pathway

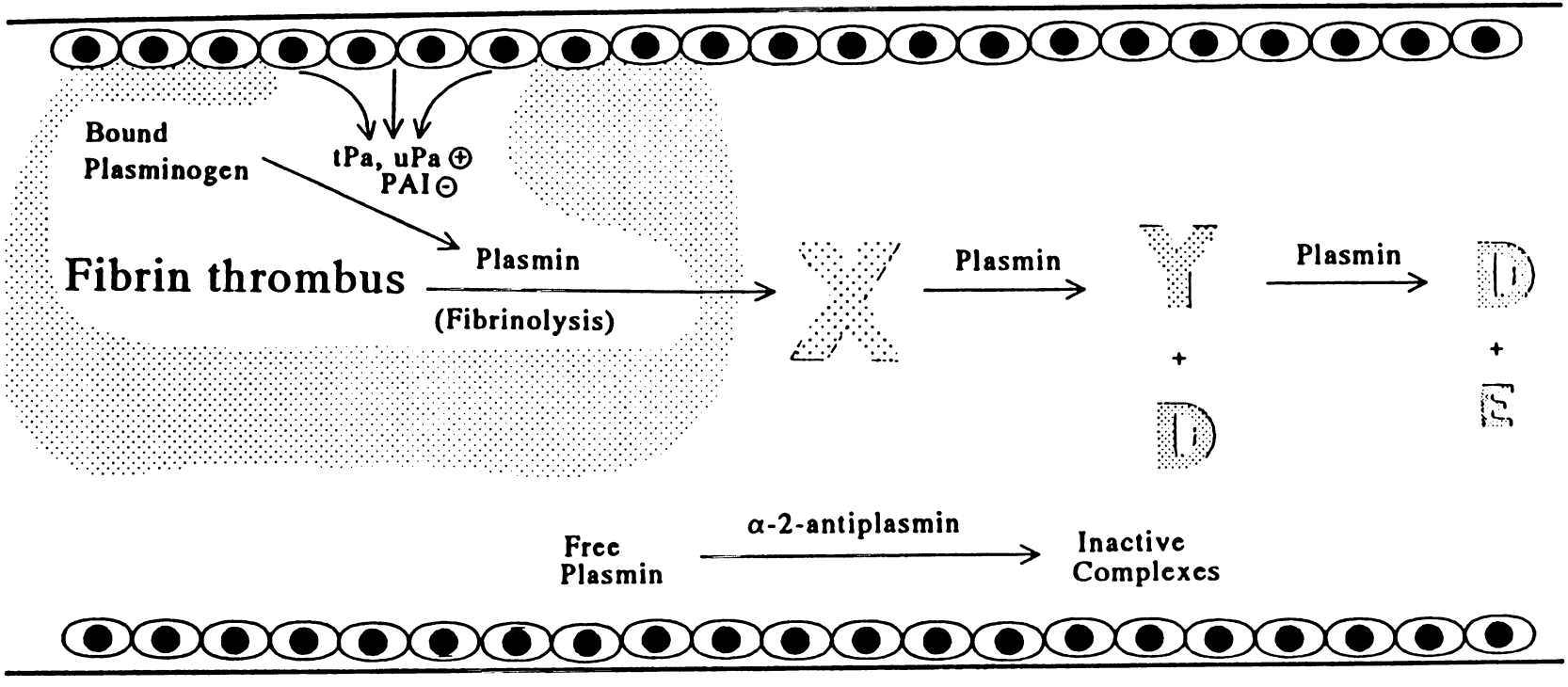
The common pathway of coagulation begins with the activation of Factor X to Factor Xa, a process induced by either Factor VIIA from the extrinsic pathway or Factor IXa and cofactors of the intrinsic system (Figure 6). Factor Xa in the presence of calcium, Factor V and phospholipid may activate prothrombin (Factor II) to thrombin (Factor IIa). Thrombin cleaves the substrate fibrinogen (Factor I) to monomers which polymerize to form an insoluble fibrin network. The network is stabilized or cross-linked by Factor XIII (Bonner, 1988).

4. The fibrinolytic system of peripheral blood

The fibrinolytic system is responsible for maintaining blood fluidity by the chemical digestion of fibrin (Takada *et al.*, 1990) (Figure 7). In addition to restoring blood flow in thrombosed vessels, the fibrinolytic system is involved in numerous biologic processes, including neovascularization, macrophage activation, ovulation, embryo implantation, tissue repair, breast involution and neoplasia (Collen, 1980; Erickson *et al.*, 1985; Bachman and Kruithof, 1984).

Plasminogen, a zymogen of peripheral blood and many other fluids, is a single chain glycoprotein of molecular weight 90,000 (Thompson and Harker, 1983). Plasminogen is converted to plasmin, an enzyme with trypsin-like activity, by the cleavage of an arg_{560}/val_{561} bond. Plasmin, a two chain molecule linked by disulfide bonds, is an active serine protease that will degrade readily several substrates, including fibrin, fibrinogen, Factor V and Factor VIII (Thompson and Harker, 1983; Curriden *et al.*, 1988).

Figure 7: Schematic representation of fibrinolysis within a blood vessel. Tissue plasminogen activator, tPa; urokinase-like plasminogen activator, uPa; plasminogen activator inhibitor, PAI; fibrin degradation products, X, Y, D and E.



Fibrinolysis
(Thrombolysis)

Substances that cause conversion of plasminogen to plasmin are termed plasminogen activators (Thompson and Harker, 1983; Takada *et al.*, 1990; Curriden *et al.*, 1988; Gimbrone and Bevilacqua, 1988). Intrinsic plasminogen activators are present in the blood and include Factor XII, prekallikrein, and high molecular weight kininogen. Extrinsic plasminogen activators are produced by cells, can be released into the blood under certain circumstances and include substances like tissue plasminogen activator from endothelial cells and urokinase produced by renal epithelial cells. Finally, exogenous activators of plasminogen include recombinant tissue plasminogen activator and streptokinase.

Protein C, a vitamin K-dependent zymogen of blood, also has profibrinolytic activity. When activated, Protein C will degrade Factors Va and VIIIa and inactivate plasminogen activator inhibitor-1, the inhibitor of endothelial cell-derived tissue plasminogen activator (Esmon, 1988).

Once active, plasmin may digest fibrin thrombi and restore blood flow. In this process, plasmin cleaves the carboxy terminal portion of crosslinked fibrin α chains to form isolated fibrin strands. These isolated strands are further degraded to fibrin degradation products (FDPs), also called fragments X and Y and smaller fragments D and E, that are readily removed from circulation by the reticuloendothelial system (Thompson and Harker, 1983).

The control of fibrinolysis occurs at two levels, inhibition of plasmin activity and inhibition of plasminogen activation. The only physiologically significant inhibitor of free plasmin in the circulation is α-2-antiplasmin which binds

rapidly to the lysine binding sites of plasmin, thereby neutralizing the activity of the molecule (Takada *et al.*, 1990). Conversion of plasminogen to plasmin can be blocked by several plasminogen activator inhibitors (PAIs) including PAI-1 from endothelial cells, PAI-2 from macrophages and monocytes, PAI-3 from urine, and protease nexin from fibroblasts. The rapidly acting PAI-1 is responsible for neutralizing tissue plasminogen activator thus preventing conversion of the zymogen, plasminogen, to the serine protease, plasmin (Curriden *et al.*, 1988; Takada *et al.*, 1990).

C. Problems of Inadequate or Excessive Function of the Hemostatic System

1. Hemorrhage

Hemorrhage may be caused by a plethora of mechanisms, most of which are associated with inadequate function of the hemostatic system. Malfunction in any one of the components of the hemostatic system may result in an abnormal tendency to bleed. Blood vessel disorders, quantitative and qualitative defects in platelets, protein clotting factors and fibrinolytic proteins may result in spontaneous bleeding or hemorrhage after trauma. These defects may be hereditary or acquired and are described in detail elsewhere (Bonner, 1988; Thompson and Harker, 1983).

2. Thrombosis

Thrombosis is defined as the formation of a solid, blood-derived mass within the heart or vessels of a living animal (Williams, 1983a; Thompson and

Harker, 1983). Thrombosis may occur within vessels of any size and at any location and may be the result of a myriad of abnormalities in hemostasis, most of which are associated with excessive function of the coagulation system. Vascular lesions, changes in blood flow, excessive procoagulant activity and defective fibrinolysis are associated with thrombosis. In addition, depletion of antithrombin III and Proteins C and S, the naturally occurring inhibitors of coagulation, may also result in thrombosis. Thrombosis may be caused by hereditary or acquired defects in hemostasis and has been reviewed in detail elsewhere (Williams, 1983a; Colman *et al.*, 1987; Bick and Kunkel, 1992).

VI. Pulmonary Endothelial Cells and Coagulation

The endothelium synthesizes and releases a vast array of factors that participate in platelet aggregation, coagulation and fibrinolysis. Under physiologic conditions, endothelial cell anticoagulant properties exist in slight excess compared to procoagulant properties. The net effect is to provide a nonthrombogenic surface for blood transport (Crutchley, 1987; Ge *et al.*, 1991). The procoagulant capabilities represent a unique mechanism to localize clot formation at sites of injured vessels (Stern *et al.*, 1983; Nawroth and Stern, 1986). The discussion that follows will emphasize the major anticoagulant, procoagulant and fibrinolytic properties of endothelial cells.

A. Anticoagulant Properties

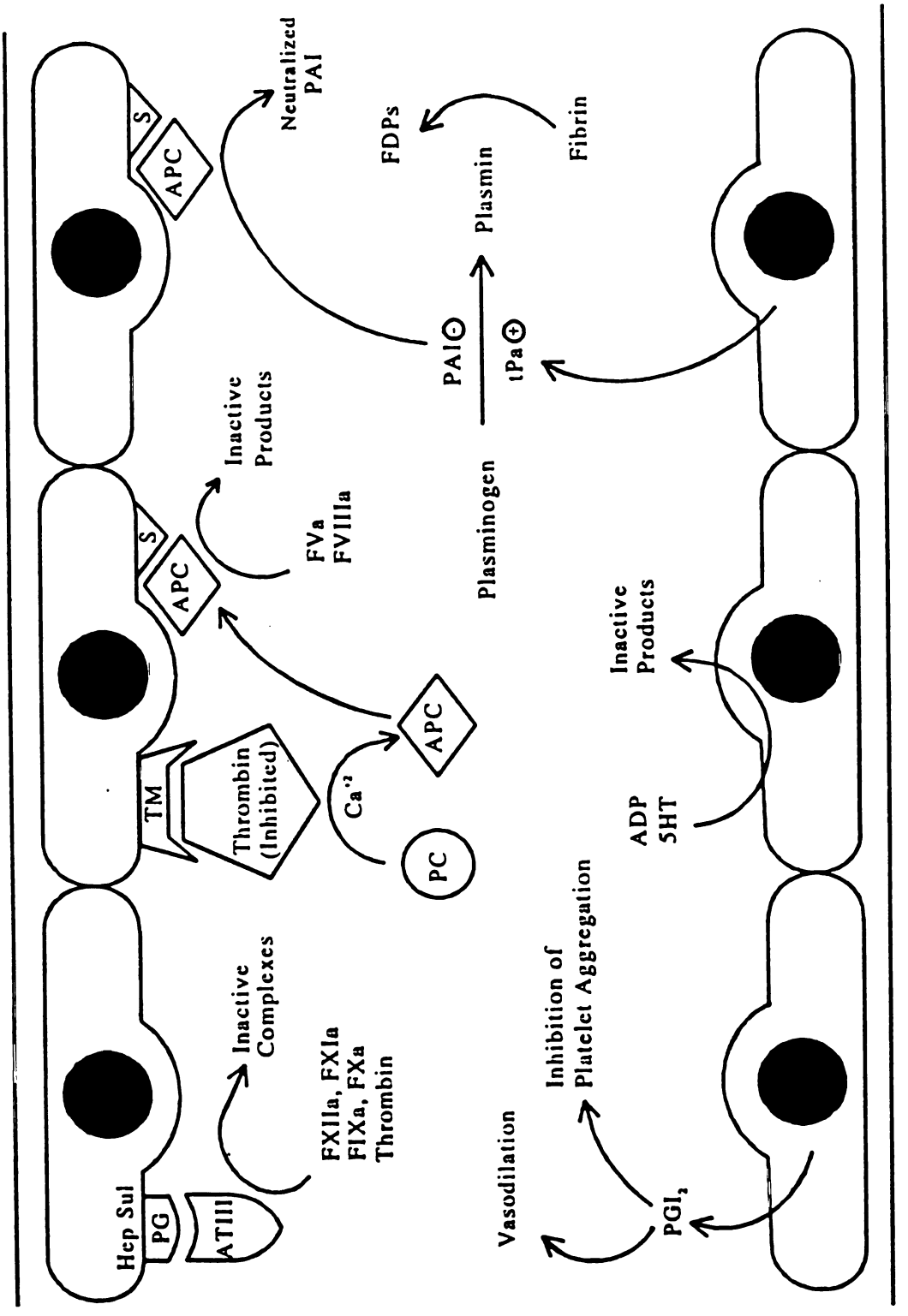
1. Heparan sulfate proteoglycan and antithrombin III

Antithrombin III (ATIII) is the major glycoprotein inhibitor of blood coagulation. It covalently binds to serine proteases such as thrombin (Factor IIa), Factor XIIa, Factor XIa, Factor IXa and Factor Xa and blocks their activities (Harpel, 1987). Heparin, a cofactor for ATIII, potentiates the binding of ATIII to serine proteases and accelerates their inactivation (Marcum and Rosenberg, 1988). Heparan sulfate proteoglycan is a heparin-like molecule present on the surface of pulmonary endothelial cells. Like heparin, heparan sulfate proteoglycan binds to ATIII and potentiates the inactivation of thrombin and other serine proteases by ATIII (Busch and Owen, 1982; Preissner, 1988; Marcum and Rosenberg, 1988) (Figure 8).

2. Thrombomodulin and Protein C

Thrombomodulin is a high affinity protein receptor on the endothelial cell surface that binds to thrombin and inhibits the procoagulant activity of the bound thrombin (Esmon *et al.*, 1982ab; Esmon, 1987; Preissner, 1988) (Figure 8). Protein C is a vitamin K-dependent, serine protease that inactivates active Factors V and VIII and potentiates fibrinolysis by degrading endothelial-derived inhibitors of plasminogen activation (Esmon, 1987, 1988). The activation of Protein C by thrombin is accelerated 20,000 fold by the binding of thrombin to thrombomodulin. Protein S, a vitamin K-dependent glycoprotein, is an important cofactor that is required for complete activation of Protein C on endothelial cell surfaces.

Figure 8: Schematic representation of the major anticoagulant properties of vascular endothelial cells. This figure depicts a cross section of a typical vessel lined by endothelial cells. Heparan sulfate proteoglycans (Hep Sul PG) present on the endothelial surface potentiate inactivation of several different serine proteases by antithrombin III (ATIII). The membrane protein receptor, thrombomodulin (TM), inactivates the procoagulant enzyme, thrombin, and the TM/thrombin complex, in the presence of calcium (Ca^{+2}), converts Protein C (PC) to activated Protein C (APC). In the presence of its cell surface cofactor, Protein S (S), APC inactivates factors Va and VIIIa and neutralizes plasminogen activator inhibitor (PAI). Prostacyclin (PGI_2), secreted by endothelial cells, causes vasodilation and inhibition of platelet aggregation. Endothelial cells metabolize adenosine diphosphate (ADP) and serotonin (5HT), mediators of platelet activation and aggregation, to inactive products thereby limiting platelet thrombus formation and subsequent coagulation. Tissue plasminogen activator (tPa), secreted by endothelial cells, causes conversion of the zymogen, plasminogen, to the active enzyme, plasmin. Fibrin is degraded by plasmin to fibrin degradation products (FDPs) that may be phagocytosed as blood flow is restored. Signs represent activation (+) or inactivation (-).



Endothelial Cell Anticoagulant Properties

Results of recent studies indicate that Protein S can also be synthesized by endothelial cells *in vitro* (Stern *et al.*, 1988).

3. Prostacyclin

Prostacyclin (PGI_2) is a potent vasodilator and inhibitor of platelet aggregation that is produced by endothelial cells (Crutchley, 1987; Ge *et al.*, 1991) (Figure 8). PGI_2 inhibits platelet aggregation by increasing the intracellular concentration of cyclic AMP in platelets (Gorman *et al.*, 1977; Tateson *et al.*, 1977). In addition, metabolism by platelets of phosphoinositides is inhibited by PGI_2 (Watson *et al.*, 1984). The release of PGI_2 from pulmonary endothelium *in vitro*, a calcium dependent process, can be stimulated by administration of bradykinin and is inhibited by calcium channel blockers, agents that block calmodulin, dexamethasone and hydrocortisone (Crutchley *et al.*, 1983, 1985; Suttorp *et al.*, 1985).

4. Metabolic capability

The pulmonary endothelium possesses the ability to metabolize adenosine nucleotides (ATP and ADP) to AMP or inosine via nucleotidases (Crutchley *et al.*, 1978; Cooper *et al.*, 1979; Ryan *et al.*, 1985) (Figure 8). Because aggregating platelets may release ADP that may cause platelet recruitment and intensify aggregation, the ability to remove ADP from the circulation must be considered a protective mechanism of endothelium to decrease or prevent platelet aggregation and coagulation. In addition, the pulmonary endothelium is able to remove and metabolize other proaggregatory biogenic amines such as serotonin and epinephrine which also stimulate platelets and cause vasoconstriction in some species.

B. Procoagulant Properties

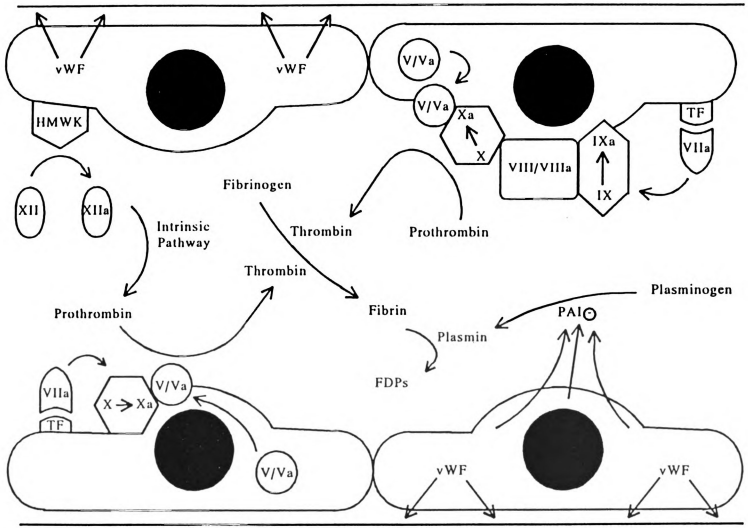
1. Tissue factor

Tissue factor (TF), also called factor 3 or tissue thromboplastin, is a membrane-bound, single chain glycoprotein found in mammalian cells. It is the main factor associated with endothelial cell procoagulant activity (Nemerson, 1988; Rapaport, 1990; Stern *et al.*, 1988). TF initiates hemostasis via both the extrinsic and intrinsic pathways of coagulation (Figure 9). In the extrinsic pathway, endothelial cell TF forms complexes with circulating Factor VII and calcium to activate factor X. In addition, the TF/Factor VIIa complex can initiate coagulation via the intrinsic pathway by activating factor IX to IXa. Once activated, both extrinsic and intrinsic pathways join in a common pathway of coagulation and result in the formation of fibrin. Under normal conditions, endothelial cells synthesize little TF, and anticoagulant properties slightly exceed procoagulant properties, thus maintaining a fluid environment. When activated by specific stimuli such as endotoxin, tumor necrosis factor or thrombin, endothelial cells increase TF activity markedly (Bevilacqua *et al.*, 1984; Jaffe, 1987).

2. Factor V

Endothelial cells synthesize and release factor V, a cofactor for the common pathway of coagulation (Cerveny *et al.*, 1984) (Figure 9). Synthesis of factor V is believed the second important mechanism for endothelial cell procoagulant activity (Gertler and Abbott, 1992). Factor V activation is increased following exposure of endothelium to interleukin 1, endotoxin, and homocysteine and after

Figure 9: Schematic representation of the major procoagulant properties of vascular endothelial cells. This figure depicts a cross section of a typical vessel lined by endothelial cells. Tissue factor (TF), a membrane bound glycoprotein, initiates coagulation by activation of factor VII to VIIa (extrinsic pathway). In turn, the TF/VIIa complex activates factor X to factor Xa. The TF/VIIa complex may also activate factor IX to IXa (intrinsic pathway). Factor V/Va, a cofactor of the common pathway of coagulation, is secreted by endothelial cells. Endothelial cells provide specific membrane receptors for factors V/Va, VIII, IX, IXa, X and Xa. They also activate FXII to XIIa via high molecular weight kininogen (HMWK). von Willebrand Factor (vWF) is secreted basolaterally where it attaches endothelial cells to the basal lamina and serves as an adhesion molecule for platelets. Thrombin, generated by any of the mechanisms described, converts the soluble plasma protein, fibrinogen, to the insoluble protein, fibrin. Under normal conditions, the enzyme plasmin will digest fibrin to fibrin degradation products (FDPs). Plasminogen activator inhibitor (PAI), secreted by endothelial cells, blocks conversion of plasminogen to plasmin thereby maintaining a fibrin network. Signs represent activation (+) or inactivation (-).



Endothelial Cell Procoagulant Properties

mechanical injury (Gertler and Abbott, 1992). In addition to secretion of factor V into plasma, endothelial cells possess specific surface binding sites for factor V, thus providing another method to localize hemostasis on endothelial cell surfaces (Maruyama *et al.*, 1984).

3. Coagulation factor binding sites

In addition to synthesis of TF and factor V, endothelial cells localize coagulation on their surface by providing specific membrane receptors for high molecular weight kininogen and factors VIII, IX, IXa, X, and Xa (Rodgers, 1988; Schmaier *et al.*, 1987; Nawroth *et al.*, 1986; Stern *et al.*, 1987; Rao *et al.*, 1987) (Figure 9). Endothelial cells also activate factor XII to XIIa on their surface via high molecular weight kininogen (Jaffe, 1987).

4. von Willebrand Factor

von Willebrand Factor (vWF) is an adhesion molecule produced in endothelial cells and megakaryocytes (Giddings, 1986; Wagner and Bonfanti, 1991). Normally, vWF is found in Weibel-Palade bodies of endothelial cells and to a lesser extent in platelet α granules and plasma. Endothelial cells secrete vWF basolaterally, where it is used to attach the endothelium to the vascular wall and for attachment of platelets to sites of damaged endothelium (Figure 9). Histamine, thrombin and fibrin are endothelial cell secretagogues that cause disruption of endothelial cell junctions and the release of vWF (Levine *et al.*, 1982; Ribes *et al.*, 1987; Hamilton and Sims, 1987).

VII. Pulmonary Endothelial Cells and Fibrinolysis

The pulmonary endothelium has a very large surface area and is the only capillary bed to receive the complete cardiac output (Crutchely, 1987). In addition to exerting a tight control over anticoagulant and procoagulant properties, the pulmonary endothelium modulates fibrinolysis by producing factors that cause or prevent formation of the enzyme, plasmin.

A. Plasminogen Activators

Endothelial cells *in vitro* and *in vivo* synthesize and release 2 activators of plasminogen, tissue plasminogen activator (tPa) and urokinase-like plasminogen activator (uPa) (Gerard and Meidell, 1989; Saksela and Rifkin, 1988). Both activators are synthesized as single chain molecules with little or no activity. When converted to their two-chain forms, each may convert readily the zymogen plasminogen to the active enzyme plasmin (Gerard and Meidell, 1989; Saksela and Rifkin, 1988). Once secreted, tPa binds to fibrin. Because fibrin binding increases the catalytic activity of tPa for plasminogen activation, tPa is considered the plasminogen activator of greatest physiologic significance (Ge *et al.*, 1991). Specific stimuli may cause endothelial cells to increase secretion of tPa. Phorbol ester, cytokines, growth factors, hormones, histamine, bradykinin and shear stress cause increased production of tPa by endothelium (Gerard and Meidell, 1989; Saksela and Rifkin, 1988; Erickson *et al.*, 1985; Dichek and Quertermous, 1989). Increased production and release of tPa relative to PAI production causes plasminogen activation. The resultant plasmin will degrade fibrin thrombi and, if unchecked,

excessive plasmin may cause a systemic lytic effect resulting in hemorrhage. Production and secretion of plasminogen activators may be considered another anticoagulant property of endothelium.

B. Plasminogen Activator Inhibitor

Endothelial cells produce plasminogen activator inhibitor-1 (PAI-1), a single-chained glycoprotein capable of rapidly inhibiting both forms of tPa and single-chained uPa (Loskutoff, 1988). In short, PAI-1 acts as an anti-activator of plasminogen by preventing conversion of the zymogen to active enzyme. PAI-1 is secreted in an active form but is quickly converted to a latent or inactive form by unknown mechanisms. Negatively charged phospholipids or detergents may cause the reactivation of latent PAI-1 (Saksela and Rifkin, 1988; Levin and Santell, 1987). Endothelial cells perturbed by endotoxin or interleukin-1 synthesize and release more PAI-1 (Colucci *et al.*, 1985; Bevilacqua *et al.*, 1986). Increased production of PAI-1 relative to plasminogen activators results in decreased fibrinolytic activity and an enhanced procoagulant condition.

VIII. Evidence for Involvement of the Hemostatic System in MCTP-induced Pneumotoxicity and Pulmonary Hypertension.

A. Evidence for Vascular Hyperreactivity

The concept that vascular remodeling contributes to the pulmonary hypertension that occurs after MCT(P) administration in rats is well accepted. The potential contribution of enhanced contractility or vasoconstriction to the initiation

or maintenance of pulmonary hypertension after exposure to these toxicants remains a topic of debate (Hilliker and Roth, 1985a; Gillespie *et al.*, 1986).

Gillespie and associates (1986) investigated vascular responsiveness in isolated, buffer-perfused lungs from rats treated with MCT. Enhanced vasopressor responses were evoked by angiotensin or hypoxia at 4 or 7 but not 14 days after MCT treatment. Since the vascular hyperresponsiveness occurred before the onset of pulmonary hypertension, Gillespie *et al.* concluded that vasoconstriction alone could not account for the prolonged increases in pulmonary arterial pressure that occur after MCT administration.

The reactivity of segments of rat pulmonary arteries was also examined at 4, 7 or 14 days after MCT treatment (Altiere *et al.*, 1986). At 4 days after MCT treatment, pulmonary artery segments had significantly increased constrictive responses to norepinephrine, angiotensin II or KCl, but at 7 days there were no differences in vascular contractility between arterial segments from MCT-treated and control rats. At 14 days, the arterial segments from rats treated with MCT had markedly reduced contractile responses compared to those of control vessels, and they were hyporesponsive when stimulated with the relaxant agonists, isoproterenol and acetylcholine. Altiere and colleagues (1986) concluded that increased contractility and vasoconstriction may occur early in the pathogenesis of MCT-induced pulmonary hypertension but would be unlikely to contribute to the sustained increases in pulmonary arterial pressure that occur late in this chronic vascular disease. In related experiments, the contractility of isolated segments of pulmonary

arteries from rats treated with MCT in drinking water was evaluated (Shubat *et al.*, 1987). MCT treatment caused decreased contractile responses to KCl and norepinephrine, but this required approximately 15-20 days to evolve. In addition, the active circumferential tension and active stress of pulmonary artery segments from rats were reduced at 3 weeks after MCT injection (Langleben *et al.*, 1988).

Although there have been several investigations of vascular reactivity due to MCT intoxication, altered vascular responses that occur after administration of the putative metabolite, MCTP, are few. The vascular reactivity of pulmonary vessels was examined in blood perfused, isolated lungs from rats treated with MCTP (Hilliker and Roth, 1985a). At 7 days post-treatment, lungs from MCTP-treated rats had a 1.5-2 fold increase in the pressor response to 5HT. At 14 days after exposure, there was a 3 fold increase in the pressor responses to 5HT and Angiotensin II in isolated lungs from MCTP-treated rats compared to controls. Hilliker and Roth (1985a) concluded that exaggerated pulmonary vascular reactivity may be involved in the development and possibly the maintenance of MCTP-induced pulmonary hypertension.

In summary, alterations in vascular responsiveness have been evaluated in isolated lungs from rats treated with MCT(P) and in isolated arterial segments from rats treated with MCT. Results indicate that vascular responsiveness to agonists is increased within several days after treatment with the toxicants. The hyper-responsiveness diminishes with time in rats treated with MCT, and vessels are hyporesponsive to both contractile and relaxant agonists late in disease progression.

Although vascular hyperresponsiveness and vasoconstriction are not synonymous, they are related since exaggerated contractility may result in inappropriate vasoconstriction. With regard to this relationship, most investigators concluded that transient vasoconstriction occurs early after treatment with MCT but does not contribute to the prolonged pulmonary hypertension that ensues weeks later. In contrast, exaggerated vasoreactivity was sustained in isolated buffer perfused lungs from rats treated with MCTP, and investigators suggested that vasoconstriction might contribute to the development and maintenance of pulmonary hypertension in this model.

Critical data are lacking in the evaluation of early vasoreactivity after MCT(P) treatment. Most investigations have focused on times several days to weeks after toxicant exposure. There is only one study of the acute effects of MCT on pulmonary vascular dynamics. The iv injection of 60 mg/kg of MCT in dogs caused a rapid decrease in cardiac output and a marked increase in pulmonary vascular resistance. Within 2 hours post-injection, there was moderate pulmonary perivascular and interstitial edema, swelling of capillary endothelial cells and leukocytosis within the alveolar capillary lumina (Miller *et al.*, 1978).

Increases in pulmonary vascular resistance may occur due to vasoconstriction, blood hyperviscosity or reduction of vascular luminal cross sectional area. Results of previous hemodynamic studies indicate hyperviscosity of blood to be unlikely in rats treated with MCT(P) (Meyrick *et al.*, 1980; Bruner *et al.*, 1983a). Most investigators have considered the possible direct effect of vasoconstriction

toward the initiation and maintenance of pulmonary hypertension but have neglected an important secondary effect that may occur. Vasoconstriction along with decreased cardiac output and increased pulmonary vascular resistance are factors that could cause significant slowing of blood flow within the lung and, under specific conditions, might promote or potentiate thrombosis. Platelet and fibrin thrombi occur very early after treatment with MCT(P) and thrombosis could, by physical obstruction, reduce cross sectional area of the pulmonary vascular bed. The potential contribution of fibrin and platelet thrombi toward initiation of pulmonary hypertension in this model was recognized by Chesney *et al.* (1974a) and Lalich *et al.* (1977) and continues to be an important subject of investigation (Hilliker *et al.*, 1982; Roth *et al.*, 1989a; Schraufnagel and Schmid, 1989).

B. Evidence for Endothelial Cell Involvement

The administration of MCT(P) to rats causes histologic and electron microscopic lesions in pulmonary vascular endothelial cells (Valdivia *et al.*, 1967ab; Butler, 1970; Plestina and Stoner, 1972; Meyrick *et al.*, 1980; Kay *et al.*, 1982ab; Molteni *et al.*, 1984; Reindel *et al.*, 1990). Within hours after administration of 60 mg MCT/kg to rats, endothelial cells swell and impinge upon the vascular lumen. Swollen mitochondria, decreased numbers of microfilaments and increases in ground substance are observed ultrastructurally (Rosenberg and Rabinovitch, 1988). Often, platelet and fibrin thromboemboli are observed within capillary lumina (Valdivia *et al.*, 1967ab; Plestina and Stoner, 1972). With time, endothelial cell lesions progress and are observed in multiple levels of the pulmonary vasculature.

The morphologic lesions in pulmonary endothelium observed *in vivo* are accompanied by alterations in endothelial cell function, including decreased removal of the biogenic amines, serotonin and norepinephrine (Gillis *et al.*, 1978; Huxtable *et al.*, 1978). In addition, production of PGI_2 and thromboxane is increased (Molteni *et al.*, 1984; Stenmark *et al.*, 1985) and production of angiotensin converting enzyme and endothelium-derived growth factor may be decreased after treatment of rats with MCT (Molteni *et al.*, 1984, 1986ab, 1987; Lafranconi and Huxtable, 1983; Ito *et al.*, 1988).

Administration of chemically synthesized MCTP to rats results in similar decreases in ability of lungs to remove biogenic amines (Hilliker *et al.*, 1983ab, 1984a) and increases in thromboxane production (Ganey and Roth, 1987a). In cultured endothelial cells, MCTP decreases angiotensin converting enzyme activity (Hoorn and Roth, personal communication 1992). Morphologic lesions and functional alterations like those detected in rats treated with MCTP have been reproduced in bovine endothelial cells grown *in vitro* and treated with MCTP (Reindel and Roth, 1991).

Endothelial cells produce several important procoagulant and fibrinolytic substances. Given the numerous other functional alterations that occur after exposure of endothelial cells to MCTP, it is likely that the delicate balance of procoagulant and fibrinolytic substances is upset after exposure to the toxicant, possibly resulting in creation of a hypercoagulable condition. There have been no

comprehensive investigations of the procoagulant and fibrinolytic properties of endothelium after exposure to MCT(P).

vWF abnormalities have been identified in some human patients with primary pulmonary hypertension (Geggel *et al.*, 1987). Although these patients had normal levels of plasma vWF antigen, they had an increase in the ratio of low molecular weight to high molecular weight multimers and in the ratio of vWF function to vWF concentration. In contrast, high plasma levels of vWF antigen were identified in people with pulmonary hypertension that occurred secondary to cardiac diseases (Rabinovitch *et al.*, 1987). In addition, these patients had fewer high molecular weight multimers of vWF compared to normal. At this time, there are not enough data to decide whether the quantitative and qualitative abnormalities in vWF of human patients are involved in pathophysiologic mechanisms of disease or are simply markers of endothelial cell or platelet dysfunction. No reports were identified that described vWF analysis in MCT(P)-induced pneumotoxicity and pulmonary hypertension. Since both endothelial cells and platelets are intricately involved in MCT(P)-induced lung injury and pulmonary hypertension, vWF analysis may provide valuable information regarding the sequestration of platelets within the lung after treatment with the toxicants.

C. Evidence for Platelet Involvement

Interest in platelets as mediators of cardiovascular dysfunction in the MCTP-treated rat model stems from two observations. Platelet thrombi have been recognized in routine histologic and electron microscopic analyses of lungs from rats

treated with MCT(P) (Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Valdivia *et al.*, 1967ab; Chesney *et al.*, 1974a; Meyrick *et al.*, 1980), and administration of the parent alkaloid, MCT, to rats causes a mild thrombocytopenia (Hilliker *et al.*, 1982).

Bruner *et al.* (1983a) observed normal platelet numbers in the blood of rats treated with MCTP, the putative, toxic metabolite of MCT. However, White and Roth (1988) demonstrated pulmonary sequestration of ^{111}In-labeled platelets at 8 and 14 days after MCTP intoxication in rats with normal blood platelet counts. These data confirm that an increased number of platelets are localized in the lung during the period of elevated pulmonary arterial pressure in MCTP-treated rats (Bruner *et al.*, 1983a; Ganey *et al.*, 1988).

Hilliker and colleagues (1984b) initially investigated the role of platelets in this model by cotreating rats with MCTP and an anti-rat platelet serum. Rats made thrombocytopenic for 48 hours beginning on days 3 or 6 after treatment with MCTP had significantly less right heart hypertrophy when killed at 14 days. However, lung injury was unaffected by the antibody-mediated platelet depletion. In addition, rats made thrombocytopenic beginning on day 0 were not protected from either lung injury or right heart hypertrophy. These results suggest that platelets are involved in the pulmonary hypertensive response after MCTP administration, but they may not mediate the early phase of lung injury.

Ganey and associates (1988) sought to define further the potential role of platelets in the MCTP-treated rat model. Rats were made moderately or

markedly thrombocytopenic with an anti-rat platelet antibody and were killed at 4, 8 or 14 days after MCTP intoxication. Moderate and marked thrombocytopenia were defined as circulating platelet numbers of 21 $\pm$ 2% or 4.1 $\pm$ 0.5% of control values, respectively. Neither degree of thrombocytopenia protected rats from lung injury at any of the designated times. Rats made moderately thrombocytopenic had no elevation in pulmonary arterial pressure at 8 or 14 days and no evidence of right cardioventricular hypertrophy at 14 days. Rats made markedly thrombocytopenic were protected from neither pulmonary hypertension nor right heart hypertrophy. Results of these experiments confirm the findings of Hilliker *et al.* (1984b) in which a moderate degree of thrombocytopenia protected rats from right cardioventricular hypertrophy but not from lung injury after MCTP administration. In addition, these data demonstrate that moderate thrombocytopenia also prevents increases in pulmonary arterial pressure, the accepted stimulus for cardiac enlargement in this model. The disparate effects of the moderate and marked degrees of thrombocytopenia in the prevention of pulmonary arterial hypertension are difficult to explain. The investigators suggested a minimal number of platelets may be necessary to maintain vascular integrity. Vascular leak induced by the marked thrombocytopenia may have actually predisposed rats to MCTP-induced vascular injury and pulmonary hypertension. An alternative explanation for the distinctly different results lies in the method of platelet quantitation. Platelets numbers were quantified in blood but not in the lungs. It is possible that peripheral blood platelet numbers may not accurately reflect pulmonary platelet sequestration in lungs of rats injured

by the toxicant and rats with the most severe lung injury actually had greater numbers of platelets removed from circulation and adhered within the lungs. These sequestered platelets may have released more mediators or growth factors associated with the observed lung injury and pulmonary hypertension.

By using ^{111}In-labeled platelets and an anti-rat platelet serum, White and colleagues (1989) examined in greater detail the pulmonary sequestration of platelets in rats treated with MCTP. As in previous studies, antibody-induced thrombocytopenia at 6-7 days after MCTP intoxication attenuated the pulmonary hypertensive response measured at 8 days but did not protect rats from lung injury even though fewer platelets were sequestered within the lung. Rats given the anti-platelet antibody and examined at 18 days after MCTP intoxication had increased pulmonary arterial pressure and right heart hypertrophy. These results suggest that antibody-mediated thrombocytopenia delays but does not prevent pulmonary hypertension in MCTP-treated rats. Lastly, White and associates (1989) observed no exacerbation of pneumotoxicity or pulmonary hypertension in MCTP-treated rats with splenectomy-induced thrombocytosis. Results of these experiments demonstrate decreased platelet sequestration in lungs of rats treated with anti-platelet serum and MCTP, and confirm the involvement of platelets in the development of pulmonary hypertension after intravenous exposures to the toxicant. The exact mechanism of platelet involvement remains to be elucidated.

In summary, platelets are sequestered in the lungs of rats treated with MCT(P). Antibody-induced thrombocytopenia attenuates pulmonary hypertension

after treatment of rats with MCTP. These data suggest that platelets may help to mediate the pulmonary vascular response. It is not clear whether platelet accumulation physically obstructs small vessels, resulting in increased vascular resistance and pulmonary hypertension, or if platelets mediate the sustained elevations in pulmonary arterial pressure via release of specific mediators or growth factors that may cause vascular remodeling (Roth and Ganey, 1987, 1988). During aggregation, activated platelets release the vasoactive substances ADP and 5HT from dense granules and several mitogens from α-granules. In addition, platelets synthesize thromboxane A_2, a vasoconstrictor. The discussion that follows will address the involvement of platelet growth factors and mediators in this model.

1. Platelet derived growth factor (PDGF)

Activated platelets release mitogens from their α granules including PDGF, epidermal growth factor and transforming growth factor *B* (Ross *et al.*, 1986; Assoian and Sporn, 1986; Oka and Orth, 1983; Habenicht *et al.*, 1990). These mitogens stimulate the growth of various cell types in culture (Keski-Oja *et al.*, 1987; Carpenter and Cohen, 1979; Ross *et al.*, 1986). In addition to promoting proliferation of mesenchymal cells, PDGF causes vasoconstriction (Berk *et al.*, 1986). Ganey and colleagues (1988) investigated the potential involvement of PDGF as a mediator in MCTP-induced lung injury and pulmonary hypertension. Rats given daily ip injections of anti-PDGF IgG for 14 days were not protected from lung injury, pulmonary hypertension or subsequent right heart hypertrophy that occurred 2 weeks after MCTP intoxication (Ganey *et al.*, 1988). These initial results suggest that

intravascular PDGF is not critical for development of pulmonary hypertension in rats treated with MCTP. Other explanations for the lack of protective effects of the anti-PDGF IgG may be found in the sources of mammalian PDGF and the treatment protocol used in this study. In addition to platelets, endothelial cells and smooth muscle cells produce PDGF-like molecules (Nilsson *et al.*, 1985; Ross *et al.*, 1986) that may mimic the effects of PDGF. The dose of anti-PDGF IgG used in these experiments was based upon a calculation of total PDGF from platelets but did not consider other sources of the growth factor. The quantity of antibody used may not have effectively eliminated all the PDGF-like molecules released in the lung after treatment with MCTP. Alternative treatment protocols need to be investigated before a definitive conclusion can be made regarding involvement of PDGF in MCTP-induced pulmonary hypertension.

2. Epidermal growth factor (EGF)

Like PDGF, EGF is a peptide mitogen found in platelet α granules and several other tissues (Oka and Orth, 1983; Mroczkowski and Ball, 1990). EGF increases transport of small molecules across cell membranes (Mroczkowski and Ball, 1990) and is associated with synthesis of protein, RNA and DNA *in vitro* (Carpenter and Cohen, 1979). *In vivo*, EGF is found in many tissue fluids and causes proliferation of epidermal and epithelial tissues. Although identified frequently in body fluids and tissues, the physiologic importance of this mitogen is unknown. Since EGF stimulates smooth muscle proliferation *in vitro* (Gospodarawicz *et al.*, 1981), and because vascular remodeling and platelet sequestration occur in

MCT(P)-induced lung injury and pulmonary hypertension, Gillespie and colleagues (1989) evaluated the potential of EGF involvement in the MCT-treated rat. At 4 days after treatment with 60 mg MCT/kg sc, rats had a diffuse perivascular pattern of EGF-like immunoreactive staining in the lungs. In addition, the iv administration of human recombinant EGF (125 pg/hr) to rats for 7 days was associated with significant increases in lung polyamine concentrations, medial thickening of pulmonary arteries of 100-200 μm dia. and pulmonary hypertension. The origin of EGF immunoreactive staining was unknown, but the investigators hypothesized phenotypically altered, pulmonary vascular fibroblasts or other vascular cell types or platelets entrapped in vascular lesions may have been sources of the staining. The significance of these results is difficult to evaluate. EGF immunoreactivity has not been detected previously in lung tissues from several species (Kasselberg *et al.*, 1985; Salido *et al.*, 1986). The morphologic data presented is of limited value due to the poor preservation of lung tissue imparted by the acetone/ethanol fixation used in these experiments. In addition, the lack of effect of exogenous EGF on remodeling of small pulmonary arteries, a hallmark of MCT(P)-induced pulmonary hypertension, was disturbing. Gillespie *et al.* suggested the lack of effect may have been due to the relatively small smooth muscle component of the small vessels or insensitivity of smooth muscle cells in this vascular region to effects of EGF. In summary, immunoreactive EGF was identified in the lungs of rats treated with MCT, and the administration of exogenous EGF mimicked some of the effects of MCT on the lung. More detailed studies, particularly those involving detailed histologic analysis, are

necessary before definitive involvement of EGF in MCT(P)-induced pulmonary hypertension can be verified.

3. Serotonin

The potential involvement of platelet-mediated 5HT release in MCTP-induced pulmonary hypertension was investigated in fawn-hooded rats. This strain of rat was selected for its platelet function defect that is characterized by decreased content of 5HT and adenine nucleotides in dense granules and diminished ability to absorb and release these compounds (Hilliker *et al.*, 1983b). Right cardio-ventricular hypertrophy and increased lung weight occurred in both fawn-hooded and Sprague-Dawley rats 14 days after MCTP intoxication. From these results, Hilliker and colleagues (1983b) concluded that platelet uptake or release of 5HT were not critical for development of MCTP-induced pulmonary hypertension.

Ganey and Roth (1987b) evaluated platelet content of 5HT from rats treated with MCTP. There were no differences in platelet content or plasma concentration of 5HT from MCTP-treated or control rats killed at 14 days after iv exposure to the toxicant. Lastly, cotreatment of rats with MCTP and either one of the 5HT receptor antagonists, ketanserin or metergoline, afforded no protection from pneumotoxicity or pulmonary hypertension (Ganey *et al.*, 1986). These results indicate that 5HT does not exclusively mediate the toxicity or compensatory lesions that occur in rats treated with MCTP.

4. Platelet Responsiveness

The responsiveness of platelets from MCTP-treated rats to normal stimuli of aggregation was evaluated by Hilliker *et al.* (1983c). There were no changes in the platelet aggregation response from rats given MCTP 1 and 4 days previously. However at 7 days, platelets from MCTP-treated rats had a decreased response to ADP, and at 14 days the response of platelets to ADP, collagen and arachidonate was depressed. The results of these studies suggest that platelets do not react in a hyperresponsive manner during MCTP-induced pneumotoxicity and pulmonary hypertension.

5. Thromboxane

Platelet production of TxB_2, a metabolite of thromboxane (TxA_2), was examined in rats treated with MCTP (Ganey and Roth, 1987b). At 1 day post-injection, the concentration of TxB_2 in unstimulated platelet rich plasma (PRP) from MCTP-treated rats was significantly greater than that of controls. However, there were no differences in the concentration of TxB_2 in unstimulated PRP from MCTP-treated and control rats killed at 4, 7 or 14 days. There was also significantly less TxB_2 released from platelets at one half the maximal arachidonic acid-induced aggregation in the PRP from MCTP-treated rats killed at 7 days compared to controls. In addition, the platelet release response to MCTP treatment of platelets *in vitro* was examined. The aggregation response to arachidonic acid and release of TxB_2 was depressed only when MCTP was added to PRP in very high

concentrations (1 mg/ml) *in vitro*. These results suggest treatment with MCTP does not affect release of platelet TxB_2.

Certain drugs that inhibit arachidonic acid metabolism in rats influence MCTP treatment effects on the lungs (Hilliker and Roth, 1984c). Dexamethasone and sulfinpyrazone prevented right heart hypertrophy in rats treated with MCTP and killed at 14 days. Dexamethasone partially attenuated lung injury but sulfinpyrazone did not. While initially interesting, these results were viewed with caution because the drugs tested have numerous other actions that may have been beneficial in reducing the pulmonary hypertensive effects of MCTP. As a potent anti-inflammatory agent, dexamethasone may have reduced the inflammation associated with MCTP-induced lung injury and thus decreased the stimulus for pulmonary vascular remodeling and secondary right heart hypertrophy. In addition, dexamethasone and sulfinpyrazone both decreased the rate of weight gain in rats treated with MCTP and, therefore, may have prevented right ventricular hypertrophy indirectly by inhibition of growth.

To elucidate further the potential involvement of thromboxane in this model, Ganey and Roth (1986) attempted to modulate the toxicity pharmacologically with drugs that interfere with thromboxane synthesis or action. Cotreatment of rats with MCTP and the cyclooxygenase inhibitor, ibuprofen, the thromboxane synthetase inhibitor, Dazmegrel, or the thromboxane receptor antagonist, L-640,035, failed to attenuate lung injury or pulmonary hypertension. The investigators concluded that thromboxane was not critical for lesion development in

MCTP-induced pneumotoxicity and pulmonary hypertension. In addition, Dazmegrel failed to protect rats treated with the parent alkaloid, MCT, from right cardio-ventricular hypertrophy (Langleben *et al.*, 1986).

6. Platelet activating factor (PAF)

PAF is a pleiotropic inflammatory mediator capable of activating and aggregating platelets and stimulating leukotriene synthesis (Benveniste *et al.*, 1972; Page *et al.*, 1984; Voelkel *et al.*, 1982). In addition, PAF can induce pulmonary edema, stimulate smooth muscle contraction and increase vascular permeability (Findlay *et al.*, 1981; Mojared *et al.*, 1983; Lichey *et al.*, 1984; Benveniste and Chignard, 1985). Treatment with MCT caused increased concentrations of lung tissue PAF at 7 and 21 days in some rats (Ono and Voelkel, 1991). The source of the increased PAF was unknown, but the investigators suggested injured endothelium or activated macrophages as potential candidates for its production. Concurrent treatment with either one of the PAF antagonists, WEB 2170 or WEB 2086, afforded protection from pulmonary hypertension and compensatory right ventricular enlargement at 3 weeks after MCT intoxication. The effects of these antagonists on protection from vascular leak, measured at 7 days, were mixed. The administration of WEB 2170 to rats treated with MCT three weeks previously obtunded the progression of right cardioventricular hypertrophy when measured 4 weeks after MCT administration. The results of these experiments are difficult to interpret because PAF has many potential sources and numerous actions. One of the beneficial effects of the PAF antagonist, WEB 2170, was to decrease vascular leak

measured at 1 week after treatment of rats with MCT. In the MCT-treated rat, vascular leak precedes the pulmonary hypertensive response and has been a postulated prerequisite for development of the pulmonary and cardiovascular lesions that ensue. In addition, WEB 2170 administration decreased the pulmonary inflammation and vascular remodeling and prevented increases in the lung tissue eicosenoids, 6-keto-$PGF_{1\alpha}$ and TXB_2, and leukotriene C_4, that are associated with MCT treatment. Ono and Voelkel (1991) also hypothesized that PAF antagonists used in this study may have acted on platelets to prevent their activation and aggregation, therefore decreasing possible release of growth factors and inflammatory mediators. The data presented neither confirm nor rule out this possibility. One problem with this report was that no data were presented describing weight gain or possible induction of cytochrome P450 enzymes in the test animals, two pieces of evidence critical for evaluation of the results. In summary, results of these studies considered *in toto* suggest a potential yet undefined role for PAF in MCT-induced lung injury and pulmonary hypertension.

D. Evidence for Involvement of the Coagulation System

The histologic and ultrastructural lesions that occur in the lungs after treatment with MCT(P) are complex. Although not reported in every investigation, pulmonary vascular thrombi are recognized with great frequency. However, thrombotic lesions are subtle. Identification may be aided by use of special stains, and the thrombi may be easily overlooked by unsuspecting or inexperienced investigators.

The significance of vascular thrombosis and its contribution to pneumotoxicity and pulmonary hypertension are the subject of continued discussion. Comparisons among studies are difficult because they differ markedly in form of toxic alkaloid administered (*C. spectabilis* or MCT(P)), dose of toxicant, route of toxicant administration and time points examined. In addition, most studies focus on the morphologic, biochemical or physiologic lesions within a single organ, usually the lung, liver or heart. Concurrent histologic examination of several major organs is cumbersome and is seldom performed. Histologic or biochemical evidence that would suggest involvement of the systemic coagulation system in lesion development after administration of MCT(P) is therefore not easily obtained from the existing literature. The discussion that follows will address these concerns.

Rats or nonhuman primates chronically fed *C. spectabilis* or given injections of MCT(P) develop fibrin and platelet thrombi in the pulmonary vasculature (Turner and Lalich, 1965; Merkow and Kleinerman, 1966; Valdivia *et al.*, 1967ab; Butler *et al.*, 1970; Allen and Carstens, 1970; Plestina and Stoner, 1972; Chesney and Allen, 1973b; Chesney *et al.*, 1974a; Heath and Smith, 1978; Lalich *et al.*, 1977; Hayashi *et al.*, 1984; Schraufnagel and Schmid, 1989; Reindel *et al.*, 1990). Most of the histologic studies of lung lesions induced by these toxicants are descriptive in nature. Thrombosis of small vessels is a common finding but is not described in extensive detail. A summary of pertinent data appears in Tables 1-3. In the majority of experiments, markers of lung injury were either not measured or they were monitored in an asynchronous fashion compared to histologic examination

Table 1: Evidence of thrombosis in lungs of rats fed *Crotalaria spectabilis*

C. spectabilis	Animals (times examined)	Thrombus location	References
0.04-0.07% in diet	M. Wistar rats (7-26 wk)	fibrin and plt. thrombi in lumen and intima of arteries, some in medial lesions	Heath and Smith, 1978
0.02-0.08% in diet	M. SD. rats 100 g (121-128 days)	fibrin thrombi occlude many alv. caps. and pulm. arteries in 23/50, fibrin present on denuded vessels in many rats	Allen and Carstens, 1970

Table 2: Evidence of thrombosis in lungs of animals treated with monocrotaline

Monocrotaline	Animals (times examined)	Thrombi location	References
60 mg/kg sc	M. SD. rats, 40-60 g (4 hr-3 wk)	small vessels and caps. contain plt. accumulations at 4 and 24 hrs; semi-quantitative study	Valdivia *et al.*, 1967ab
10-75 mg/kg diet (↑ MCT increment with time)	F. SD. and Wistar rats, 37-49 g (26-232 days)	11/23 had cap. occlusion by fibrin thrombi, 12/19 had fibrin on damaged intimal surfaces of small arteries	Turner and Lalich, 1965
25 mg/kg diet or 1.25 mg/0.5 ml ip daily X 15 days	M. and F. SD. rats 36-110 g (24 hr-7 wk)	small arterial and cap. hyaline thrombi seen frequently	Merkow and Kleinerman, 1966
2% in drinking water	stumptail monkeys (duration unknown)	fibrin and plt. thrombi in cap. lumen	Chesney and Allen, 1973b
60 mg/kg sc	SD. rats, 5 wks age (1-4 wk)	focal hyaline thrombi in caps. and small arteries, 1 wk (5/10), 2 wk (9/10), 3 and 4 wks (10/10); ↑ lung weight, ↓ alv. surface area, ↓ lung ACE activity at all 4 time points examined	Hayashi *et al.*, 1984
60 mg/kg ip	F. SD. rats 250 g, 35 days age (16 days)	pulm. vascular density ↓ due to fibrin cap. thrombosis of alveoli & small vessels	Schraufnagel and Schmid, 1989
120 mg/kg sc	M. SD. rats, 14 days age (20-47 days)	fibrin thrombi in alv. caps. of all treated rats at all times	Hayashi and Lalich, 1967

Table 3: Evidence of thrombosis in lungs of rats treated with monocrotaline pyrrole

MCTP	Animals (times examined)	Thrombus location	References
5-30 mg/kg iv	M. Fisher rats 300-350 g (9 hr-3 wk)	plt. thrombi at 9 hr in cap. lumen, between and within intimal endothelial cells; lung weight ↑ at 6 hr, vasc. leak ↑ (^{125}I cpm/g bloodless lung) at 15 hr	Plestina and Stoner, 1972
2-4 mg/kg iv	M. SD. rats 40-80 g (1-49 days)	fibrin thrombi in alv. caps. at 1-7 days, semi-quantitative study	Lalich *et al.*, 1977
2-4 mg/kg iv	M. L.E. rats 250 g (4 wk)	numerous pulmonary caps. & arterioles partially or completely occluded by fibrin and plt. thrombi; ↓ body weight; ↓ [SVS/RV], ↑ lung weight, ↑ systolic & diastolic blood pressure	Chesney *et al.*, 1974a
3.5 mg/kg iv	M. SD. rats 230-280 g (1-14 days)	vascular thrombosis not widespread, some plt. thrombi observed but no reference to time of occurrence given	Reindel *et al.*, 1990
5 mg/kg iv	M. Porton rats 55-95 g (2 days-4 wk)	fibrin present in "oedematous areas"	Butler *et al.*, 1970

of lung tissue. In addition, many studies focussed on times days to weeks after toxicant administration, and the early times before animals developed clinical signs of illness or times before there were overwhelming changes in markers of lung injury were all but ignored. Therefore, it is very difficult to assess accurately the temporal relationship between thrombosis and the onset of lung injury. Few studies concurrently measured markers of lung injury and pulmonary hypertension, and those studies that included these markers appear in Tables 1-3 (Plestina and Stoner, 1972; Chesney *et al.*, 1974a; Hayashi *et al.*, 1984; Reindel *et al.*, 1990).

Several investigators performed semi-quantitative analysis of the pulmonary thrombotic lesions that occur after MCT administration (Valdivia *et al.*, 1967a; Lalich *et al.*, 1977; Schraufnagel and Schmid, 1989). Valdivia *et al.* (1967a) treated rats with 60 mg MCT/kg and evaluated changes in the pulmonary alveoli. Rats were killed at 4, 24 or 48 hours, 1, 2 or 3 weeks after MCT intoxication, and detailed histologic and ultrastructural analyses of the lungs were performed. Thrombotic lesions were graded as 1-4+. At 4 hours, fibrin and platelet thrombi (1+) were observed in the alveolar capillaries and small arterioles and were accompanied by mild interstitial edema. The number of thrombi in small vessels remained constant at 24 and 48 hours but several endothelial lesions were noted to occur at these times. At 1 week after MCT intoxication, the severity of thrombotic lesions increased to 2+, and marked interstitial edema, moderate endothelial lesions, marked elastolysis and hypertrophy of granular pneumocytes were observed. The severity of thrombosis increased with time, and at 2 and 3 weeks interstitial edema, endothelial lesions and

thrombi were graded as 4+. In this study, capillary thrombosis preceded overt endothelial cell injury. Since right heart enlargement often occurs without histologic evidence of marked alterations in small pulmonary arteries, Valdivia and colleagues (1967a) commented that capillary destruction alone was sufficient to cause right cardioventricular hypertrophy in rats treated with MCT.

Lalich *et al.* (1977) evaluated fibrin thrombosis in rats after administration of MCTP. Rats received a single, bolus injection of MCTP (2-4 mg/kg) and were killed at various times thereafter. Fibrin thrombosis of alveolar capillaries and arterioles was observed in 8/8 rats killed at 1-7 days after MCTP intoxication. Thrombi were diffuse in distribution and occurred in the absence of right heart enlargement. Other lesions included focal edema, alveolar fibrosis and subpleural cellular hyperplasia. Fibrin thrombosis was detected in 17/24 rats treated with MCTP and killed at 21-49 days. Of the 17 rats that developed significant right ventricular hypertrophy, 14 had fibrin thrombosis. Fibrin thrombi in DMF control rats were few and transient. Lalich and colleagues (1977) hypothesized that fibrin thrombi obliterate alveolar capillaries, cause reduced pulmonary blood flow and, due to their great incidence in MCTP-treated rats with right heart enlargement, may cause *cor pulmonale.*

Schraufnagel and Schmid (1989) demonstrated a decrease in pulmonary capillary density in rats given 60 mg MCT/kg ip and killed at 16 days. Lungs of MCT-treated and control rats were infused with methlymethacrylate, and corrosion casts were analyzed by scanning electron microscopy with image analysis.

This method clearly demonstrated a decrease in vascular density. A review of histologic sections revealed fibrin thrombosis of small capillaries as the reason for the decreased capillary filling.

In summary, administration of MCT(P) to rats causes pulmonary vascular thrombi to form in alveolar capillaries and small arterioles. Thrombi appear before overt endothelial cell injury, and their number and severity increase with time. Eventually thrombi are found in larger vessels at sites of denuded endothelium. The incidence of thrombosis is high enough that several investigators have proposed that thrombi may cause pulmonary hypertension and *cor pulmonale*.

The presence of thrombi only in the lungs would suggest an organ-specific dysfunction of the hemostatic system. Thrombotic lesions have been identified in several other organs of rats treated with MCT. In addition to the alveolar capillary thrombosis and pulmonary edema observed at 20-47 days after treatment with 120 mg MCT/kg, some rats had renal arterial and glomerular thrombi that were observed at 31-47 days after intoxication (Hayashi and Lalich, 1967). Similar degrees of alveolar capillary and glomerular thrombosis were observed in rats at 30-60 days during continuous feeding of *C. spectabilis* (0.025-.1%) in the diet (Masugi *et al.*, 1965). Lastly, thrombi in lungs and liver were reported in rats 4-6 weeks after they were given several dermal applications of MCT in alcohol or were chronically fed MCT in drinking water (Schoental and Head, 1955). Although these studies varied somewhat in toxicant, dose and route of administration, a common finding was thrombosis of small vessels, often in the absence of necrosis. The

presence of thrombosis in several organs suggests the deleterious effects of MCT on the hemostatic system may not be strictly limited to the lung endothelium.

Laboratory investigations of the coagulation system and MCT-induced pneumotoxicity and pulmonary hypertension are few and of limited scope. Marked prolongations of the prothrombin time were observed in rats given large doses of MCT (120 mg/kg) and killed at 24 hours (Rose *et al.*, 1945). Rats given a lower dose of MCT (40 mg/kg) had no change in the activated partial thromboplastin time or thrombin time when killed at 21 days (Fasules *et al.*, 1987). In addition, turkeys chronically fed *Crotalaria sp.* developed hemorrhagic lesions and elongated prothrombin times relatively late in the disease process (Allen *et al.*, 1963).

The preceding discussion suggests that animals treated with low or moderate doses of MCT(P) develop conditions in the lungs and other organs that promote thrombosis relatively early after exposure. After high doses of MCT, or prolonged exposures to MCT or *Crotalaria sp.*, animals may develop hemorrhagic lesions accompanied by prolongations in the prothrombin time (Rose *et al.*, 1945; Allen *et al.*, 1963). A systematic evaluation of the coagulation system after treatment with MCT(P) has not been performed. Such a study is necessary to determine a mechanism for the thrombosis and to elucidate the role of pulmonary thrombi in MCT(P)-induced pneumotoxicity and pulmonary hypertension.

The effect of anticoagulant and non-anticoagulant heparin on MCT-induced pneumotoxicity and pulmonary hypertension was explored by Fasules *et al.* (1987). Rats cotreated with 60 mg MCT/kg sc and 300 U/heparin/kg sc twice

daily had prolonged activated partial thromboplastin times and thrombin times compared to controls, indicating an anticoagulant effect measured three hours after the heparin was administered. Administration of 300 or 600 U/heparin/kg sc twice daily to rats treated with 40-60 mg MCT/kg sc did not attenuate the vascular leak, vascular remodeling, increase in pulmonary arterial pressure or cardioventricular hypertrophy that occur after MCT treatment. The potential beneficial effects of a synthetic non-anticoagulant heparin fragment, an antiproliferative agent, were also investigated in this model. Rats cotreated with MCT and 2 mg non-anticoagulant heparin fragment/kg sc twice daily were not protected from vascular leak or cardiovascular lesions associated with MCT administration. At first glance, results of these experiments suggest neither anticoagulant nor non-anticoagulant heparin will attenuate the vascular leak and pulmonary hypertension that occur after treatment of rats with MCT. However, there were several problems with these experiments that warrant consideration. The anticoagulant effects of heparin were measured only at three hours after its administration. Since a time course of anticoagulant effect was not measured, one can not assume that the anticoagulant effect was present throughout the duration of the study. The investigators merely stated that heparin was administered twice daily but did not specify the duration of heparin treatment. Any period of inadequate heparinization may have permitted thrombi to form in alveolar capillaries. In addition, the results of specific coagulation tests listed in the materials and methods section were not reported in the text and some data appear to have been analyzed by inappropriate statistical tests. In the experiments of the

antiproliferative effects of non-anticoagulant heparin, no positive control was included in the study, so one cannot judge whether non-anticoagulant heparin had an antiproliferative effect in this investigation. In previous experiments using an hypoxic mouse model (Hales *et al.*, 1983), heparin prevented pulmonary vascular remodeling and development of pulmonary hypertension. Heparin treatment also attenuated pulmonary vascular thickening in chronically hypoxic rats and guinea pigs (Geggel *et al.*, 1986; Hassoun *et al.*, 1986). Therefore, Fasules and colleagues (1987) postulated that heparin administration may prevent vascular remodeling and possibly attenuate the pulmonary hypertension associated with MCT treatment. Alternatively, they hypothesized that the anticoagulant effects of heparin may worsen pneumotoxicity associated with administration of MCT. The authors cited several possible reasons for heparin's lack of protection in these experiments. Differences in the extent of chemical-induced lung injury, inflammatory response, growth factors produced and cellular response to growth factor mediators were all potential explanations given for the differences in response between the hypoxic mouse and MCT-treated rat. Probably more importantly, the investigators recognized that heparin kinetics may differ between rats and mice. It is clear that more detailed investigations, particularly those involving analysis of heparin kinetics in rats, are necessary before definitive evidence of effect or lack thereof can be verified in the MCT-treated rat model.

E. Evidence for Involvement of the Fibrinolytic System

Deficits in fibrinolysis may cause thrombosis. With the exception of Molteni and colleagues (1984, 1985, 1986ab, 1987, 1988ab, 1989), who demonstrated a decrease in plasminogen activator activity of lung tissue slices relatively late after MCT intoxication in rats, there have been no reports of fibrinolysis in MCT-induced pneumotoxicity and pulmonary hypertension. In these investigations, Molteni and associates used the decrease in plasminogen activator activity along with decreases in activity of lung ACE and increases in PGI_2 and thromboxane concentration in the lungs as nonspecific markers of injury to pulmonary endothelium. Most measurements of plasminogen activator activity were made at times 6 wks or later after continuous administration of MCT in drinking water. In a limited time course study in which plasminogen activator activity was measured at 1, 2, 4, 6 and 12 wks after treatment with MCT in drinking water (Molteni *et al.*, 1984), plasminogen activator activity did not decrease significantly until 6 wks after the toxicant administration. The method used to measure plasminogen activator activity in these studies, the fibrin plate technique (Astrup and Albrechtsen, 1957), is the subject of some controversy and is viewed by some investigators a more accurate reflection of net fibrinolytic capacity of the tissue analyzed vs. plasminogen activator activity specifically (Ts'ao *et al.*, 1983). There have been no studies of fibrinolytic activity in rats after treatment with MCTP.

F. Summary

There is evidence that dysfunction of the hemostatic system is involved in MCT(P)-induced pneumotoxicity and pulmonary hypertension. Two components of the hemostatic system, blood vessels and platelets, have been the focus of intensive research. The contribution of the coagulation and fibrinolytic systems to the lesions that occur after MCT(P) administration has not been evaluated.

IX. Research Goals/Specific Aims

The MCTP-treated rat is a useful animal model for the study of chronic pulmonary vascular disease. Much research of the pulmonary hypertension induced by MCTP administration has centered on vascular remodeling and the loss of small to medium sized pulmonary arteries/arterioles. It is recognized that rats treated with MCTP develop pulmonary vascular thrombotic lesions. The relationship of the fibrin and platelet thrombi to the subsequent lung injury and pulmonary hypertension has not been examined, and the exact cause of the thrombosis has not been elucidated. A thorough investigation of the hemostatic system of MCTP-treated rats has not been performed. The purpose of this study was to determine whether dysfunction of the hemostatic system contributes to the lung injury, vascular remodeling and pulmonary hypertension that occur in the MCTP-treated rat. This investigation involved analysis of procoagulant and fibrinolytic pathways in blood and tissue. Attention focussed on the pulmonary vascular endothelial cells and the production of fibrinolytic substances *in vitro* and *in vivo*. The hypothesis that irregularities in coagulation and fibrinolysis

contribute to the pulmonary vascular thrombi and resultant pneumotoxicity and pulmonary hypertension was tested by performing experiments to:

A. Evaluate lung injury during the early time period after administration of MCTP, when previous histologic evaluations reported fibrin thrombi to occur in the lung. These experiments involved evaluation of pneumotoxicity at times within hours to several days after MCTP administration. The injury assessment involved several markers of lung injury including the wet lung-to-body weight ratio, protein concentration and lactate dehydrogenase activity in the bronchoalveolar lavage fluid, and cytologic examination of the bronchoalveolar lavage fluid.

B. Evaluate the procoagulant activity in the peripheral blood of rats treated with MCTP. These experiments were designed to assess changes in the coagulation system of rats treated with MCTP, to correlate them with markers of lung injury and cardioventricular hypertrophy and to determine if excess procoagulant activity in the systemic circulation is likely to contribute to the lung injury and resultant right ventricular hypertrophy that occur in this model.

C. Evaluate the fibrinolytic activity in peripheral blood and lung tissue of rats treated with MCTP. These experiments were designed to assess changes in the fibrinolytic system of rats treated with MCTP, to correlate them with markers of lung injury and cardioventricular hypertrophy, and to determine if deficient fibrinolytic

activity is likely to contribute to the lung injury and resultant right ventricular hypertrophy that occur in this model.

D. Evaluate the major procoagulant and fibrinolytic properties produced by bovine endothelial cells in culture after treatment with MCTP. These experiments were designed to assess changes in tissue factor and factor V activity and correlate changes with routine markers of cellular toxicity. In addition, these experiments were designed to evaluate changes in the ratio of plasminogen activators and plasminogen activator inhibitors to access fibrinolytic capacity of cells treated with MCTP and to correlate changes in these parameters with markers of cellular toxicity.

In general, the preceding experiments were designed to identify specific malfunctions in the coagulation and fibrinolytic systems after treatment of rats with MCTP. The ultimate goal is to modulate the coagulation and fibrinolytic pathways pharmacologically to alter the development of lung injury and pulmonary hypertension in rats treated with MCTP.

CHAPTER TWO

EARLY INDICATIONS OF MONOCROTALINE PYRROLE-INDUCED LUNG INJURY IN RATS

ABSTRACT:

Monocrotaline pyrrole (MCTP), a putative metabolite of the naturally occurring plant toxin, monocrotaline (MCT), causes lung injury, pulmonary hypertension and right cardioventricular hypertrophy when administered intravenously to rats. The lesions caused by MCTP administration are similar to those caused by MCT but appear to occur on a slightly accelerated time course. To explore the onset and development of lung lesions, male, Sprague-Dawley rats were treated with a single, intravenous injection of MCTP, and several markers of lung injury were evaluated at early times after administration. Rats received 3.5 mg MCTP/kg or an equal volume of the vehicle, N,N-dimethylformamide (DMF), via the tail vein at time 0 and were killed at 4, 12, 24, 48, 72, or 120 hours after treatment. Beginning at 4 hours, MCTP-treated rats had increased wet lung-to-body weight ratios (LW/BW). The LW/BW remained elevated at 12 hours, returned to baseline at 24 hours, then increased steadily over the next few days. At 24 hours, the protein concentration of cell-free bronchoalveolar lavage fluid (BALF) was slightly elevated. Lactate dehydrogenase activity in cell-free BALF samples was moderately increased 48 hours after MCTP. Changes in these markers were modest initially but became much more pronounced by 120 hours. Total nucleated cell counts in BALF were variable but were moderately elevated at 120 hours. Cytologic examination of the BALF samples revealed small but significant infiltrates of segmented neutrophils at 4 hours and relatively large infiltrates of segmented neutrophils and small lymphocytes at 120 hours post-treatment. Mature neutrophils had degenerate cytomorphologic characteristics at both 4 and 120 hours. These results confirm that pronounced lung injury is delayed for several days after a single, intravenous administration of MCTP, but they also indicate that subtle lung injury can be detected using quantitative markers quite early after MCTP administration.

INTRODUCTION:

Pyrrolizidine alkaloid-containing plants are distributed worldwide, and their consumption has been associated with considerable mammalian morbidity and mortality. When ingested by animals grazing pasture or humans drinking home-brewed beverages, some pyrrolizidine alkaloids cause fatal necrosis or veno-occlusive disease of the liver, pulmonary parenchymal and vascular lesions, and pulmonary hypertension (Huxtable, 1980; Mattocks, 1986).

Monocrotaline (MCT), a pyrrolizidine alkaloid derived from plants of the genus, *Crotalaria*, produces hepatic lesions when administered to rats in high doses. If given in low doses, MCT causes pulmonary vascular lesions and lung injury (Mattocks, 1986). To cause toxicity, MCT must first be converted to an active pyrrolic metabolite by the cytochrome P450 monooxygenase system of the liver (Mattocks and White, 1971). When administered to rats intravenously, monocrotaline pyrrole (MCTP), a putative metabolite of MCT, causes lung injury, right cardioventricular hypertrophy, and pulmonary hypertension that are similar to those caused by MCT itself (Butler, 1970; Butler *et al.*, 1970). The pathogenesis of MCT- and MCTP-induced lung injury and pulmonary hypertension has been investigated but not completely elucidated.

Central to identifying specific mechanisms of disease is the assessment of observations made throughout the course of illness. Subtle changes that occur early in the course of illness may have great influence on the development of lesions later in the progression of disease (Hill, 1980). Although development of MCT- and

MCTP-induced lung injury and pulmonary hypertension has been investigated, most quantitative studies have focused on times several days after toxin administration, since pronounced lung injury is delayed for several days and progresses thereafter. The time period before animals develop clinical illness has received less attention.

The objective of this study was to examine early times in the course of MCTP-induced lung injury to determine if subtle injury could be detected and quantified before damage was observed as pronounced changes in biologic markers of injury or development of clinical signs.

MATERIALS AND METHODS:

Animals. Male, Sprague-Dawley (CD-Crl:CD(R)(SD)BR VAF/Plus(TM)) rats, purchased from Charles River Laboratories (Portage, MI) were delivered in air filtered crates. Animals weighing 230-280 grams were randomly assigned to groups and housed three per plastic cage on aspen chip bedding (Northeastern Products Corp., Warrensburg, NY) under conditions of controlled temperature (70° ± 2° F), humidity (40-70%) and light cycle (L:D, 12:12). They were allowed food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and tap water *ad libitum*. Cages were maintained within animal isolators (Contamination Control Inc., Lansdale, PA); therefore, test animals breathed only high efficiency particulate (HEPA) filtered air for the duration of the study.

MCTP synthesis. Monocrotaline pyrrole was synthesized from monocrotaline (Trans World Chemicals, Washington, DC) via an N-oxide intermediate (Mattocks, 1969) as previously described by Bruner *et al.* (1986). It was dissolved in the vehicle N,N-dimethylformamide (DMF) and stored in the dark under nitrogen gas at 0° C.

Treatment protocol. Rats were immobilized in a plastic restrainer (Plas-Labs, Baxter Scientific, Romulus, MI) and given a single injection of MCTP (3.5 mg/kg) or an equal volume of DMF vehicle (0.5 ml/kg) in a tail vein. The animals were killed at 4, 12, 24, 48, 72 or 120 hours post-treatment, and several markers of lung injury were evaluated.

Bronchoalveolar lavage. Each rat was weighed, and anesthetized with pentobarbital sodium (50 mg/kg ip) and the trachea was cannulated. A midline laparotomy was performed, the viscera were retracted and 0.1 ml heparin sulfate (1000 U/ml, Sigma Chemical Co., St. Louis, MO, No. H-7005) was injected into the vena cava. One minute later, the vena cava and aorta were severed and the rat was exsanguinated The trachea, lungs and heart were excised *en bloc* and rinsed with saline. The lungs were lavaged twice with isotonic saline as described previously (Roth, 1981). Lavage fluid aliquots were pooled.

Total nucleated cell counts were performed by direct enumeration of well-mixed lavage fluid in a hemocytometer (Reichert-Jung Cambridge Instruments Inc., Buffalo, NY). Lavage fluid (200 μl) was concentrated on a 1 X 3 glass microscope slide by spinning in a Cytospin 2 centrifuge (Shandon Southern Instruments, Inc., Sewickley, PA). Slides were air dried and stained with a modified Wright's stain (Hema-Tek 1000 automatic slide stainer, Miles Laboratories Inc., Elkhart, IN). Stained slides were coded and randomized, and a detailed cytologic examination, including a 300 unit differential cell count, was performed. Absolute counts were determined by multiplying the relative fraction of each cell type by the total number of nucleated cells/μl. Cell-free supernatant fluids were prepared by spinning bronchoalveolar lavage (BALF) samples in a centrifuge at 600*g* for 10 minutes. Lactate dehydrogenase (LDH) activity and protein concentration of cell-free lavage fluid were determined by the methods of Bergmeyer and Bernt (1974) and of Lowry *et al.* (1951), respectively.

Wet lung-to-body weight ratio (LW/BW). Prior to lavage, the excised block of trachea, lungs and heart was weighed. Following lavage, the lung lobes were trimmed free from the trachea by severing major bronchi. The remaining heart, trachea and bronchial remnants were reweighed. Lung weights were determined by subtracting the weight of the heart, trachea and bronchial remnants from that of the total weight of the original block. LW/BW ratios were determined by comparing the lung weight to body weight on day of kill.

Statistical analysis. All data were expressed as means $\pm$ SE. Data were compared using a two-way analysis of variance (Steel and Torrie, 1980). Homogeneity of variance was assessed with the F-max test. A log transformation was performed on any nonhomogeneous data. Individual comparisons were made using the least significant difference test (Steel and Torrie, 1980). Cell counts were compared by the Rank Sums test (Steel and Torrie, 1980). The criterion for significance was $p < 0.05$.

RESULTS:

Wet lung-to-body weight ratio. The LW/BW of DMF-treated rats was essentially constant throughout the experiment, except at 24 hours, at which time it was slightly elevated. To investigate whether the DMF vehicle might have affected LW/BW, we performed a comparison in which rats were randomly assigned to one of 3 groups (n=6/group). Each rat received either no treatment or a single injection of DMF or isotonic saline in the tail vein. At 24 hours, there were no significant differences in the LW/BW between any of the groups (data not shown).

The LW/BW of MCTP-treated rats was significantly increased as compared to control rats as early as 4 hours after injection (Figure 1). The LW/BW remained elevated at 12 hours, returned to the control value at 24 hours, then showed a slow, but steady increase between 48 and 120 hours after MCTP treatment. To determine whether differences in body weight may have influenced the LW/BW results, lung weights of treated and control rats were compared by ANOVA. The pattern of differences (data not shown) was similar to that seen in LW/BW comparisons. A second statistical comparison was made by analyzing lung weight as a covariant of body weight. The data indicated that differences in body weight were not responsible for the increases in lung weight of MCTP-treated rats.

Protein concentration and LDH in BALF. The protein concentration of BALF from control rats remained unchanged at all six times. Beginning at 24 hours and continuing through 72 hours, MCTP-treated rats had protein concentrations that were slightly but significantly increased (Figure 2A). BALF protein was elevated

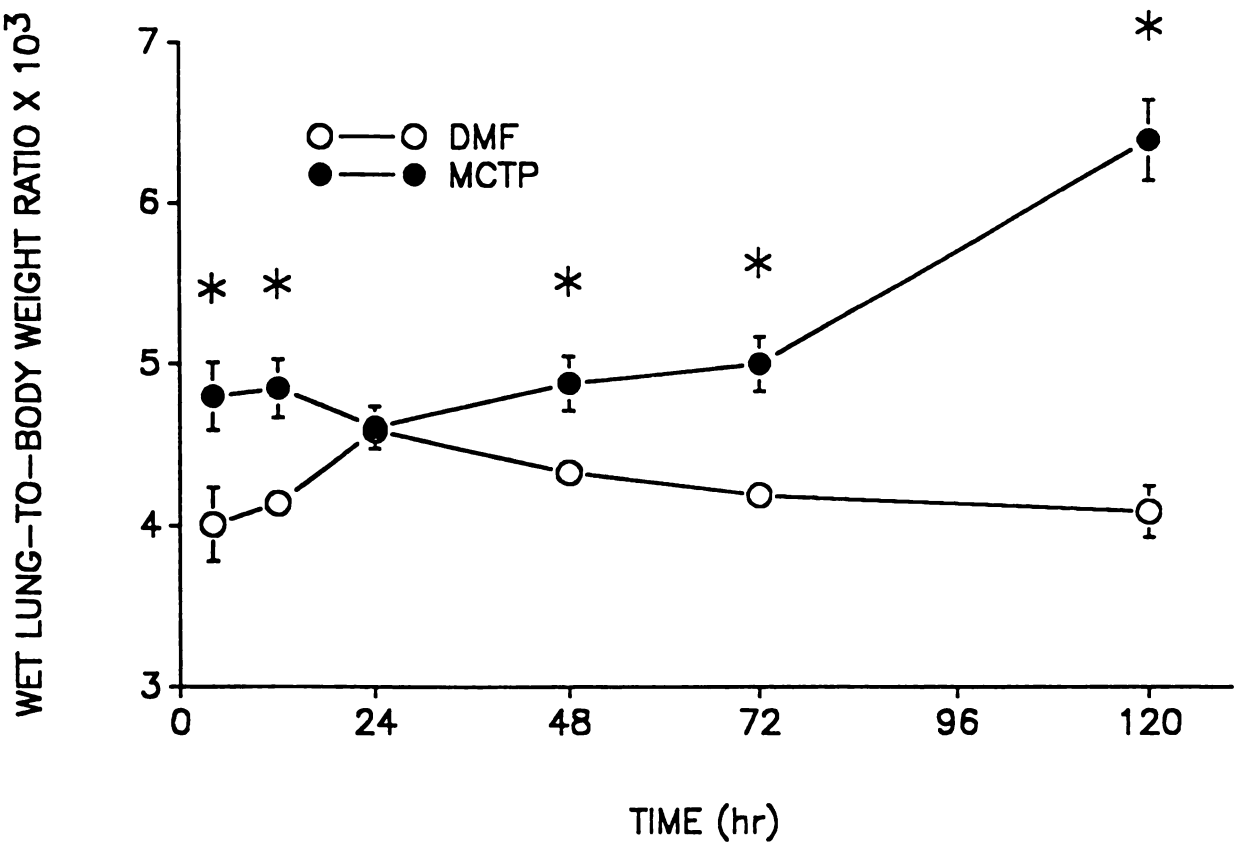


Figure 1: Effect of monocrotaline pyrrole (MCTP) on wet lung-to-body weight ratios (LW/BW). Rats received a single iv injection of MCTP (3.5 mg/kg) or N,N-dimethylformamide (DMF) vehicle at time 0. Values represent mean $\pm$ SE of 8 rats. *Significantly different from DMF control.

markedly at 120 hours post-treatment. DMF-treated control rats had relatively low and constant levels of LDH activity in BALF at all times. At 48 hours, MCTP-treated rats had significantly greater LDH activity in cell-free BALF as compared to controls (Figure 2B). The LDH activity continued to increase progressively thereafter.

Cytologic examination of BALF. The total nucleated cell counts in BALF of MCTP- and DMF-treated rats varied somewhat with time. At 48 hours post-injection, MCTP-treated rats had slightly but significantly fewer cells compared to controls. At 120 hours, the MCTP-treated rats had a pronounced elevation in total nucleated cell counts in BALF (Figure 3).

The BALF cytospin preparations from DMF-treated rats contained a normal array of cells (DeNicola *et al.*, 1981; Henderson, 1988) at all times (Figures 3 and 4A). The concentrated preparations were characterized by many quiescent macrophages on a clear background with few erythrocytes. There were few small lymphocytes and normal-appearing, segmented neutrophils scattered within the preparations. In certain BALF samples there were a few (less than 1%) eosinophils, mast cells, ciliated columnar epithelial cells or, occasionally, cuboidal epithelial cells.

The cytologic evaluations performed on BALF of MCTP-treated rats indicated numerous changes in cellular composition and cytomorphologic characteristics. The concentrated preparations collected at 4 hours were characterized by mixed populations of cells (Figure 4B). There were many macrophages, some of which appeared activated, on a clear background with few erythrocytes. The activated

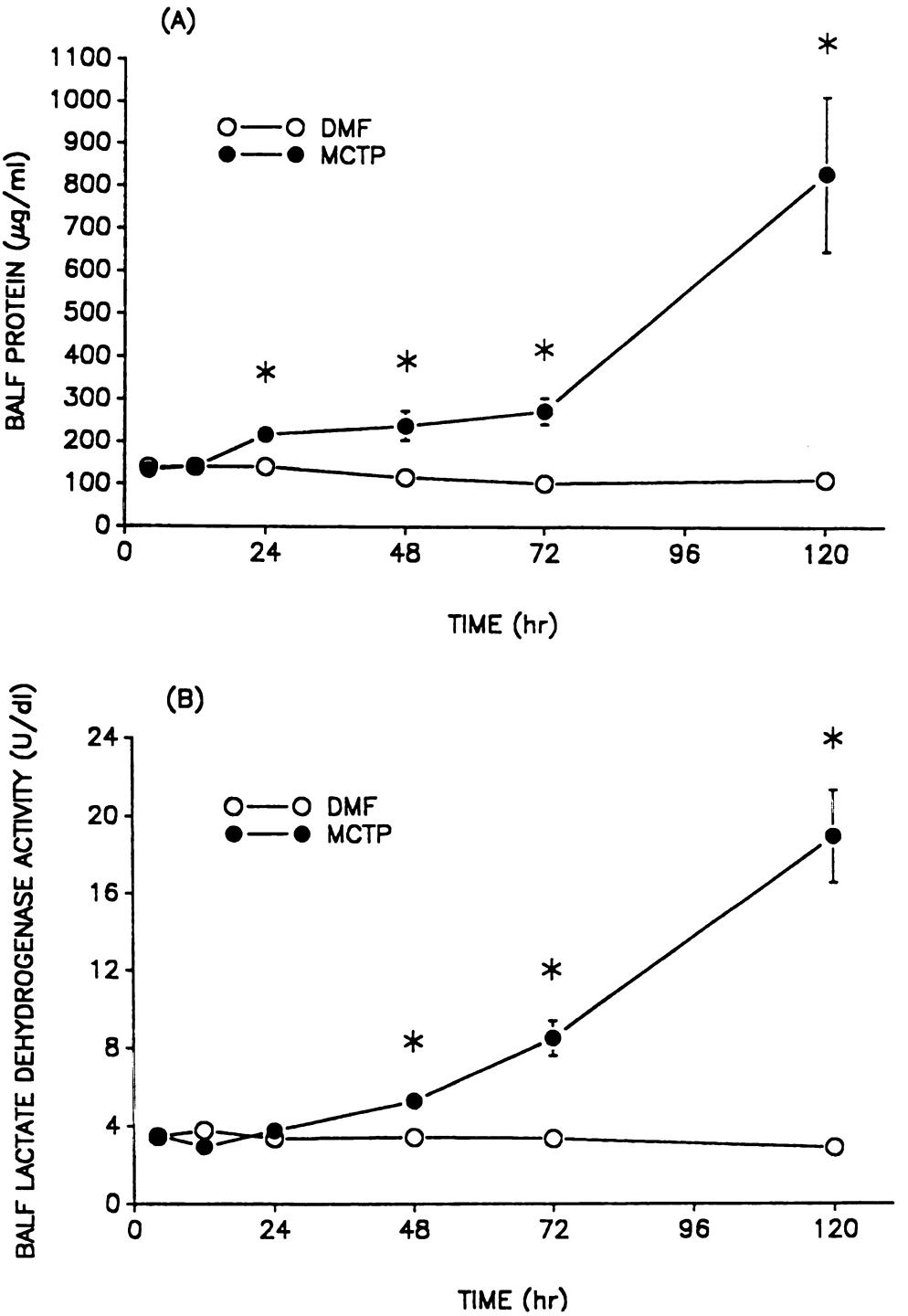


Figure 2: Effect of monocrotaline pyrrole (MCTP) on bronchoalveolar lavage fluid (BALF) **A**: protein concentration and **B**: lactate dehydrogenase (LDH) activity. Rats received a single iv injection of MCTP (3.5 mg/kg) or N,N-dimethylformamide (DMF) vehicle at time 0. Values represent means $\pm$ SE of 8 rats. Those points without SE bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

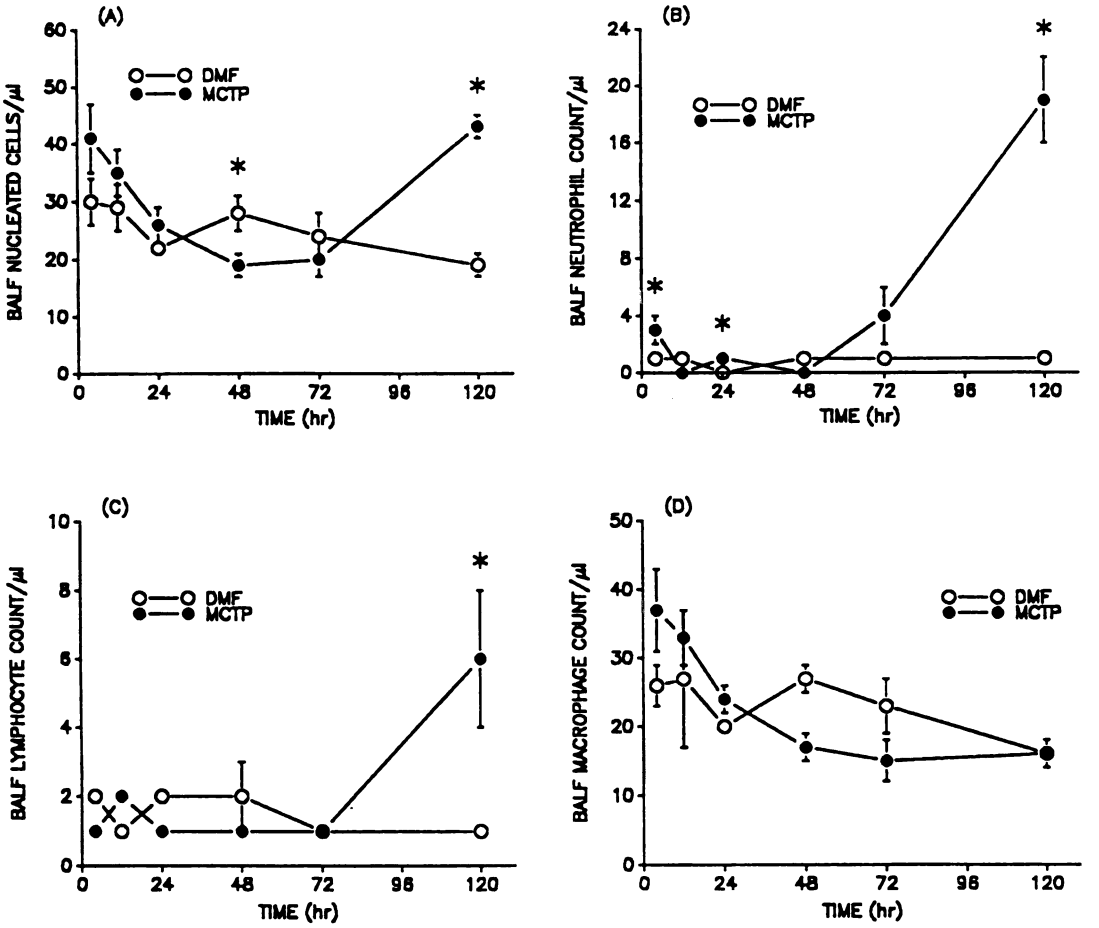


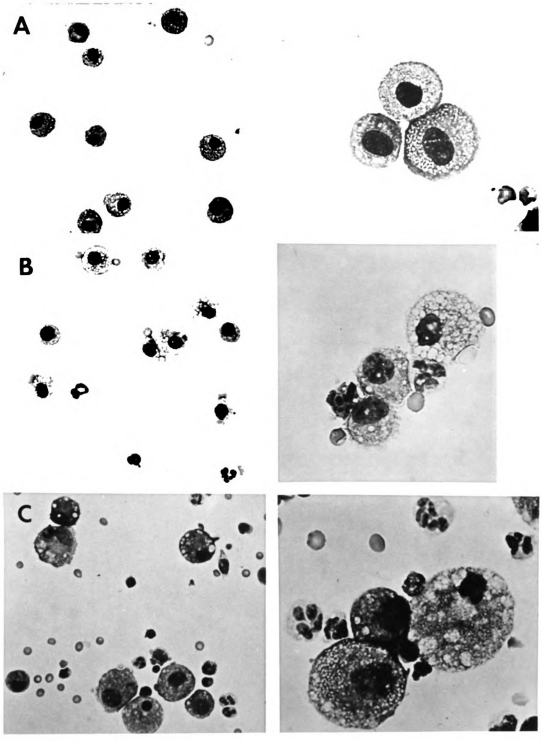
Figure 3: Effect of monocrotaline pyrrole (MCTP) on bronchoalveolar lavage fluid (BALF) total nucleated cell count (TNCC), neutrophil count, lymphocyte count, and macrophage count. Rats received a single iv injection of MCTP (3.5 mg/kg) or N,N-dimethylformamide (DMF) vehicle at time 0. Values represent means $\pm$ SE of seven to 8 rats. *Significantly different from DMF control.

macrophages were > 20 μm in diameter and had large amounts of cytoplasm that was less basophilic than that of normal-appearing cells. Some of the activated macrophages had many cytoplasmic vacuoles. There were also numerous segmented neutrophils and fewer small lymphocytes. Many segmented neutrophils had mild to moderate degrees of degenerate changes, including pyknosis, karyorrhexis and karyolysis (Duncan and Prasse, 1986; Perman *et al.*, 1979). Evidence of erythrophagocytosis was an inconsistent finding. Numbers of neutrophils were significantly greater at 4 hours in MCTP-treated than in control rat BALF. The numbers of other cell types were not different in BALF from control and MCTP-treated rats at this time.

At 12 hours, the segmented neutrophil count in BALF from MCTP-treated rats had decreased to that of controls, and cytomorphologic characteristics of neutrophil degeneration were not observed. The numbers of macrophages and lymphocytes were also similar to those from controls. Cytologic evidence of macrophage activation was a slightly more consistent finding in BALF from MCTP-treated rats. The cytologic composition of BALF from MCTP-treated rats killed at 24 and 48 hours was essentially unchanged from that obtained at 12 hours. At 72 hours, the cytologic composition was also similar, with the exception of an elevated number of normal-appearing, segmented neutrophils.

The BALF samples obtained at 120 hours post MCTP-injection had the most dramatic alterations in cell quantity (Figure 3) and morphology (Figure 4C). The concentrated preparations were characterized by mixed populations of cells on a

Figure 4: Low (left) and high (right) magnifications of photomicrographs of concentrated bronchoalveolar lavage fluid (BALF). **A:** Sample from a control rat treated with N,N-dimethylformamide (DMF) vehicle. **Left**, notice the cytologically quiescent macrophages on a clear background with few erythrocytes; **right**, the pulmonary macrophages were small, had eccentric round or oval nuclei and had abundant granular cytoplasm. **B:** Sample collected at 4 hours post-monocrotaline pyrrole (MCTP) treatment. **Left**, the preparations were characterized by many macrophages and lesser numbers of segmented neutrophils; **right**, several neutrophils had degenerate cytomorphologic characteristics and numerous macrophages had cytoplasmic vacuoles. **C:** Sample collected at 120 hours post-monocrotaline pyrrole (MCTP) treatment. **Left**, notice the approximately equal numbers of activated macrophages and degenerate neutrophils; **right**, some neutrophils had extremely pyknotic nuclei. The activated macrophages were very large, had many cytoplasmic vacuoles, and some contained phagocytic debris. Modified Wright's Stains, **left**: X332, **right**: X798.


A
B
C

clear background with a moderate number of erythrocytes. There were approximately equal numbers of segmented neutrophils and activated macrophages. Many neutrophils had degenerate morphology, and numerous macrophages contained phagocytized erythrocytes or other debris. In addition, there were numerous small lymphocytes scattered throughout the preparations. The numbers of neutrophils and lymphocytes were greater than those of control rat BALF. The numbers of macrophages were equal to those of controls.

To characterize further the neutrophil kinetics of BALF at early times, we performed another experiment in which groups of rats were killed at 1, 4, or 8 hours after treatment. The cytologic composition of control rat BALF appeared as described above. At 1 hour after MCTP treatment, the number of segmented neutrophils in BALF was significantly greater than that of controls (Figure 5). The number of segmented neutrophils from MCTP-treated animals tended to remain greater at 4 and 8 hours, but the trends were not statistically significant. Degenerate cytomorphologic changes were observed in the neutrophils of some MCTP-treated rats at all three times, but these were not observed in the neutrophils of control animals.

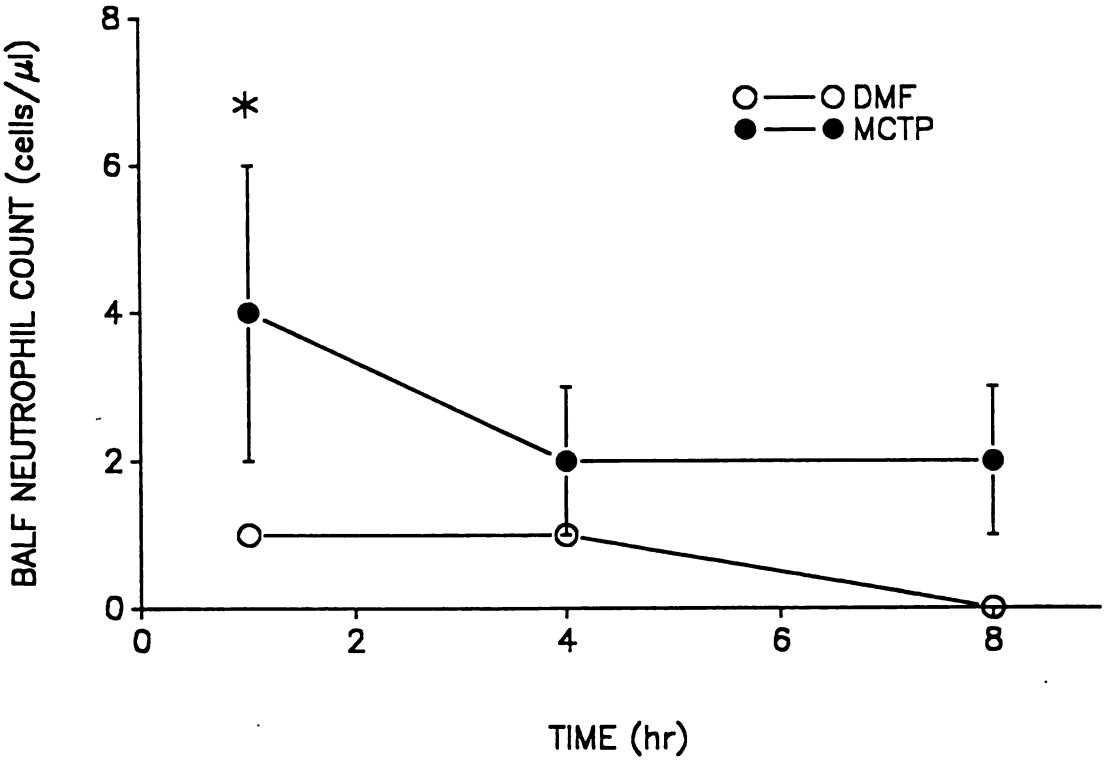


Figure 5: Effect of monocrotaline pyrrole (MCTP) on segmented neutrophil counts of bronchoalveolar lavage fluid (BALF). Rats received a single iv injection of MCTP (3.5 mg/kg) or N,N-dimethylformamide (DMF) vehicle at time 0. Values represent means $\pm$ SE of 4 rats. *Significantly different from DMF control.

DISCUSSION:

Cardiopulmonary lesions induced by administration of MCT to laboratory animals are well documented and have been the subject of numerous investigations (Hilliker *et al.*, 1982; Roth, 1981; Vincic *et al.*, 1989). Evidence of lung injury was observed within 7 days after toxin administration (Hilliker *et al.*, 1982; Shale *et al.*, 1986; Vincic *et al.*, 1989), and pulmonary hypertension and/or death occurred within 14-28 days (Stenmark *et al.*, 1985; Hilliker *et al.*, 1982; Sugita *et al.*, 1983). The overt lung injury that follows administration of pneumotoxic doses (ie. 60-105 mg/kg) is delayed in onset. Once manifest, the injury worsens progressively over a period of days to weeks.

MCT requires bioactivation by liver to pyrrolic metabolite(s) that are pneumotoxic (Mattocks and White, 1971). When injected iv into rats, one putative toxic metabolite, MCTP, causes lung injury and pulmonary hypertension similar to those caused by MCT itself (Butler, 1970). Lung injury from intravenous MCTP occurs at lower doses than from MCT, and the onset of overt lung injury may be somewhat earlier, perhaps because the time needed for absorption and bioctivation is eliminated. Time course studies describing the early lung injury induced by MCTP are relatively few in number. Typical structural lesions present 2-3 days after injection of a low dose of MCTP included multifocal areas of alveolar edema, congestion and/or hemorrhage. At 7 days, the edema and congestion were reduced slightly (Butler *et al.*, 1970). Ultrastructural studies of MCTP-induced lung lesions in rats revealed progressive endothelial cell lesions first observed at approximately

1 week post-injection (Butler, 1970; Reindel *et al.*, 1990). Bruner *et al.* (1983a) treated rats with MCTP and found that LDH activity and protein concentration in cell-free BALF were significantly elevated at 4 days post-treatment. Wet lung-to-body weight ratios and BALF total nucleated cell counts became elevated at 7 days. In most studies describing lung injury after exposure to low doses (3-5 mg/kg) of MCTP either very early times in the course of illness were not examined or few changes were reported.

In this investigation we evaluated several markers to detect the onset of lung injury. The earliest increase in LW/BW was detected at 4 hours post-MCTP. The most probable explanations for this increase were pulmonary edema, hemorrhage, and/or cellular inflammatory infiltrates. Vascular endothelial cells are injured following exposure to pyrrolizidine alkaloids (Huxtable *et al.*, 1978; Hilliker *et al.*, 1982; Ito *et al.*, 1988; Molteni *et al.*, 1984, 1989; Reindel *et al.*, 1990), and this may allow leakage of proteinaceous fluid into the pulmonary parenchyma. Pulmonary edema, hemorrhage, and congestion were observed in histologic analyses of rat lungs after treatment with MCTP (Butler, 1970; Butler *et al.*, 1970; Reindel *et al.*, 1990). Hurley and Jago (1975) also noted pulmonary edema within 6 hours in rats treated with larger doses of MCTP. Cellular inflammatory infiltrates must also be considered a possible cause for increases in LW/BW after MCTP treatment. Indeed, mild influxes of segmented neutrophils were detected in the BALF at 1 and 4 hours. These inflammatory cells may have interacted with endothelial cells or other indigenous lung cells to amplify edema via production of chemical mediators or

release of cellular enzymes. Cellular infiltrates may have made a greater contribution to increases in LW/BW as lung lesions intensified and as infiltrates increased in magnitude at 72 and 120 hours.

Increased protein content of BALF has been shown generally to be one of the more sensitive indicators of pulmonary inflammation (Henderson *et al.*, 1978b, 1985). In MCTP-treated rats we observed a mild increase in lavage fluid protein beginning at 24 hours. Increases in protein were subtle until 72 hours but were much more pronounced at 120 hours post-treatment. The excess protein in cell-free BALF of MCTP-treated rats probably resulted from increases in alveolar-capillary permeability (Henderson, 1988), again suggesting endothelial cell dysfunction.

LDH is a cytoplasmic enzyme which when released into the fluid lining the airways gives a sensitive but nonspecific indication of cellular injury (Henderson *et al.*, 1978a, 1979ab, Henderson, 1988; Roth, 1981). The lavage fluid of MCTP-treated rats had significantly greater LDH activity beginning at 48 hours. The LDH activity continued to increase at 72 and 120 hours post-treatment, suggesting continuing cellular leakage. The delayed and progressive release of LDH into BALF was also observed in endothelial cell monolayers treated with MCTP (Roth *et al.*, 1989b; Reindel and Roth, 1991). That the elevation in LDH activity in BALF was sustained suggests that injury to lung cells continues long after the initial insult by MCTP. On the other hand, that LDH activity in BALF was unaffected at earlier times when LW/BW and BALF protein concentration were elevated suggests that vascular leak occurs early, perhaps in the absence of overt cellular injury.

An evaluation of quantity, identity and morphologic characteristics of cells in body fluids or organ lavage fluids may detect developing lesions that may not be clinically apparent or easily identified by other methods (Koss and Durfee, 1961). The influx of neutrophils into BALF was shown to be the most sensitive indicator of early pulmonary inflammation in numerous other models of lung injury (DeNicola *et al.*, 1981; Henderson *et al.*, 1979ab, Henderson, 1988). Mild influxes of segmented neutrophils have been detected in the lungs of animals treated with the parent compound, MCT (Heath and Smith, 1978; Miller *et al.*, 1978; Czer *et al.*, 1986). As early as 2 hours after iv injection of MCT into dogs, the margination and penetration of segmented neutrophils into the pulmonary arterial and arteriolar walls was observed (Czer *et al.*, 1986). In this study of MCTP, mild infiltrates of segmented neutrophils were detected by cytologic analysis of BALF from MCTP-treated rats as early as one hour after MCTP injection. The specific stimulus for neutrophil infiltration into the lungs in this model remains unknown.

The neutrophilic infiltrates of rats killed at 1 and 4 hours were mild compared to those seen at 72 and 120 hours, the times when histologic evidence of lung injury has been readily apparent. Although statistically significant and consistently observed, the clinical significance of these early, mild infiltrates is open to debate. The influx of moderate to large numbers of neutrophils into the BALF is a well accepted and sensitive indicator of acute lung injury (DeNicola *et al.*, 1981; Henderson *et al.*, 1979ab, Henderson, 1988). Since MCTP causes a progressive form

of pulmonary damage, early and subtle cellular influxes may deserve additional attention.

The segmented neutrophils observed in the BALF at 4 and 120 hours post-MCTP treatment had some degree of degenerate cytomorphology which is usually associated with an environment hostile to neutrophil survival. These morphologic changes may result either from direct exposure to pathogens or from chemical insult (Perman *et al.*, 1979; Duncan and Prasse, 1986; Tvedten and Till, 1985; Cowell *et al.*, 1989). No infectious agents were observed in any of the cytologic preparations at any time, and septic pneumonia is not a direct consequence of MCTP administration. The significance of the degenerate cytomorphologic characteristics identified at 4 hours post-MCTP treatment is not entirely clear, but it suggests a pulmonary milieu antagonistic to the health and longevity of neutrophils. The degenerate cytomorphologic characteristics observed at 120 hours are more easily attributed to toxicity associated with foci of pulmonary parenchymal necrosis (Reindel *et al.*, 1990) and are associated with increased release of protein and LDH into the BALF at that time (Bruner *et al.*, 1983a; Reindel *et al.*, 1990).

Since endothelial cell dysfunction occurs in animals exposed to pyrrolizidine alkaloids, it is not unreasonable to hypothesize that compromised endothelial cells release mediators of neutrophil chemotaxis and activation, and that in excess these or other mediators may cause neutrophil cytotoxicity in rats treated with MCTP. Activated neutrophils are an excellent source of oxygen radicals capable of damaging tissue (Fantone and Ward, 1982). In addition, stimulated or damaged neutrophils

may release enzymes such as elastases, proteinases, and hydrolases which may amplify tissue destruction. Neutrophils were shown to be intricately involved in numerous other models of lung injury (Brigham and Meyrick, 1984; Till *et al.*, 1982, 1983). In the MCTP model, a role for neutrophils in the early lung injury has not been established. However, our data indicate that increases in LW/BW and BALF protein are associated with the presence of neutrophils in the BALF.

In conclusion, the results from this investigation indicate that MCTP-induced lung inflammation begins much earlier than has been reported previously. Pronounced changes in markers of lung injury, however, are delayed for several days after a single injection of a low dose of MCTP. Neutrophil kinetics in BALF of MCTP-treated rats are consistent with results of histologic analyses of lungs from rats treated with the parent compound, MCT. The involvement, if any, of early and mild influxes of segmented neutrophils into the lungs of rats treated with MCT or MCTP in the subsequent development of pulmonary hypertension remains to be determined.

CHAPTER THREE

AN EVALUATION OF PROCOAGULANT ACTIVITY IN THE PERIPHERAL BLOOD OF RATS TREATED WITH MONOCROTALINE PYRROLE

ABSTRACT:

Monocrotaline pyrrole (MCTP), a putative toxic metabolite of the naturally occurring, pyrrolizidine alkaloid, monocrotaline, causes pulmonary vascular thrombi that are associated with vascular remodeling, pulmonary hypertension and right cardioventricular hypertrophy in rats. The thrombi are composed of platelets and fibrin and occur in the absence of vascular necrosis. Since thrombosis may result from excessive procoagulant activity in the systemic circulation, we evaluated the hemostatic system of rats treated with MCTP to determine if a hypercoagulable state developed in the peripheral blood. Male, Sprague-Dawley rats received a single, bolus injection of MCTP (3.5 mg/kg) or an equal volume of the N,N-dimethyl-formamide (DMF) vehicle in the tail vein. Rats were killed at 1, 3, 5, 8, 11, or 14 days after toxin administration, and several markers of lung injury and hemostasis were evaluated. The protein concentration of cell-free bronchoalveolar lavage fluid (BALF) from MCTP-treated rats was slightly elevated at 1 and 3 days. At 5 days the elevation had become more pronounced, and values increased markedly thereafter. The wet lung-to-body weight ratio and lactate dehydrogenase activity of cell-free BALF from rats treated with MCTP were mildly increased at 3 days. Increases in these markers progressed at 5 days and values reached a plateau thereafter. MCTP-treated rats had moderately increased total nucleated cell counts in BALF at 5 days, and counts increased markedly thereafter. Right cardioventricular hypertrophy was first detected at 8 days in MCTP-treated rats and became more pronounced with time. The prothrombin time and modified prothrombin time of MCTP-treated rats were consistently greater than those of controls. Although statistically different, these values never exceeded the range of normal values. The activated partial thrombo-plastin time of MCTP and control rats was variable. Only at day 14 did MCTP-treated rats have significantly longer activated partial thromboplastin times than those of controls, and these values were within the normal range. Rats treated with MCTP had statistically significant elevations in antithrombin III activity at day 8 and of fibrinogen concentration and antithrombin III activity at day 11 that exceeded the normal range. Platelet numbers of both MCTP- and DMF-treated rats were greater than normal on day 1 but quickly returned to baseline. The plasminogen values of rats treated with MCTP were lower than controls on day 5 only. These results suggest that the pulmonary vascular thrombi induced by administration of MCTP to rats are not mediated by an excess in procoagulant activity in the peripheral blood.

INTRODUCTION:

Plants containing toxic pyrrolizidine alkaloids are ubiquitous, and their consumption by people or animals results in serious illness and death (Huxtable, 1980; Mattocks, 1986). Monocrotaline, perhaps the most studied pyrrolizidine alkaloid, is found in the seeds and leaves of the plant *Crotalaria spectabilis* (Bull *et al.*, 1968; Kay and Heath, 1969; Mattocks, 1986). Monocrotaline consumption by animals causes progressive lung injury associated with pulmonary hypertension and compensatory right cardioventricular hypertrophy (Valdivia *et al.*, 1967ab; Kay and Heath, 1969; Mattocks, 1968). The lesions induced by monocrotaline are similar to those observed in chronic pulmonary vascular diseases of humans (Voelkel and Reeves, 1979; Snow *et al.*, 1982; Meyrick and Reed, 1979a).

Following ingestion by rats, monocrotaline is bioactivated by the cytochrome P450 monooxygenase system of the liver to a pyrrolic metabolite that is responsible for the development of lesions (Mattocks and White, 1971). Administration of chemically synthesized monocrotaline pyrrole (MCTP) causes pulmonary and cardiovascular lesions similar to those of monocrotaline (Butler, 1970; Butler *et al.*, 1970; Bruner *et al.*, 1983a). Although subtle lung injury can be detected within hours after a single, iv administration of low doses (2-5 mg/kg) of MCTP (Schultze *et al.*, 1991a), major lung injury does not occur until 3-5 days after administration and becomes progressively more pronounced thereafter (Bruner *et al.*, 1983a, 1986; Reindel *et al.*, 1990). At 6-8 days increased pulmonary artery pressure is observed, and right cardioventricular hypertrophy occurs within 8-14 days (Bruner *et al.*, 1983a,

1986; Roth and Ganey, 1988; White *et al.*, 1989). Typical histologic lesions that develop over the 14 day course include muscularization of previously nonmuscular pulmonary arteries, medial hypertrophy of pulmonary arteries, endothelial cell swelling, and capillary thrombosis (Schoental and Head, 1955, Valdivia *et al.*, 1967ab; Merkow and Kleinerman, 1966; Hayashi and Lalich, 1967; Masugi *et al.*, 1965; Kay and Heath, 1969; Lalich *et al.*, 1977; Meyrick and Reid, 1982; Kay *et al.*, 1982b; Ghodsi and Will, 1981).

The pathogenesis of MCT(P)-induced lung injury and pulmonary hypertension is not completely understood. Dysfunction of the hemostatic system is associated with the development of lung injury, pulmonary hypertension and vascular remodeling in the MCTP-treated rat. Fibrin and platelet thromboembolic lesions, occurring in the absence of vascular necrosis, have been identified in the lungs of monocrotaline- and MCTP-treated rats by light microscopic and electron microscopic analysis (Merkow and Kleinerman, 1966; Valdivia *et al.*, 1967ab; Plestina and Stoner, 1972; Chesney *et al.*, 1974a; Lalich *et al.*, 1977; Meyrick *et al.*, 1980; Schraufnagel and Schmid, 1989). White and Roth (1988) demonstrated that ^{111}In-labeled platelets were sequestered in lungs of MCTP-treated rats at 8 and 14 days post-toxin injection. Pulmonary hypertension and right cardioventricular hypertrophy were attenuated by depletion of rat platelets (Hilliker *et al.*, 1984b; Ganey *et al.*, 1988), although lung injury was unaffected by platelet depletion (White *et al.*, 1989). These data suggest that platelets, a constituent part of the hemostatic system, are involved in the development of pulmonary hypertension. The endothelial cell is also an important

component of the hemostatic system, and endothelial cell degeneration and dysfunction result from exposure to monocrotaline or MCTP (Molteni *et al.*, 1986b; Reindel *et al.*, 1990; Kay *et al.*, 1982b; Meyrick *et al.*, 1980). Since endothelial cells produce several procoagulant and fibrinolytic substances, it is reasonable to hypothesize that MCTP may have a deleterious effect on the normal balance of these substances and, therefore, may contribute to dysfunction of the hemostatic system.

The occurrence of fibrin and platelet thrombi in lungs of rats treated with monocrotaline or MCTP suggests that dysfunction of the hemostatic system may be involved in the development of lesions. The formation of a thrombus within a blood vessel suggests either excessive procoagulant activity or defective fibrinolysis (Schafer, 1985; Haake and Berkman, 1986; Bauer and Rosenberg, 1987). The objectives of this study were to describe changes in the hemostatic system of rats treated with MCTP, to correlate them with markers of lung injury and cardioventricular hypertrophy, and to determine if excess procoagulant activity in the systemic circulation likely contributes to the lung injury and resultant right ventricular hypertrophy that occur in this model.

MATERIALS AND METHODS:

Animals. Male, Sprague-Dawley (CD-Crl:CD(R)(SD)BR VAF/Plus(TM)) rats purchased from Charles River Laboratories (Portage, MI) were delivered in air filtered crates. Animals weighing 200-250 grams were randomly assigned to groups and housed three per plastic cage on aspen chip bedding (Northeastern Products Corp., Warrensburg, NY) under conditions of controlled temperature (70° ± 2°F), humidity (40-70%) and light cycle (L:D 12:12). They were allowed food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and tap water *ad libitum*. Cages were maintained within isolators (Contamination Control Inc., Lansdale, PA) so that test animals breathed only high efficiency particulate (HEPA) filtered air for the duration of the study.

MCTP synthesis. Monocrotaline pyrrole, synthesized from monocrotaline (Trans World Chemicals, Washington, DC) via an N-oxide intermediate (Mattocks, 1969), was dissolved in the vehicle, N,N-dimethylformamide (DMF), and stored in the dark under nitrogen at 0°C.

Treatment protocol. Rats were immobilized in a plastic restrainer (Plas-Labs, Baxter Scientific, Romulus, MI) and given a single injection of either MCTP (3.5 mg/kg) or an equal volume of DMF vehicle (0.5 ml/kg) in a tail vein. Groups of 8-10 animals were evaluated at 1, 3, 5, 8, 11 or 14 days. Each rat was weighed and anesthetized with pentobarbital sodium (50 mg/kg ip), and the trachea was cannulated as described previously (Roth, 1981). A midline laparotomy was performed, and blood was drawn into a syringe containing 3.8% sodium citrate (9:1,

blood:sodium citrate). Well mixed blood was dispensed into plastic tubes seated in an ice bath. Blood samples were spun in an IEC Centra-7R refrigerated centrifuge at 600*g* and 4°C for 20 minutes. Plasma was removed and assays requiring immediate action were performed. Plasma aliquots were frozen at -20°C for later analysis.

Following phlebotomy, 0.1 ml heparin sulfate (1000 U/ml, Sigma Chemical Co., St. Louis, MO, No. H-7005) was injected into the vena cava. One minute later, the vena cava and aorta were severed and the rat was exsanguinated. The trachea, lungs and heart were excised *en bloc* and rinsed with saline. Rat lungs were lavaged twice with isotonic saline as described previously (Roth, 1981). Bronchoalveolar lavage fluid (BALF) aliquots were pooled. Total nucleated cell counts were performed using a hemocytometer. Cell-free supernatant fluid was prepared by spinning BALF samples in a centrifuge at 600*g* for 10 minutes. Protein concentration and lactate dehydrogenase activity were determined by the methods of Lowry *et al.* (1951) and Bergmeyer and Bernt (1974), respectively.

Lung weights were determined as described by Bruner *et al.* (1988). An indirect marker of pulmonary hypertension was determined by comparing the ratio of right cardiac ventricular weight to the combined weights of the septum and left cardiac ventricular wall (RV/(LV+S)) as described previously (Fulton *et al.*, 1952).

Platelet count. Well mixed, citrated, whole blood samples were diluted in a unopette microcollection system (Becton-Dickinson Division of Becton, Dickinson and Company Rutherford, NJ), and platelets were enumerated in a hemocytometer by using light microscopy.

Prothrombin time. The prothrombin time was measured by the method of Quick *et al.* (1935). Aliquots of tissue thromboplastin (Dade Thromboplastin-C, Baxter Healthcare Corp., Dade Division, Miami, FL) were used to activate Factor VII. The clotting times were measured on a BBL fibrometer coagulation timer (BBL Microbiology Systems, Baxter Healthcare Corp., McGaw Park, IL), reported in seconds and compared to those of 50 normal rats. A control of commercially prepared normal citrated plasma (Ci-Trol Coagulation Control, Level 1, American Dade, Aguada, Puerto Rico) and abnormal citrated plasma (Ci-Trol Coagulation Control, Level 3, American Dade, Aguada, Puerto Rico) were run with each set of specimens.

Modified prothrombin time. Normal rats have very short prothrombin times, and small variations in the concentrations of coagulation factors may be difficult to detect. To increase the sensitivity of the traditional prothrombin time, the test procedure was modified so that citrated rat plasma samples were analyzed using activator diluted at one half strength to give a longer clotting time.

Activated partial thromboplastin time. Rat activated partial thromboplastin times were determined using standard methods (Henry, 1979; Sirridge, 1974). A standardized volume of phospholipid (Thrombosil I Activated Partial Thromboplastin Time Reagent, Ortho Diagnostic Systems, Raritan, NJ) was added to each sample. Plasma aliquots were recalcified ($CaCl_2$ solution, 25 mmol/liter, Sigma Diagnostics, St. Louis, MO), and the clotting times were measured on a fibrometer. A normal

plasma and abnormal plasma control (Ci-Trol Coagulation Controls Levels 1 and 3, respectively, American Dade, Aguada, Puerto Rico) were run with each set of tests.

Fibrinogen concentration. The fibrinogen concentration of citrated plasma was determined by a modification of the thrombin clotting time as described by Clauss (1957). Rat plasma was diluted with Owrens's Buffer (American Dade, Aguada, Puerto Rico) and warmed to 37°C. An excess of thrombin (Data-Fi Thrombin Reagent, American Dade, Aguada, Puerto Rico) was added to the reaction mixture, and the clotting time was measured on a fibrometer. The times were compared to those of a standard fibrinogen solution (Data-FI Fibrinogen Calibration Reference, American Dade, Aguada, Puerto Rico) treated similarly. The thrombin clotting time of plasma was inversely proportional to the fibrinogen concentration (Clauss, 1957; Borgstrom, 1945; Jacobson, 1955). An aliquot of previously analyzed, pooled, normal rat plasma was run with each batch of test samples as a control.

Antithrombin III activity. The antithrombin III activity was determined with a chromogenic substrate assay (Coatest Antithrombin, Helena Laboratories, Beaumont, TX) that is a modification of the amidolytic heparin cofactor assay of Odegard (1975). The reaction was measured spectrophotometrically at 405 nm on a Flexigem analyzer (Electro-Nucleonics Inc., Fairfield, NJ). An aliquot of previously analyzed, pooled, normal rat plasma was run with each batch of test samples as a control.

Plasminogen concentration. The plasminogen concentration of citrated plasma was assayed using a modification of a chromogenic substrate assay (Owens

and Cimino, 1985). The reaction was measured at 405 nm on a Beckman spectrophotometer (Beckman Instruments Inc., South Pasadena, CA). An aliquot of previously analyzed, pooled, normal rat plasma was run with each batch of test samples as a control.

Normal values. Fifty male, Sprague-Dawley rats, weighing 200-300 grams that were obtained and housed as described above were anesthetized with pentobarbital sodium, and citrated plasma was collected for coagulation studies. Prothrombin time, modified prothrombin time and activated partial thromboplastin time were performed immediately. Remaining plasma was frozen in aliquots at -20°C. Antithrombin III activity and plasminogen and fibrinogen concentrations were determined at a later time. The normal range was defined as those values within two standard deviations from the mean.

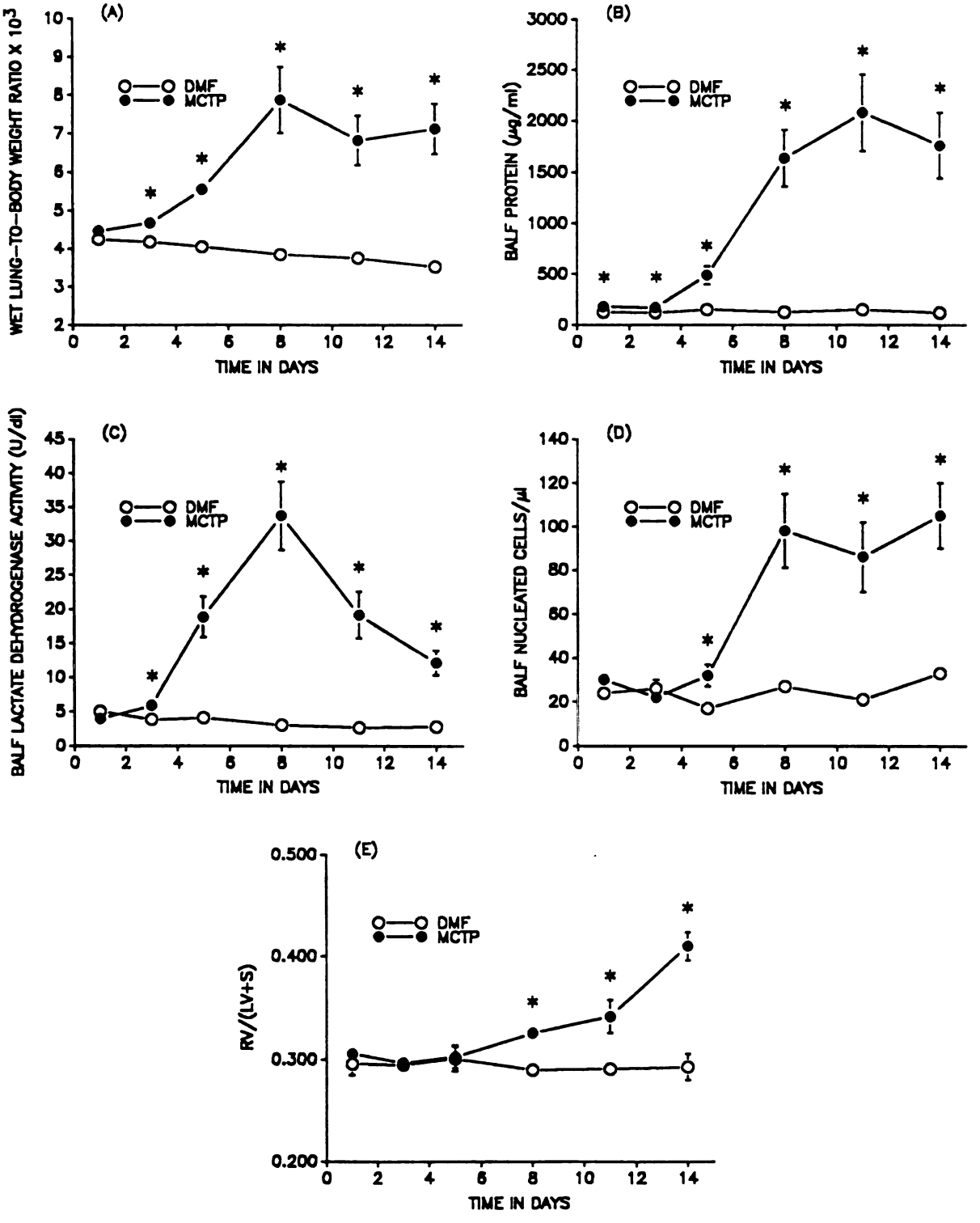
Statistical analysis. All determinations were run in duplicate and values were averaged. Data are expressed as means $\pm$ SE. Homogeneity of variance was assessed with the F-max test (Sokal and Rohlf, 1969). A log transformation was performed on any data sets for which variances were nonhomogeneous. If variances were homogeneous, data were compared using a two-way analysis of variance. Individual means were compared using the least significant difference test. Data with heterogeneous variances were analyzed with the Student *t* test for independent means (Steel and Torrie, 1980). The criterion for significance was $p < 0.05$.

RESULTS:

Lung Injury and Cardioventricular Hypertrophy

Rats treated with a single injection of MCTP had a mild increase in wet lung-to-body weight ratio at three days compared to DMF controls. The wet lung-to-body weight ratio increased further through day 8 and remained elevated thereafter (Figure 1). Rats treated with MCTP had slight but significant elevations in protein concentrations of cell-free BALF at 1 and 3 days (Figure 1). At 5 days, the protein in BALF was moderately elevated, and marked elevations were observed thereafter. MCTP-treated rats had mildly increased lactate dehydrogenase activity in cell-free BALF at 3 days compared to DMF controls (Figure 1). Lactate dehydrogenase activity increased markedly until 8 days, and then decreased steadily until 14 days, when it was still significantly elevated compared to that of controls. The total nucleated cell count of DMF-treated rats varied little throughout the time course of the experiment. MCTP-treated rats, however, had a slight increase in total nucleated cell counts of BALF at 5 days (Figure 1). Marked increases in total nucleated cell count were observed thereafter. A moderate increase in RV/(LV+S) was observed at 8 and 11 days in rats treated with MCTP (Figure 1), and this increase became pronounced by 14 days.

Figure 1: Effect of monocrotaline pyrrole (MCTP) on (A) wet lung-to-body weight ratio, (B) protein concentration of cell-free bronchoalveolar lavage fluid (BALF), (C) lactate dehydrogenase activity of cell-free BALF, (D) total nucleated cell counts of BALF, and (E) right cardioventricular hypertrophy. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 8-24 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.


(A)
WET LUNG-TO-BODY WEIGHT RATIO X 10^{-3}
DMF
MCTP
TIME IN DAYS
(B)
BALF PROTEIN (μg/ml)
DMF
MCTP
TIME IN DAYS
(C)
BALF LACTATE DEHYDROGENASE ACTIVITY (U/dl)
DMF
MCTP
TIME IN DAYS
(D)
BALF NUCLEATED CELLS/μl
DMF
MCTP
TIME IN DAYS
(E)
RV/(LV+S)
DMF
MCTP
TIME IN DAYS

Procoagulant Activity

Platelet numbers of MCTP-treated and DMF control rats were higher than normal at day 1 after treatment (data not shown). However, no significant differences between MCTP- and DMF-treated rats were observed at any time. MCTP-treated rats had a fairly consistent, but extremely mild, elevation in prothrombin time compared to controls (Figure 2). Although these elevations were statistically significant at several times, at no time did the prothrombin time exceed the normal range (Table 1). Since rats have extremely short clotting times, a modification of the prothrombin time was performed to increase the sensitivity of the test and enable detection of minor changes in the levels of specific procoagulant factors. The pattern and interpretation of the modified prothrombin time were similar to that of the prothrombin time (Figure 3). The test modification did identify a few rats with low levels of specific coagulation factors, but their values were deleted as outliers after the test for extreme means was performed (Dixon and Massey, 1969).

Activated partial thromboplastin times of MCTP-and DMF-treated rats were similar and varied little throughout the time course of the experiment (Figure 4). Only at day 14 was the activated partial thromboplastin time of MCTP-treated rats elevated; values approached but did not exceed the upper limit of the normal range (Table 1).

TABLE 1

NORMAL COAGULATION VALUES IN UNTREATED RATS

Test	Mean	Standard Deviation	Normal Range
Activated partial thromboplastin time	22.0 seconds	2.8	16.4 - 27.6
Antithrombin III	101%	8	85 - 117
Fibrinogen	175 mg/dl	17	141 - 209
Modified pro-thrombin time	17.5 seconds	2.9	11.7 - 23.3
Prothrombin time	11.9 seconds	0.6	10.7 - 13.1
Plasminogen	112%	37	38 - 186

Normal values——based on assay of citrated plasma from 50 clinically normal, male, Sprague-Dawley rats, weighing 200-300 grams

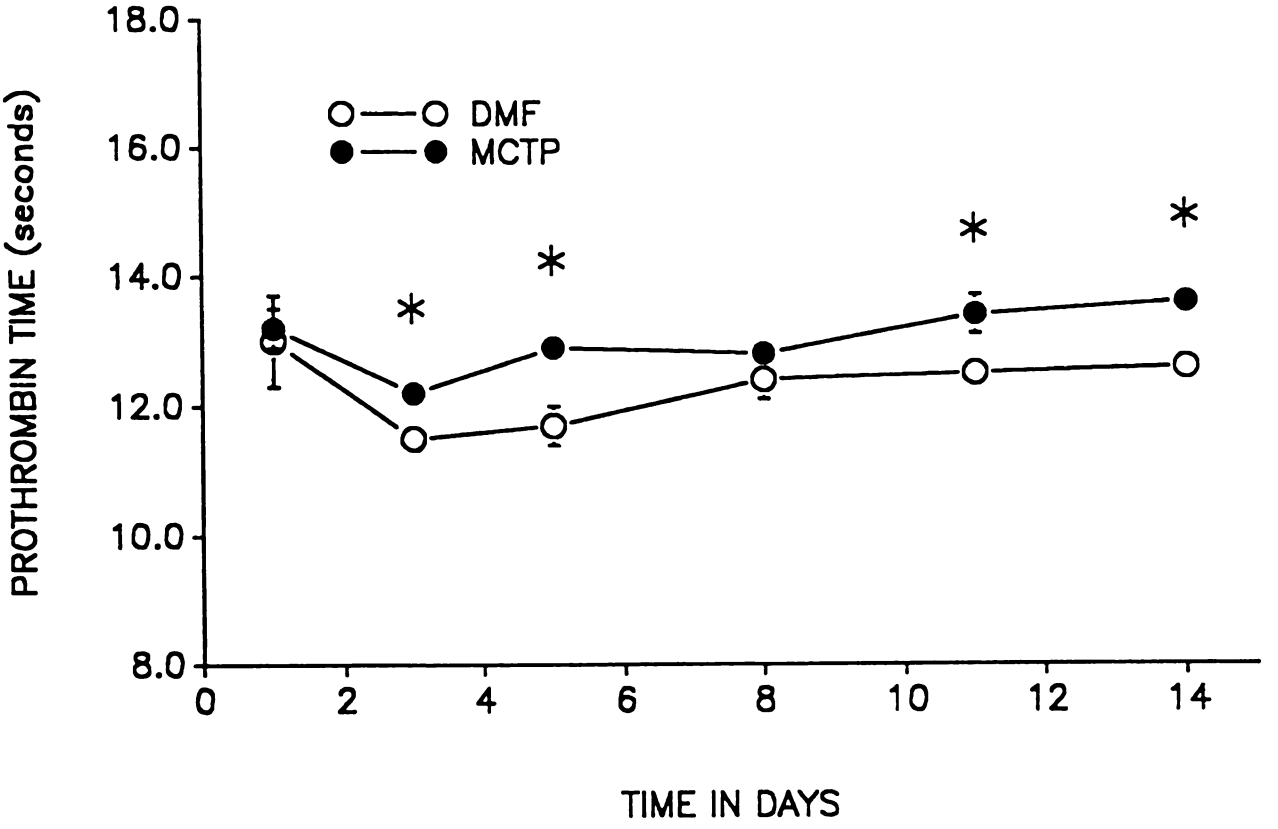


Figure 2: Effect of monocrotaline pyrrole (MCTP) on prothrombin time. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 8-24 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

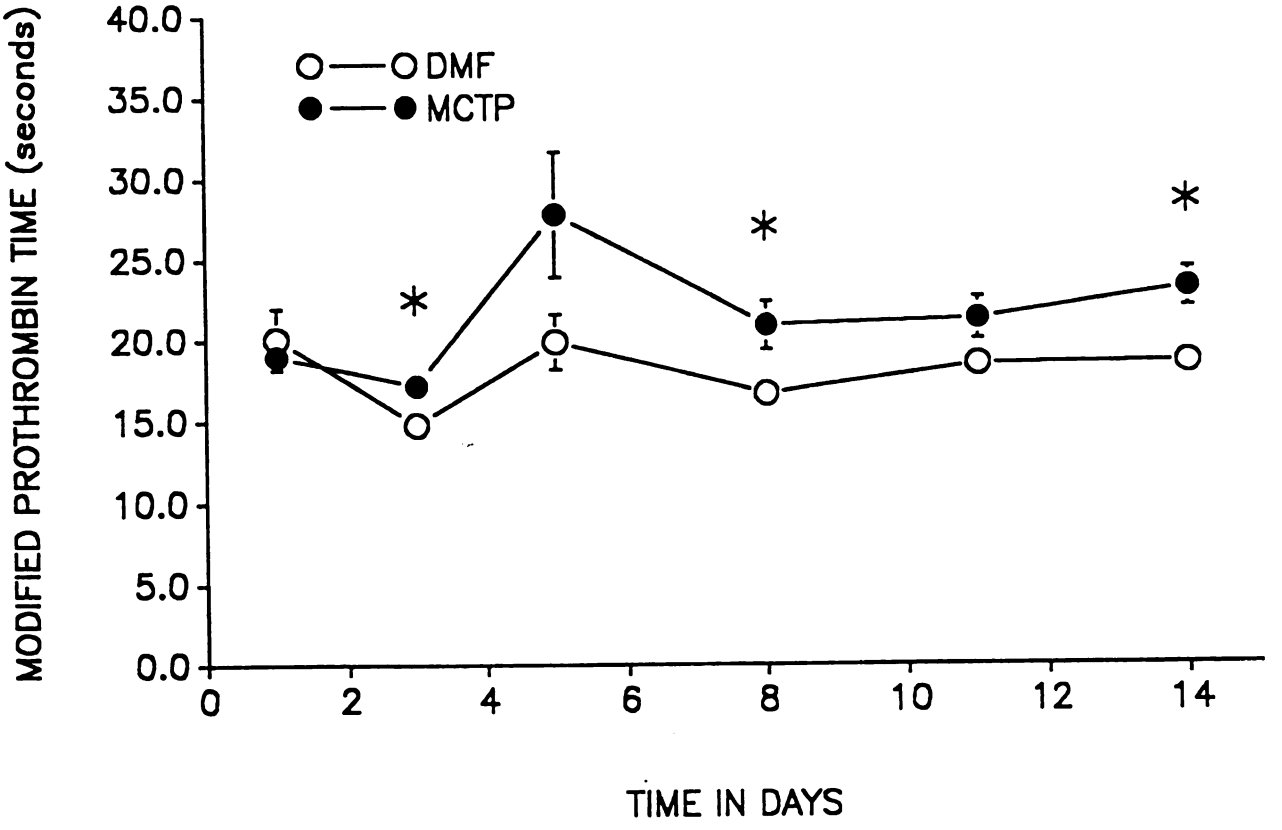


Figure 3: Effect of monocrotaline pyrrole (MCTP) on modified prothrombin time. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 8-24 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

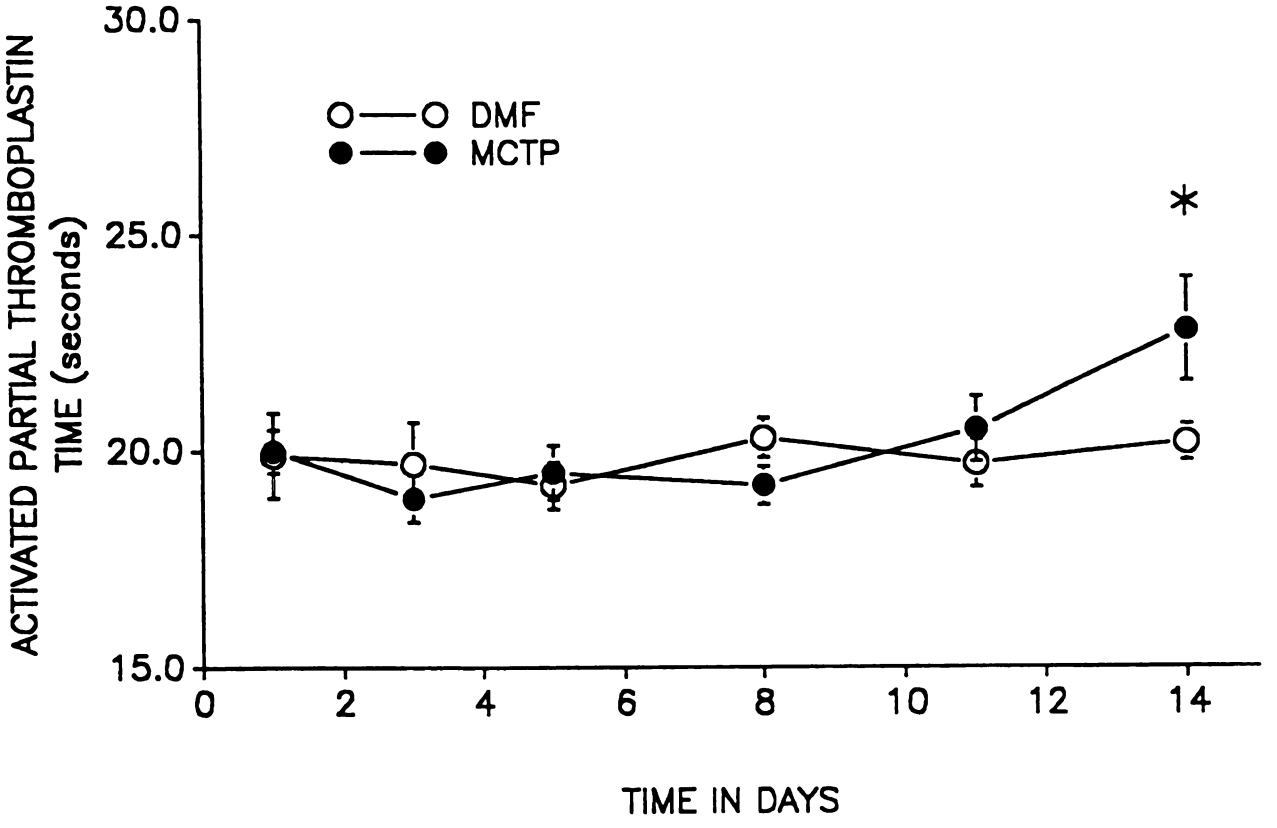


Figure 4: Effect of monocrotaline pyrrole (MCTP) on activated partial thromboplastin time. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 8-24 rats. *Significantly different from DMF control.

MCTP-treated rats showed a consistent trend for slightly decreased fibrinogen concentration during the first 4 days after MCTP administration (Figure 5). Beginning at day 5, the fibrinogen concentration of MCTP-treated rats began a rapid and steady elevation that continued through day 11. At day 11, the elevation became statistically significant, and the fibrinogen concentration exceeded the range of normal values (Table 1). By day 14, the fibrinogen level of MCTP-treated rats had decreased slightly.

The antithrombin III activity of MCTP-treated rats rose after day 3 and became statistically significant at days 8 and 11, at which time the activity level also exceeded the range of normal values (Figure 6 and Table 1). In a manner similar to the fibrinogen concentration, the antithrombin III activity of MCTP-treated rats decreased slightly at day 14.

The plasminogen concentration of both DMF- and MCTP-treated rats was lower than the mean of the normal values on day 1 (Figure 7). The plasminogen concentration of DMF-treated rats showed a rapid return toward the mean normal value on day 3, and concentrations varied only moderately thereafter. The plasminogen concentration of MCTP-treated animals showed a similar trend except that values were significantly decreased when compared to controls on day 5. From days 8 to 14, MCTP-treated plasminogen values did not differ from controls.

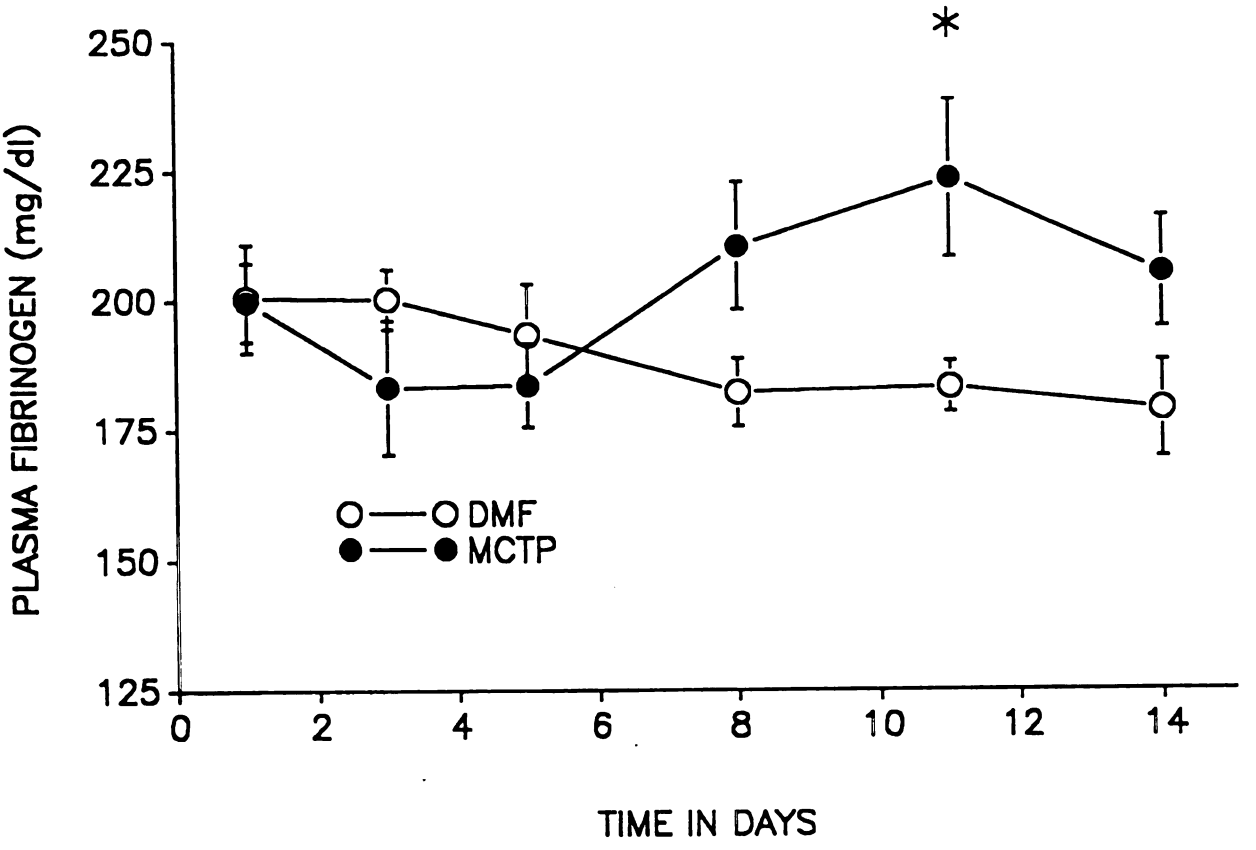


Figure 5: Effect of monocrotaline pyrrole (MCTP) on plasma fibrinogen concentration. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 8-24 rats. *Significantly different from DMF control.

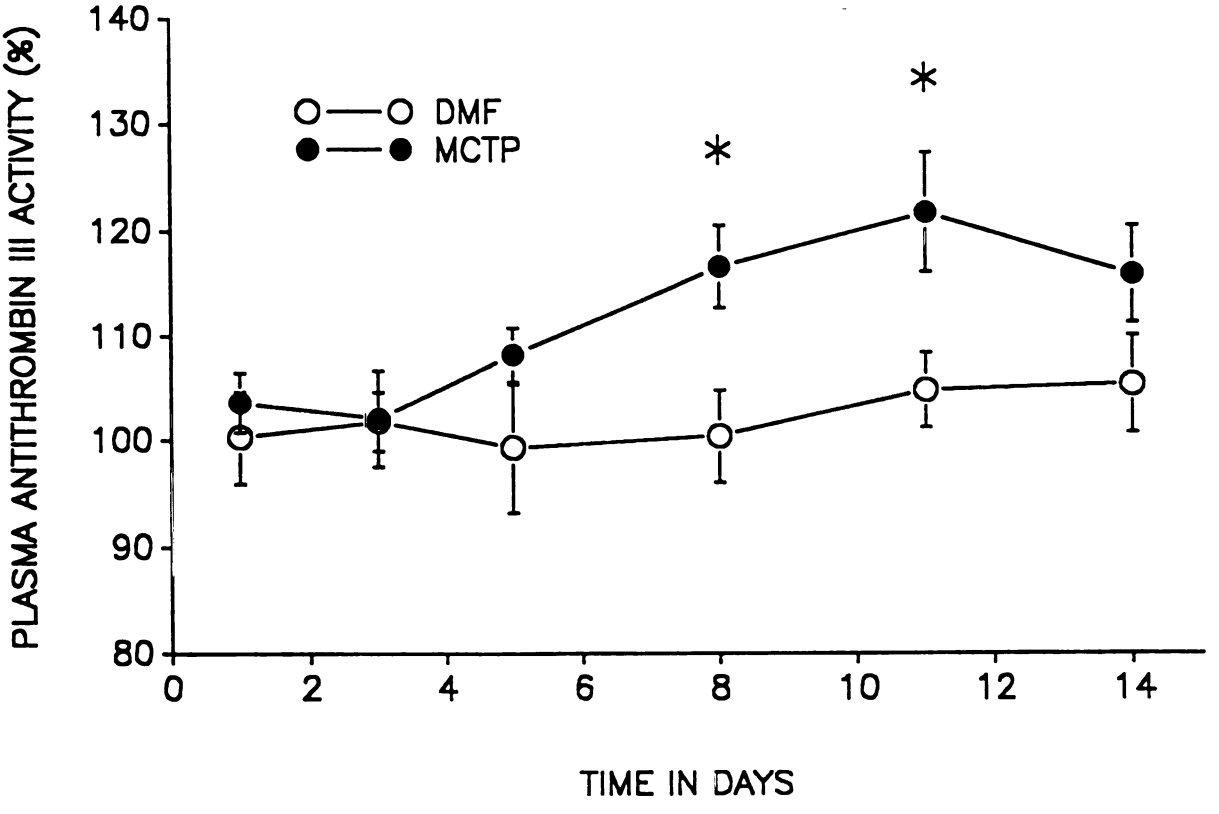


Figure 6: Effect of monocrotaline pyrrole (MCTP) on plasma antithrombin III activity. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 8-24 rats. *Significantly different from DMF control.

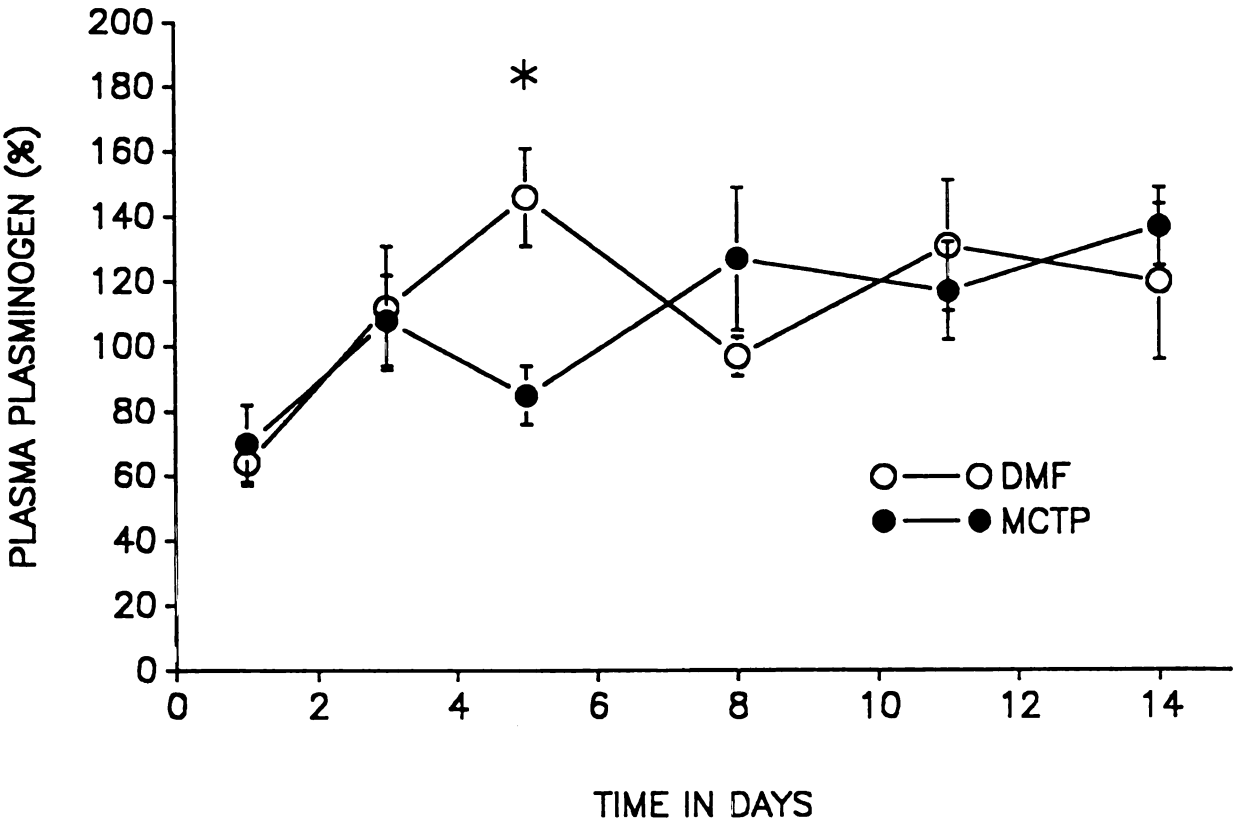


Figure 7: Effect of monocrotaline pyrrole (MCTP) on plasma plasminogen concentration. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean ± SE of 8-24 rats. *Significantly different from DMF control.

DISCUSSION:

Fibrin and platelet thrombi occur in lungs of animals with monocrotaline- or MCTP-induced lung injury and pulmonary hypertension (Valdivia *et al.*, 1967ab; Butler, 1970; Plestina and Stoner, 1972; Chesney *et al.*, 1974a; Lalich *et al.*, 1977; Merkow and Kleinerman, 1966; Meyrick *et al.*, 1980). Recently, Schraufnagel and Schmid (1989) demonstrated a decrease in pulmonary capillary density in rats given monocrotaline. Analysis of histologic sections revealed fibrin thrombosis of small capillaries as the reason for the decreased capillary filling (Schraufnagel and Schmid, 1989). Lalich *et al.* (1977) have suggested that fibrin thrombosis was critical for development of pulmonary hypertension in rats treated with MCTP, and thrombi and pulmonary emboli have been suggested as a cause for primary pulmonary hypertension in humans (Harrison *et al.*, 1966; Wyngaarden and Smith, 1985).

The formation of fibrin thrombi in the absence of frank vascular necrosis may be due to an excess in systemic procoagulant activity or decreased fibrinolytic activity within the blood. We sought to determine if rats treated with MCTP developed a systemic coagulation disorder such as disseminated intravascular coagulation or a secondary or acquired hypercoagulable state in the peripheral blood (Schafer, 1985; Haake and Berkman 1986; Bauer and Rosenberg, 1987). In addition to a battery of markers to evaluate lung injury and cardioventricular hypertrophy, we selected a variety of screening tests (prothrombin time, modified prothrombin time, activated partial thromboplastin time) as well as several specific tests (antithrombin III activity, plasminogen concentration, fibrinogen concentration, platelet concentration) to

provide an overview of the hemostatic system and to assess the probability that a hypercoagulable state existed in the peripheral blood. Standard test methods were modified if necessary for use in rats, and a normal range for reference values and determination of biologic significance was established (Table 1). For this study, those values that exceeded the normal range ($\pm$ 2 standard deviations from the mean) were taken to represent "biologically meaningful differences" in coagulant activity in peripheral blood.

Several markers of lung injury were monitored to compare the development of pneumotoxicity with temporal changes in coagulant activity. Alterations in wet lung-to-body weight ratio, protein concentration and lactate dehydrogenase activity in cell-free BALF and BALF total nucleated cell counts (Figure 1) confirmed that marked lung injury is delayed for several days after a single exposure to MCTP. Right cardioventricular hypertrophy occurred in rats as early as 8 days after MCTP treatment and increased through day 14 (Figure 1).

Platelets have been closely linked to the development of pulmonary hypertension in MCTP-treated rats and may serve as one source of mediators that trigger procoagulant activity. Because both MCTP- and DMF-treated rats had similar increases in blood platelet counts at day 1, and since rats treated with DMF do not form significant numbers of pulmonary thrombi (Lalich *et al.*, 1977), the initial increase in platelet numbers in MCTP- and DMF-treated rats at day 1 (data not shown) was likely due to handling or some other common stress and was likely of little consequence.

The prothrombin time was used as a measure of the activity of the extrinsic and common pathways of coagulation, including Factors I, II, V, VII, and X (Williams, 1983b; Thompson and Harker, 1983; Francis and Marder, 1987). Developing lung lesions are excellent sources of tissue thromboplastin that may trigger the extrinsic coagulation cascade. Excessive cellular inflammatory infiltrates into the lung could also contribute to procoagulant activity in this model and shorten the prothrombin time of rats with significant lesions. The activated partial thromboplastin time was used as a rapid screen of the activity of the intrinsic and common pathways of coagulation, including Factors XII, XI, IX, VIII, X, V, II and I (Williams, 1983b; Thompson and Harker 1983; Francis and Marder, 1987). Although MCTP-treated rats had mild elevations in the prothrombin time and activated partial thromboplastin time the clinical significance is doubtful, since at no time did the prothrombin time or activated partial thromboplastin time of the MCTP-treated rats exceed the normal range (Figures 2, 4 and Table 1).

The early trend for slightly lower fibrinogen levels in MCTP-treated rats is consistent with the conversion of fibrinogen to fibrin and deposition of small thrombi in capillaries of the lung (Figure 5). The rise in fibrinogen that began after day 5 may have been due to the increased production and mobilization of interstitial fibrinogen that occurs during inflammation (Haake and Berkman, 1986). The plasma antithrombin III activity was measured to see if MCTP treatment would deplete the pool of inhibitors of coagulation. Treatment with MCTP did not deplete antithrombin III activity. Like fibrinogen, antithrombin III is an acute phase inflam-

matory protein, and its activity followed a similar course throughout the 14 day duration of the study (Figure 6). The initial decrease in plasminogen concentration in both DMF- and MCTP-treated animals is consistent with a conversion of the zymogen, plasminogen, to the active enzyme, plasmin, in response to deposition of fibrin within the lung (Figure 7). The longer time period required for normalization of plasminogen concentrations in the MCTP-treated animals suggested the possibility of ongoing conversion of fibrinogen to fibrin and subsequent conversion of plasminogen to plasmin.

Results obtained in this investigation indicated that there were functional alterations in the hemostatic system of rats treated with MCTP. Several of these alterations, while statistically significant, were likely not of great biologic importance in regard to the development of a hypercoagulable condition in the systemic circulation since they were only modest in magnitude and therefore unlikely to be the cause of or result from fibrin and platelet thrombi formed in the lungs. Although prolonged elevations in plasma fibrinogen, thrombocytoses and decreased plasminogen concentration have been associated with hypercoagulable states and thrombosis in human beings, the transient and modest changes in these markers in the peripheral blood of rats treated with MCTP do not suggest systemic hypercoagulability. We conclude that treatment with MCTP does not cause excessive procoagulant activity in the peripheral blood of rats. These results raise the possibility that pulmonary thrombi in this model may result from a deficit in the fibrinolytic system or from

effects that increase coagulation locally in the pulmonary microcirculation but may not be reflected in the peripheral blood as a condition of systemic hypercoagulability.

CHAPTER FOUR

FIBRINOLYTIC ACTIVITY IN BLOOD AND LUNGS OF RATS TREATED WITH MONOCROTALINE PYRROLE

ABSTRACT:

Monocrotaline pyrrole (MCTP), a putative toxic metabolite of the pyrrolizidine alkaloid, monocrotaline, causes pulmonary vascular thrombi that are associated with vascular remodeling, pulmonary hypertension and right cardioventricular hypertrophy in rats. It is possible that such thrombi contribute to the lung injury and pulmonary hypertension in this model. A previous study indicated that rats treated with MCTP did not have excessive procoagulant activity in the peripheral blood. Since thrombosis may also result from insufficient fibrinolytic activity in the systemic circulation, we evaluated the fibrinolytic system of rats given MCTP. Male, Sprague-Dawley rats were given a single injection of MCTP (3.5 mg/kg) or an equal volume of N,N-dimethylformamide (DMF) vehicle in the tail vein and were killed at 1, 3, 5, 8, 11 or 14 days after toxin administration. Several markers of lung injury and fibrinolysis were measured. Lung injury was evident approximately three days after administration of MCTP and became more pronounced with time. In MCTP-treated rats, right heart hypertrophy was observed at 11 days and became more pronounced at 14 days. There was no change in the plasminogen concentration or in the activities of tissue plasminogen activator or α-2-antiplasmin in blood throughout the time course. Beginning at Day 8 and continuing through Day 14, there was an increase in the activity of plasminogen activator inhibitor in blood of rats that received MCTP. In addition, we evaluated the fibrinolytic activity of lung tissue slices. Rats treated with MCTP had a significant decrease in fibrinolytic activity of lung tissue at Day 3. On Day 5, the fibrinolytic activity returned toward normal, but beginning at Day 8 and progressing through Day 14, MCTP-treated rats had a marked decrease in fibrinolytic activity in lung tissue. In summary, MCTP treatment of rats decreases the fibrinolytic activity of lung tissue relatively early after exposure to the toxicant and before the onset of pulmonary hypertension. The change is reflected slightly later in plasma. These alterations in fibrinolytic activity may explain why fibrin thrombi form in the lungs of rats treated with MCTP.

INTRODUCTION:

The seeds and foliage of the plant, *Crotalaria spectabilis*, contain the toxic pyrrolizidine alkaloid, monocrotaline (MCT) (Bull *et al.*, 1968; Heath, 1969; Kay and Heath, 1969; Mattocks, 1986). When administered to rats, MCT causes pneumotoxicity and pulmonary hypertension that are delayed and progressive (Valdivia *et al.*, 1967a; Meyrick *et al.*, 1980; Hayashi *et al.*, 1984). The pulmonary lesions are characterized by perivascular edema, fibrin and platelet thrombosis of the pulmonary microvasculature and endothelial cell hypertrophy and hyperplasia (Valdivia *et al.*, 1967ab). These lesions worsen with time and, eventually, hypertrophy and hyperplasia of the medial layer of pulmonary vessels and extension of smooth muscle to normally nonmuscular pulmonary arterioles occur (Meyrick *et al.*, 1980; Ghodsi and Will, 1981; Meyrick and Reid, 1982). The vascular remodeling is accompanied by sustained increases in pulmonary arterial pressure and compensatory right cardioventricular hypertrophy (Chesney *et al.*, 1974a; Meyrick *et al.*, 1980; Hilliker *et al.*, 1982). The pathophysiology of the pneumotoxicity and pulmonary hypertension is similar to certain pulmonary vascular diseases of people, such that the MCT-treated rat provides an animal model for the study of primary pulmonary hypertension (Voelkel and Reeves, 1979) and the proliferative phase of the adult respiratory distress syndrome (Snow *et al.*, 1982).

To cause toxicity, MCT must be bioactivated by the hepatic cytochrome P450 monooxygenase system (Mattocks, 1968; Mattocks and White, 1971). Intravenous administration of chemically synthesized monocrotaline pyrrole (MCTP), the putative

toxic metabolite of MCT, causes pneumotoxicity, pulmonary hypertension and right cardioventricular hypertrophy in rats similar to those caused by the parent compound (Butler, 1970; Butler *et al.*, 1970; Bruner *et al.*, 1983a).

The mechanisms by which MCT- or MCTP-induced pnuemotoxicity and pulmonary hypertension occur have not been elucidated completely. Numerous investigators have recognized fibrin and platelet accumulations in the pulmonary microvasculature and have hypothesized that they may be critical for the pulmonary hypertension and *cor pulmonale* that ensue (Turner and Lalich, 1965; Valdivia *et al.*, 1967ab; Chesney *et al.*, 1974a; Lalich *et al.*, 1977; Schraufnagel and Schmid, 1989).

Thrombosis implies some alteration in the balance between one or more components of the hemostatic system. Analyses of the components of hemostasis in MCT- or MCTP-induced pulmonary hypertension are few. A recent investigation of blood from rats treated with MCTP (Schultze *et al.*, 1991b) revealed only small and transient alterations in procoagulant activity, indicating that treatment with MCTP was not a likely cause of the fibrin and platelet microvascular thrombosis in the lungs. Since thrombosis may also result from decreased fibrinolysis (Schafer, 1985; Haake and Berkman, 1986; Bauer and Rosenberg, 1987), we evaluated the fibrinolytic system of rats given MCTP.

The objectives of this investigation were to understand the development of changes in the fibrinolytic system of rats treated with MCTP, to relate them to markers of lung injury and cardioventricular hypertrophy and to determine if

alterations in fibrinolysis are consistent with a potential role for the fibrinolytic system in the genesis of pneumotoxicity and pulmonary hypertension in this model.

MATERIALS AND METHODS:

Animals. Male, Sprague-Dawley (CD-Crl:CD*(SD)BR VAF/Plus*) rats purchased from Charles River Laboratories (Portage, MI) were delivered in air filtered crates. Animals weighing 200-225 grams were randomly assigned to groups and housed three per plastic cage on aspen chip bedding (Northeastern Products Corporation, Warrensburg, NY) under conditions of controlled temperature (70° ± 2°F), humidity (40-70%) and light cycle (Light:Dark 12:12). They were allowed food (Wayne Lab-Blox, Allied Mills, Chicago, IL) and tap water *ad libitum*. Cages were maintained within isolators (Contamination Control Inc., Lansdale, PA) so that test animals breathed only high efficiency particulate (HEPA) filtered air for the duration of the study.

MCTP synthesis. Monocrotaline pyrrole, synthesized from monocrotaline (Trans World Chemicals, Washington, DC) via an N-oxide intermediate (Mattocks, 1969), was dissolved in the vehicle, N,N-dimethylformamide (DMF), and stored in the dark under nitrogen at 0°C.

Treatment protocol. Rats were immobilized in a plastic restrainer (Plas-Labs, Baxter Scientific, Romulus, MI) and given a single injection of either MCTP (3.5 mg/kg) or an equal volume of DMF vehicle (0.5 ml/kg) in a tail vein. Groups of 6 animals were evaluated at 1, 3, 5, 8, 11 or 14 days. Each rat was weighed and anesthetized with pentobarbital sodium (50 mg/kg ip), and the trachea was cannulated as described previously (Roth, 1981). A midline laparotomy was performed, and blood was drawn into a syringe containing 3.8% sodium citrate (9:1,

blood:sodium citrate). Well mixed blood was dispensed into plastic tubes seated in an ice bath. Blood samples were spun in an IEC Centra-7R refrigerated centrifuge at 600*g* and 4°C for 20 minutes. Plasma was removed and aliquots were frozen at -20°C for later analysis. For assays of tissue plasminogen activator (tPa), 1 ml anticoagulated blood was mixed immediately with 1 ml of cold acetate buffer working solution (sodium acetate, 0.2 mol/l, pH 3.9) and spun in a centrifuge as described above. The acidified plasma was removed and 10 μl 1M HCl was added to each 150 μl aliquot of acidified plasma. Samples were then frozen for later analysis.

Following phlebotomy, 0.1 ml heparin sulfate (1000 U/ml, Sigma Chemical Co., St. Louis, MO, No. H-7005) was injected into the vena cava. One minute later, the vena cava and aorta were severed and the rat was exsanguinated. The trachea, lungs and heart were excised *en bloc* and rinsed with saline. Rat lungs were lavaged twice with isotonic saline as described previously (Roth, 1981). Lavage aliquots were pooled. Total nucleated cell counts were performed by direct enumeration of well-mixed bronchoalveolar lavage fluid (BALF) in a hemocytometer. Cell-free supernatant fluid was prepared by spinning BALF samples in a centrifuge at 600*g* for 10 minutes. Protein concentration and lactate dehydrogenase (LDH) activity were determined by the methods of Lowry *et al.*, (1951) and Bergmeyer and Bernt (1974), respectively.

Lung weights were determined as described by Bruner *et al.* (1988). An indirect marker of pulmonary hypertension was determined by comparing the ratio

of right cardiac ventricular weight to the combined weights of the septum and left cardiac ventricular wall (RV/(LV+S)) as described previously (Fulton *et al.*, 1952).

Rats from which lungs were used for measurement of fibrinolytic activity were treated as above with the following exception. At death, the thoracic cavity was incised on the ventral midline, a cannula was placed in the pulmonary artery and secured with a simple cerclage suture. The lungs were excised *en bloc* and rinsed as described above. After lavage, the pulmonary vasculature was cleared of blood by flushing the cannula with 10 ml of saline while the lungs were gently inflated and deflated with room air for a 30 second period. The lung lobes were severed from the bronchial tree, placed in plastic centrifuge tubes and frozen rapidly by immersion of the tube in a bath of acetone and dry ice. Samples were stored at -20°C for later analysis.

Plasminogen concentration. The plasminogen concentration of citrated plasma was assayed using a modification of a chromogenic substrate assay (Owens and Cimino, 1985). The reaction was measured at 405 nm on a Beckman spectrophotometer (Beckman Instruments Inc, South Pasadena, CA). An aliquot of previously analyzed, pooled normal rat plasma and a commercially prepared control sera (Chrom-Trol, Kabi Diagnostica, Stockholm, Sweden) was run with each batch of test samples as controls.

α-2-antiplasmin activity. The activity of α-2-antiplasmin in citrated plasma was determined in microtiter plates (Corning Glass Works, Corning, NY) with a chromogenic substrate assay (COATEST Antiplasmin, Helena Laboratories,

Beaumont, TX). A commercially available control serum (Chrom-Trol, Helena Laboratories, Beaumont, TX) was analyzed with each set of specimens. The reaction was measured at 405 nm on an EIA microplate reader (BIO-TEK Instruments, Inc., Queue Systems, Parkersburg, WV).

Tissue plasminogen activator (tPa) activity. The tPa activity was determined in microtiter plates using a chromogenic substrate assay (COA-SET t-Pa, Helena Laboratories, Beaumont, TX). An aliquot of previously analyzed, pooled normal rat plasma was run with each batch of samples as an assay control. In short, aliquots of acidified plasma had plasminogen activated to plasmin by the addition of a standard amount of tPa. Conversion of plasminogen to plasmin was increased markedly by the addition of t-Pa stimulator. The amidolytic action of plasmin on the chromogenic substrate S-2251 was monitored to determine the final activity of tPa in the samples. The generation of p-nitroaniline was monitored at 405 nm on an EIA microplate reader.

Plasminogen activator inhibitor (PAI) activity. The activity of PAI in citrated plasma was assayed with a chromogenic substrate assay (COATEST PAI, Kabi Diagnostica, Stockholm, Sweden). A normal plasma and high plasma control (PAI Control Normal and PAI Control High, Kabi Diagnostica Stockholm, Sweden) were run with each set of tests. Using microtiter plates, a standard amount of tPa was added to each sample well of citrated plasma. The excess of tPa, in the presence of tPa stimulator, caused conversion of plasminogen to plasmin. The release of p-nitroaniline from the chromogenic substrate, S-2403, was monitored at 405 nm on an

EIA microplate reader. The activity of plasminogen activator inhibitor in the sample was inversely proportional to the amount of plasmin formed.

Fibrinolytic activity of lung tissue. The fibrinolytic activity of lung tissue was measured by a modification of the assay of Astrup and Mullertz (1952). In summary, sections of frozen lung tissue from the right cranial and left lobes were cut at a uniform thickness and diameter using a 3 mm biopsy punch (Baker Cummins, Miami, FL). The punches of lung tissue were placed upon plates of fibrin prepared from bovine fibrinogen (Miles Lab., Elkhart, IN). The plates were incubated for 4 hours at 37°C. The sections of lung tissue were removed and the zone of lysis was measured. Fibrinolytic activity was expressed as the diameter of the clear lytic zone that appeared in the fibrin plate.

Statistical analysis. All determinations were run in duplicate and values were averaged. Data are expressed as means $\pm$ SE. Homogeneity of variance was assessed with the F-max test. A log transformation was performed on nonhomogeneous data. If variances were homogeneous, data were compared using an analysis of variance. Individual means were compared using the least significant difference test. Data with heterogeneous variances were analyzed with the Student's t test for independent means (Steel and Torrie, 1980). The criterion for significance was $p < 0.05$.

RESULTS:

Lung injury and cardioventricular hypertrophy. Several markers of pneumotoxicity were monitored to compare the onset of lung injury with temporal changes in fibrinolytic activity. Rats treated with a single injection of MCTP had a mild increase in the wet lung-to-body weight ratio at 3 days compared to DMF controls. Values for the wet lung-to-body weight ratio increased progressively thereafter (Figure 1). The LDH activity and protein concentration of cell-free BALF from MCTP-treated rats were slightly increased compared to control values at Days 3 and 5, respectively (Figures 2 and 3). Increases in these markers progressed until Day 8 after which they reached a plateau. Rats that received MCTP had marked elevations in the number of nucleated cells in BALF from Days 8-14 (Figure 4). Right cardioventricular hypertrophy, characterized by a significant increase in RV/(LV+S), was first observed at Day 11 in rats treated with MCTP. This increase became more pronounced by Day 14 (Figure 5). These changes in markers of cardiopulmonary injury are consistent with previous studies of MCTP-induced pneumotoxicity and pulmonary hypertension (Bruner *et al.*, 1983a, 1986; Reindel *et al.*, 1990).

Fibrinolytic activity in plasma. We measured changes in the major components of the fibrinolytic system to determine if this system was activated in response to treatment of rats with MCTP. Conversion of the zymogen, plasminogen, to the enzyme, plasmin, decreases the concentration of plasminogen in blood and suggests an increase in fibrinolytic activity. There were no significant changes in the

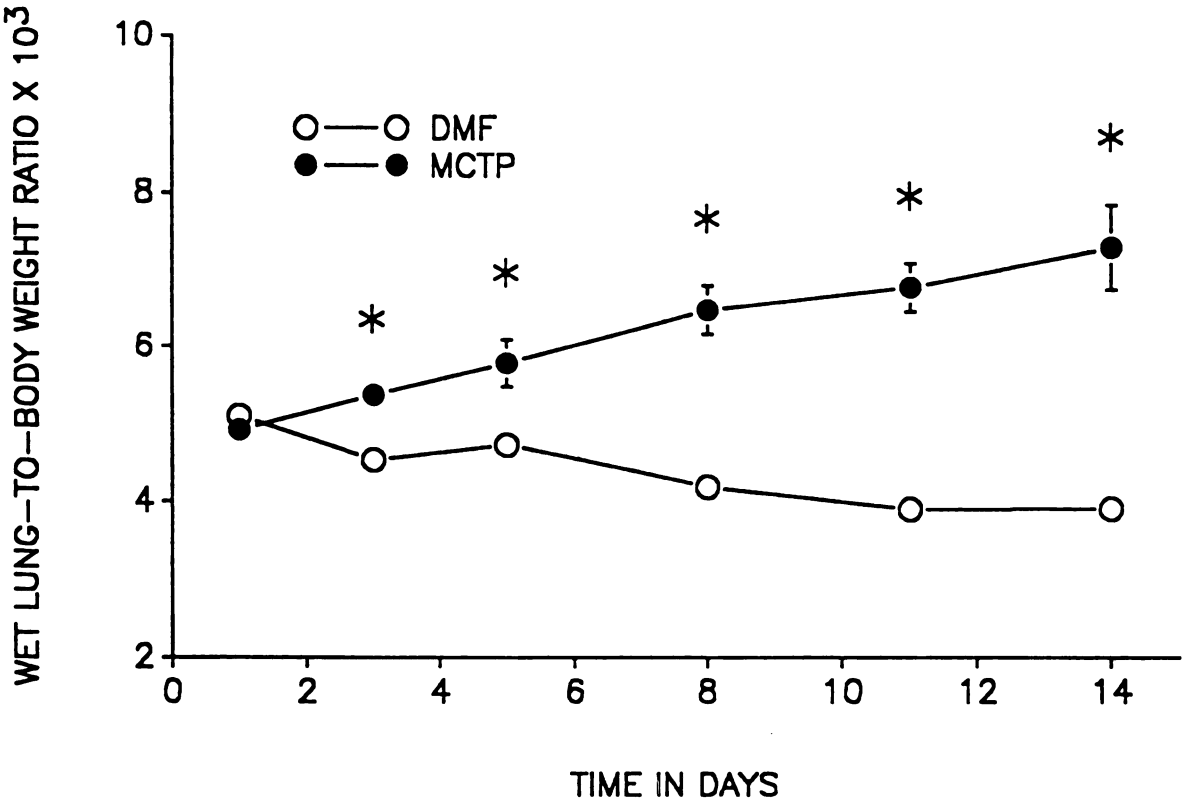


Figure 1: Effect of monocrotaline pyrrole (MCTP) on wet lung-to-body weight ratio. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

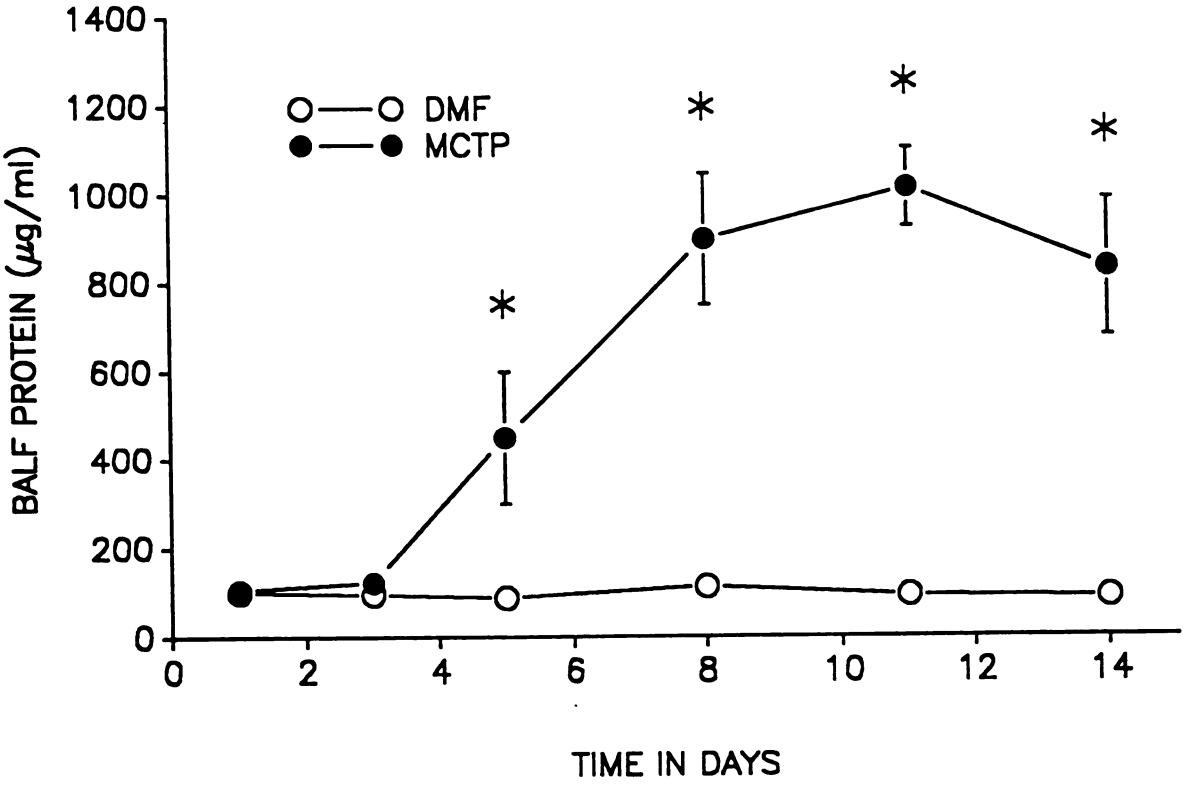


Figure 2: Effect of monocrotaline pyrrole (MCTP) on the protein concentration of cell-free bronchoalveolar lavage fluid (BALF). Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

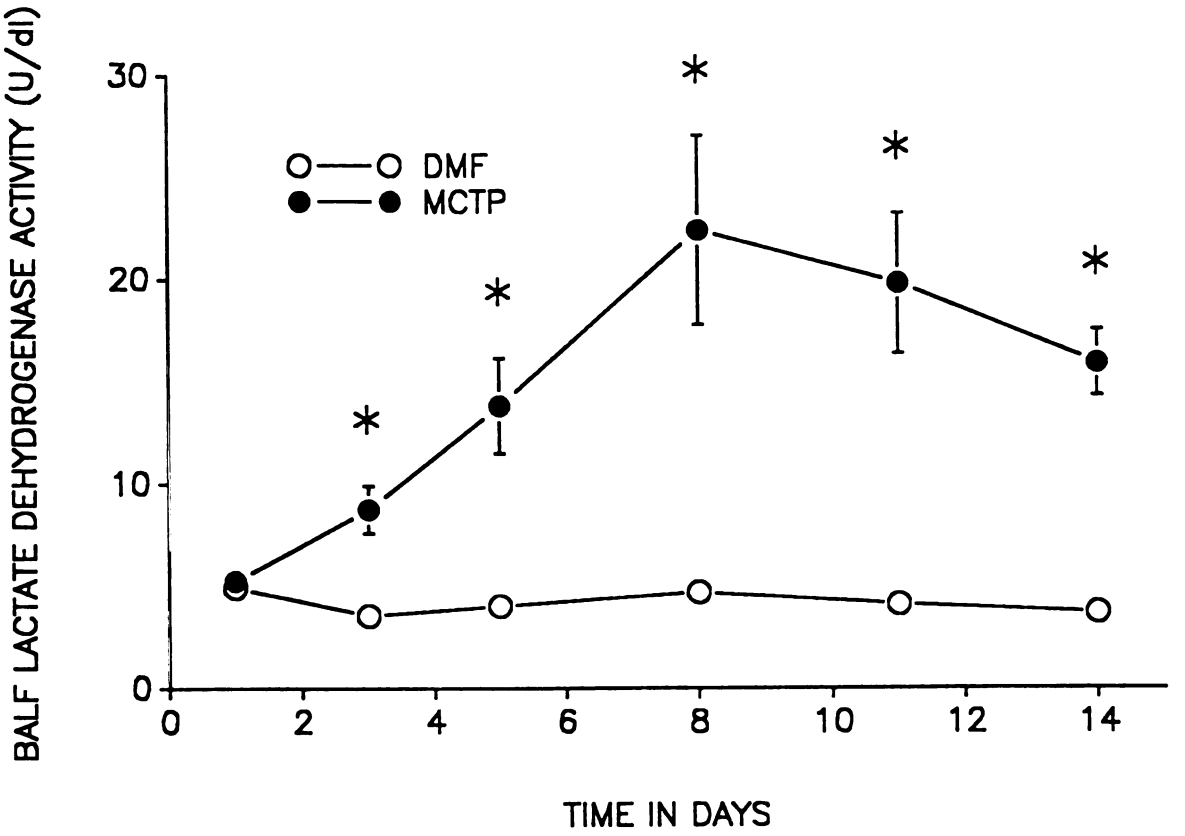


Figure 3: Effect of monocrotaline pyrrole (MCTP) on lactate dehydrogenase (LDH) activity of cell-free bronchoalveolar lavage fluid (BALF). Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

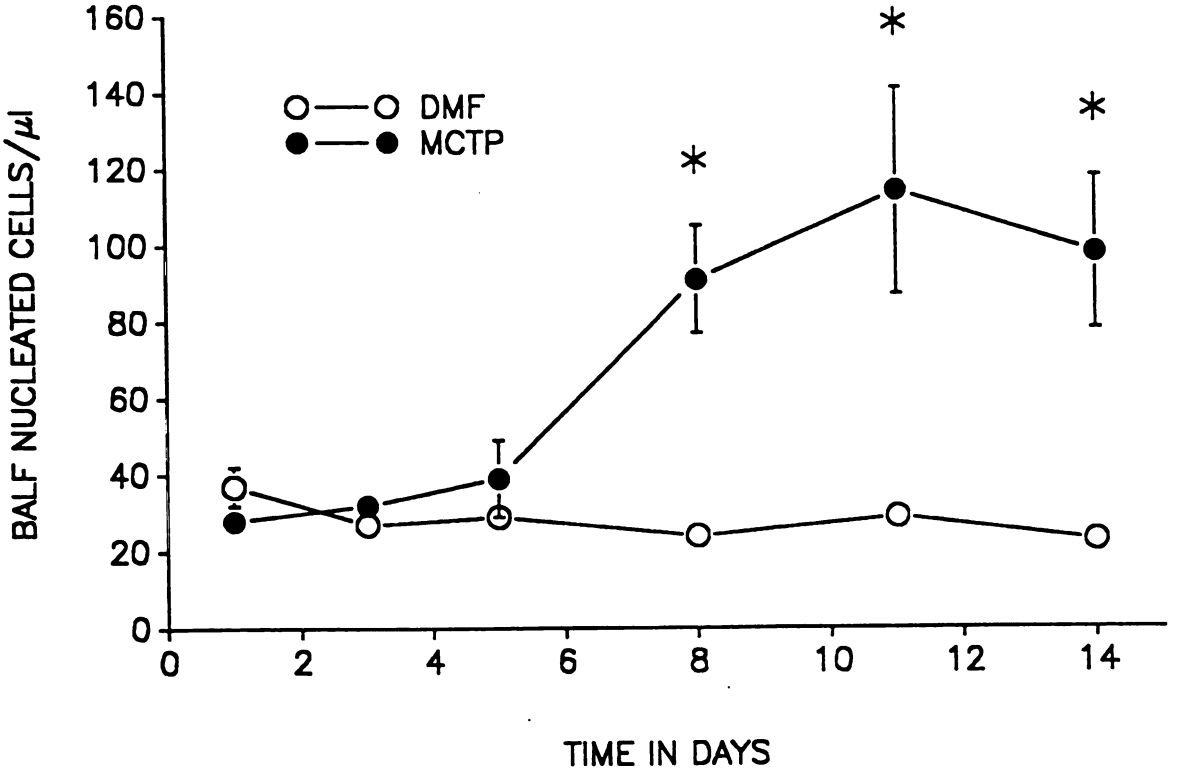


Figure 4: Effect of monocrotaline pyrrole (MCTP) on total nucleated cell counts of BALF. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean ± SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

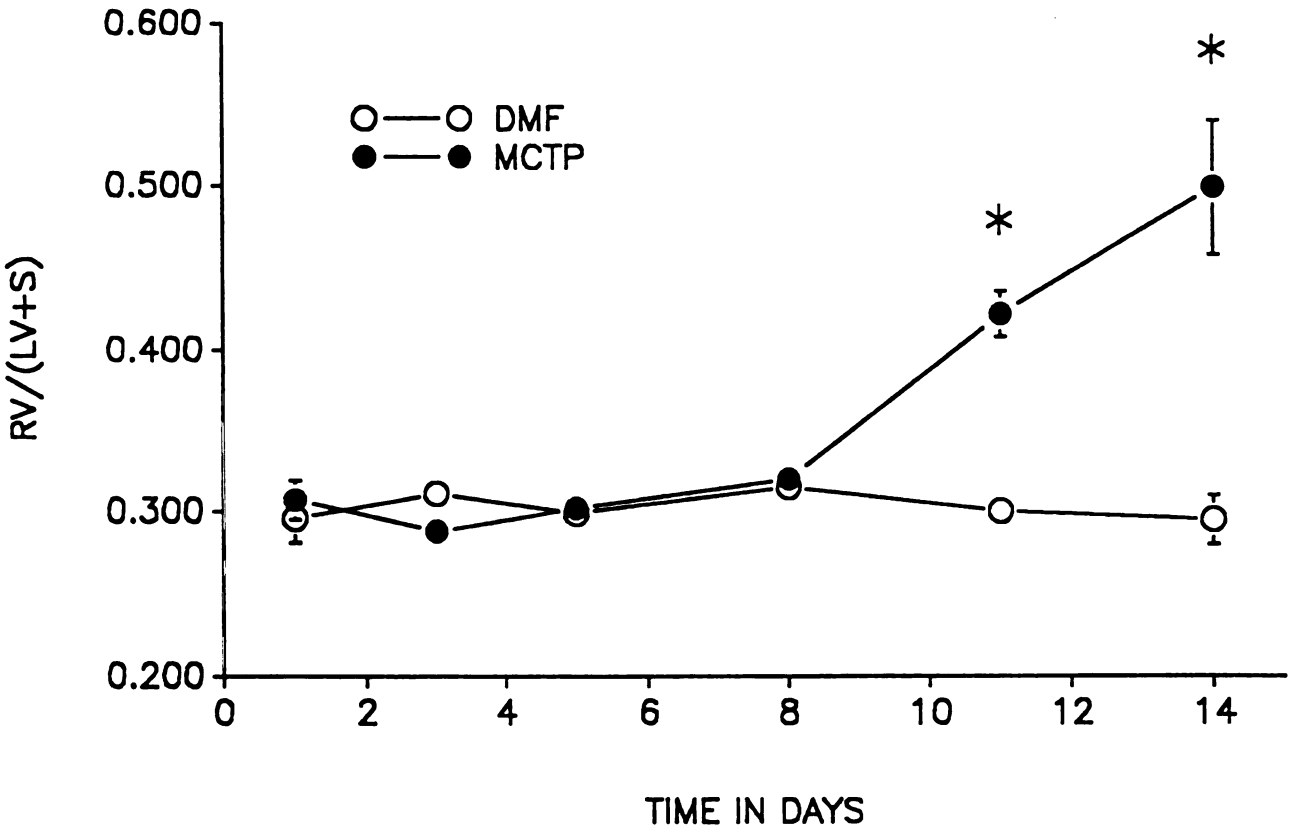


Figure 5: Effect of monocrotaline pyrrole (MCTP) on right cardioventricular hypertrophy. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

plasminogen concentration in plasma as a result of MCTP treatment at any time (Figure 6).

The major inhibitor of free plasmin in the blood is α-2-antiplasmin. Tissue plasminogen activator (tPa) is an endothelial cell-derived glycoprotein that causes conversion of plasminogen to plasmin. A decrease in the activity of α-2-antiplasmin or an increase in activity of tPa also suggests increased fibrinolysis. In contrast, increased activity of α-2-antiplasmin or decreased activity of tPa would suggest conditions that would not favor fibrinolysis. The activities of α-2-antiplasmin and tPa in plasma from rats treated with MCTP were not different from those of rats treated with vehicle (Figures 7 and 8).

Plasminogen activator inhibitor (PAI) is an endothelial cell-derived glycoprotein that inactivates tPa, and if secreted in increased quantities, PAI may inhibit fibrinolysis. The PAI activity in plasma from control rats was essentially constant over time. In contrast, the plasminogen activator inhibitor activity in plasma of rats treated with MCTP was significantly increased from Days 8-14 (Figure 9).

Fibrinolytic activity of lung tissue. The fibrinolytic activity of lung tissue from DMF control rats was relatively constant throughout the experiment. A significant decrease in fibrinolytic activity was observed at Day 3 in lung tissue of rats treated with MCTP. The fibrinolytic activity returned toward control at Day 5, and then decreased markedly from Days 8-14 (Figure 10). Within individual rats treated with MCTP or DMF vehicle, there was no difference in the fibrinolytic activity between sections of lung taken from right cranial and left lobes at any time (data not shown).

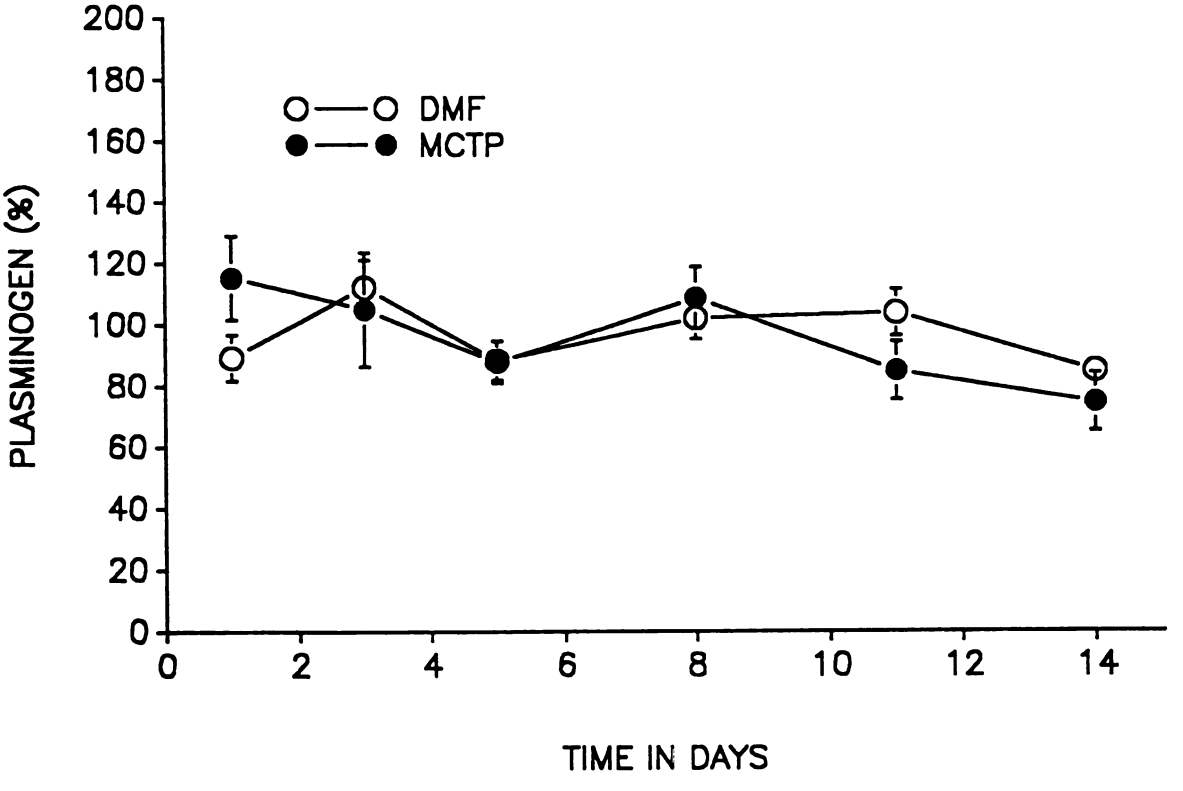


Figure 6: Effect of monocrotaline pyrrole (MCTP) on plasminogen concentration in plasma. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

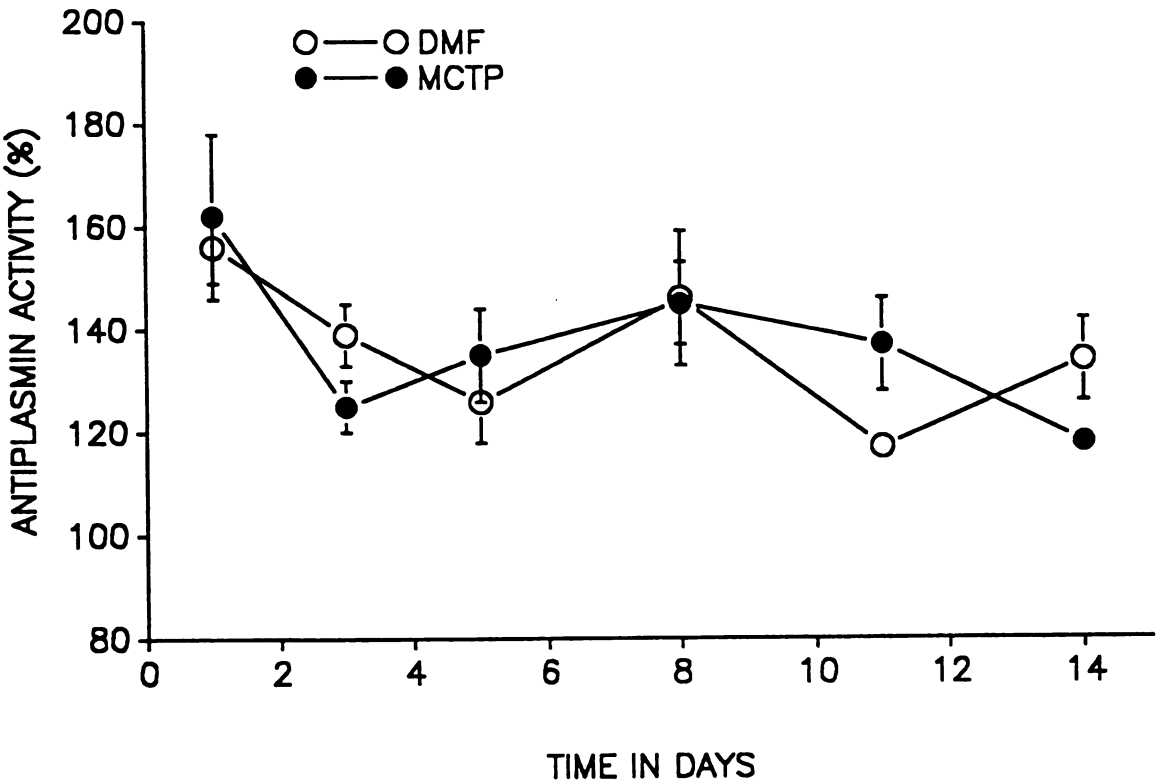


Figure 7: Effect of monocrotaline pyrrole (MCTP) on α-2-antiplasmin activity in plasma. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean ± SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

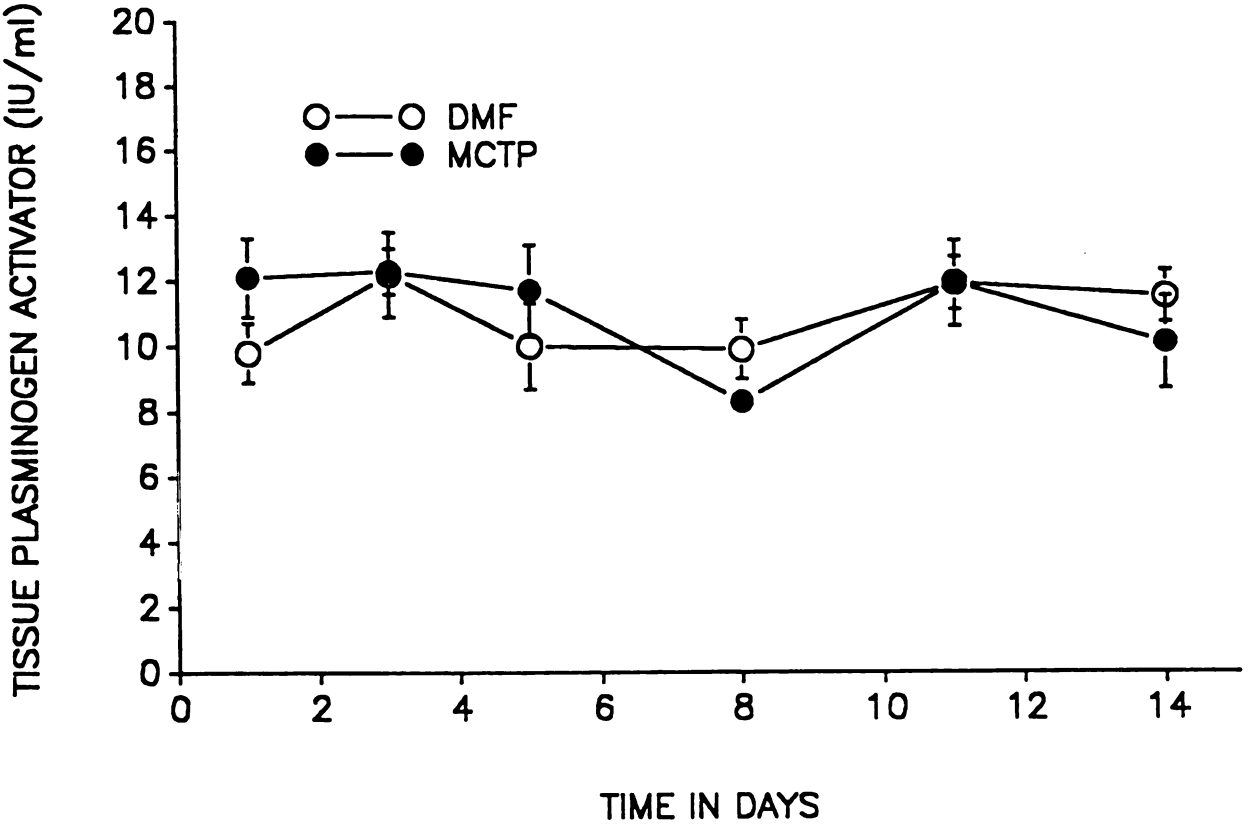


Figure 8: Effect of monocrotaline pyrrole (MCTP) on tissue plasminogen activator activity in plasma. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

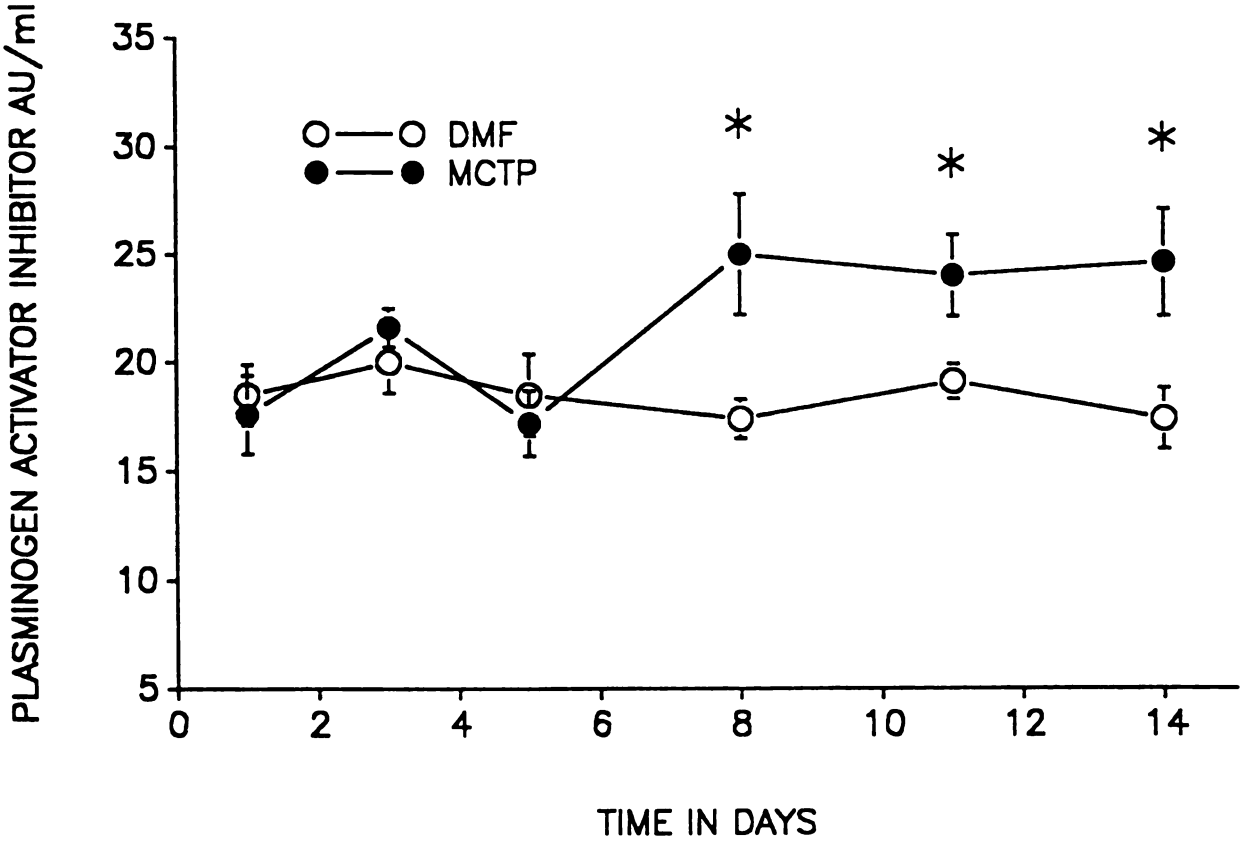


Figure 9: Effect of monocrotaline pyrrole (MCTP) on plasminogen activator inhibitor activity in plasma. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 5-6 rats. Those points without bars had SE less than the area covered by the symbol. *Significantly different from DMF control.

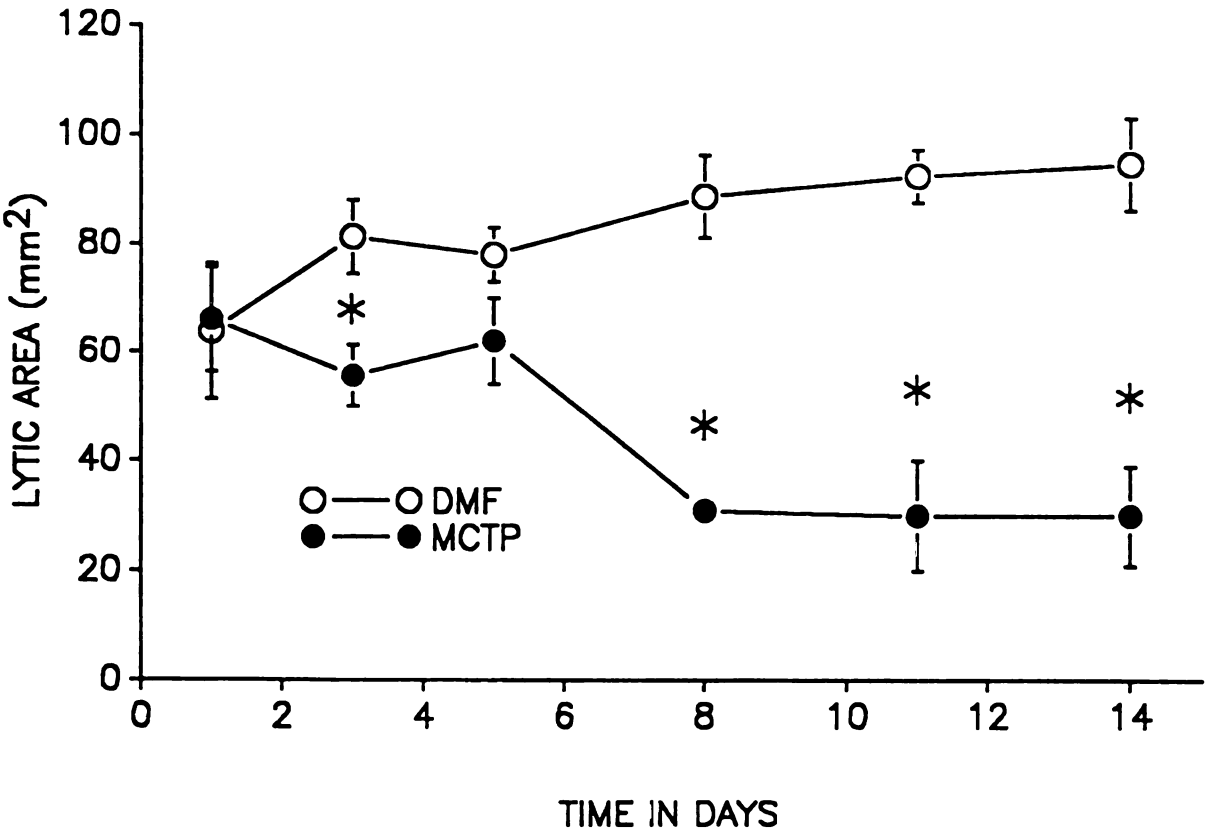


Figure 10: Effect of monocrotaline pyrrole (MCTP) on fibrinolytic activity of lung tissue. Rats received a single iv injection of MCTP (3.5 mg/kg) or DMF vehicle at time 0. Values represent mean $\pm$ SE of 5-6 rats. *Significantly different from DMF control.

DISCUSSION:

Recurrent pulmonary thromboembolism has been suggested as a cause for primary pulmonary hypertension in people (Harrison *et al.*, 1966; Wyngaarden and Smith, 1985). Thrombosis of the lung microvasculature is also a lesion that occurs commonly in people with the adult respiratory distress syndrome (Tomashefski *et al.*, 1983; Heffner *et al.*, 1983, 1987). Due to the numerous similarities in pathophysiology, the MCT- or MCTP-treated rat is a useful animal model for these human pulmonary vascular diseases. Pulmonary microvascular thrombosis has been observed by numerous investigators after administration of MCT or MCTP to rats (Turner and Lalich, 1965; Valdivia *et al.*, 1967ab; Chesney *et al.*, 1974a; Lalich *et al.*, 1977; Schraufnagel and Schmid, 1989). The cause of the thrombosis and its relationship to the pneumotoxicity and pulmonary hypertension that occur after treatment of rats with these toxicants has not been determined. Several investigators performed semi-quantitative analysis of fibrin thrombi in rats treated with MCT or MCTP (Valdivia *et al.*, 1967a; Lalich *et al.*, 1977; Schraufnagel and Schmid, 1989). In summary, the fibrin and platelet thrombi occurred relatively early in the lungs after administration of the toxicants and increased in number and severity with time. It was postulated that these thrombi may decrease vascular cross sectional area and cause an increase in vascular resistance that in turn results in *cor pulmonale* (Turner and Lalich, 1965; Valdivia *et al.*, 1967ab; Chesney *et al.*, 1974a; Lalich *et al.*, 1977; Schraufnagel and Schmid, 1989).

Normal blood fluidity is dependent upon a delicate balance between coagulation and fibrinolysis. Studies of the past two decades revealed that vascular endothelial cells participate in hemostasis by synthesizing procoagulant, anticoagulant, profibrinolytic and antifibrinolytic substances (Stern *et al.*, 1988; Ge *et al.*, 1991; Rodgers, 1988; Gertler and Abbott, 1992). In addition, they influence these processes by producing receptors that localize coagulation and fibrinolytic reactions on their surfaces. Since the endothelium of the lungs comprises a large surface area that is exposed to the entire cardiac output, it is in a unique position to influence these reactions in the peripheral blood. Treatment of rats with MCT or MCTP results in endothelial cell dysfunction (Gillis *et al.*, 1978; Hilliker *et al.*, 1983a, 1984a; Molteni *et al.*, 1984, 1987). Given the complex nature of hemostasis and the intricate involvement of the endothelium in hemostatic reactions, it is reasonable to hypothesize that exposure to MCTP may upset the delicate balance of coagulation and fibrinolysis and thereby contribute to thrombosis in this model.

In these experiments, markers of lung injury were monitored to compare the development of pneumotoxicity with temporal changes in fibrinolysis. Alterations in the wet lung-to-body weight ratio, protein concentration and LDH activity of cell-free BALF and BALF total nucleated cell counts confirmed that marked lung injury is delayed for several days after a single exposure to a low dose of MCTP (Figures 1-4). Right cardioventricular hypertrophy was first observed at 11 days after treatment with MCTP and progressed through day 14 (Figure 5). Alterations in markers of car-

diopulmonary injury of this type are typical for rats treated with MCTP (Bruner *et al.*, 1983a, 1986).

We sought to determine if rats treated with MCTP developed changes in fibrinolysis in the peripheral blood that may result in thrombosis. Several tests of the major components of the fibrinolytic system were selected to provide an overview of fibrinolysis in the blood. We observed no differences in plasminogen concentration or activity of α-2-antiplasmin in blood of rats treated with MCTP or DMF vehicle. We hypothesized that rats may develop decreased activity of plasma tPa, increased activity of plasma PAI or both after treatment with MCTP. However, there were no significant differences in the tPa activity in blood of rats treated with MCTP or DMF vehicle. Rats that received MCTP did have significantly increased activity of PAI in peripheral blood from days 8-14 after treatment with the toxicant (Figure 9). The increase in activity of PAI was modest and first occurred at times when lung injury was well established but before right cardioventricular hypertrophy occurred.

The significance of the increase in PAI activity is difficult to determine. *In vitro*, marked increases in PAI activity are observed in the culture media of endothelial cells treated with endotoxin or interleukin-1 (Emeis and Kooistra, 1986). Since MCTP causes a progressive form of pulmonary damage and since thrombi increase in severity and number with time, mild to moderate increases in the activity of PAI or other changes in the blood that would promote thrombosis may deserve additional attention.

Results obtained from analysis of components of fibrinolysis in peripheral blood suggested a second hypothesis: rats treated with MCTP may develop decreased fibrinolytic activity in the lung microenvironment that promotes thrombosis but is not reflected by early changes in the peripheral blood. To address this hypothesis, we measured the fibrinolytic activity of lung tissue slices. As early as 3 days after treatment with MCTP, rats had a significant decrease in fibrinolytic activity of lung tissue. This result suggests that early decreases in fibrinolysis may occur in the lungs of rats treated with MCTP that are not reflected in the peripheral blood. The early decrease occurred at the time major lung injury was beginning, as confirmed by markers of lung injury (Figures 1-4). The initial decrease in fibrinolytic activity observed at day 3 corresponds to small trends toward lower fibrinogen concentration in peripheral blood of rats treated with MCTP (Schultze *et al.*, 1991b). We suggested previously that this trend might reflect conversion of fibrinogen to fibrin and deposition of small thrombi in capillaries of the lungs. The results of previous histologic examinations are consistent with this interpretation.

Abnormalities in fibrinolysis have been associated with certain human pulmonary vascular diseases. Kingdon *et al.* (1966) postulated that inherited deficiencies in components of the fibrinolytic system may result in inability to lyse recurrent pulmonary thromboemboli and could be a cause of familial pulmonary hypertension. Abnormal fibrinolysis has been identified in a kindred of people with familial pulmonary hypertension (Inglesby *et al.*, 1973). Eight of ten members of the kindred had abnormally increased antiplasmin activity and five of the ten had

physical or electrocardiographic evidence of pulmonary hypertension at the time of the case study. Investigation of fibrinolysis in a second kindred of eight people with familial pulmonary hypertension revealed no detectable deficiencies in components of fibrinolysis (Tubbs *et al.*, 1979). The authors suggested that differences in methodology may have caused the inability to detect abnormal fibrinolysis in the second kindred.

Other abnormalities in the fibrinolytic system have also been associated with development of pulmonary hypertension in people. Increased PAI activity was observed in the plasma of nineteen of twenty seven people with primary pulmonary hypertension (Eisenberg *et al.*, 1990) suggesting that a limited fibrinolytic response to thrombosis may occur. Increased PAI activity has been identified in people with other thrombotic disorders including deep venous thromboembolism and myocardial infarction (Hamsten *et al.*, 1985; Wiman *et al.*, 1985; Juhan-Vague *et al.*, 1987; Chmielewska and Wiman, 1986). It is possible that the increase in the plasma PAI activity in rats treated with MCTP contributes to the lung injury and pulmonary hypertension in this model.

Decreased fibrinolytic activity was observed in radiation induced-lung injury in rats (Ts'ao *et al.*, 1983). The decrease in net fibrinolytic capacity of the irradiated lung tissue was determined to be due to a decrease in plasminogen activator activity but not to an increase in PAI activity. Law (1981) proposed that radiation injury causes increased vascular permeability and resultant extravasation of fibrinogen-rich fluid. Once converted to fibrin, the proteinaceous strands persist due to inadequate

fibrinolysis and serve as stimuli for fibrogenesis. The pathogenesis of MCTP-induced pneumotoxicity and pulmonary hypertension may share distinct similarities with this mechanism.

In conclusion, results of this investigation indicate that MCTP treatment caused functional alterations in the fibrinolytic system of rats. The decrease in fibrinolytic activity of lung tissue slices began at 3 days and became severe at 8-14 days after a single exposure to MCTP. PAI activity increased in the peripheral blood beginning at 8 days. These results suggest that pulmonary vascular thrombi in this model may be the result of deficits in fibrinolysis in the lung. If deficiencies in fibrinolytic activity prove to be important in the pathogenesis of pulmonary hypertension, then these deficits may be a potential target for pharmacologic manipulation in an effort to delay, attenuate or prevent thrombosis and subsequent pulmonary hypertension.

CHAPTER FIVE

PROCOAGULANT AND FIBRINOLYTIC PROPERTIES OF BOVINE ENDOTHELIAL CELLS TREATED WITH MONOCROTALINE PYRROLE

ABSTRACT:

The administration of chemically synthesized monocrotaline pyrrole (MCTP) causes pulmonary vascular thrombi that are associated with vascular remodeling, pulmonary hypertension and compensatory right cardioventricular hypertrophy in rats. Some investigators hypothesize that the thrombi contribute to the lesions that occur after MCTP treatment. The pulmonary endothelium receives the entire cardiac output and is in a unique position to influence procoagulant and fibrinolytic reactions locally in the lungs and in the peripheral blood. In these experiments, we examined changes in the procoagulant and fibrinolytic properties of cultured pulmonary artery endothelial cells exposed to MCTP to see if they might favor thrombosis. Monolayers of bovine pulmonary arterial endothelial cells received a single administration of MCTP or N,N-dimethylformamide (DMF) vehicle, and the media or lysates were examined at 4, 24, 48 or 120 hours after treatment. MCTP caused a time dependent cell detachment and an increase in release of lactate dehydrogenase into culture medium. Although cell numbers decreased dramatically, the protein concentration of cell monolayers was unchanged by treatment. MCTP treatment caused no change in the amount of tissue factor activity/μg protein in bovine endothelial cell lysates and only a very subtle increase in the activity of Factor V/cell in the culture medium at 120 hours. In addition, the medium from bovine endothelial cells treated with MCTP had a time dependent increase in the activity of plasminogen activators and a decrease in activity of plasminogen activator inhibitors. Thus, bovine endothelial cells exposed to MCTP *in vitro* do not produce changes in these procoagulant or fibrinolytic properties that would explain the thrombosis observed *in vivo*.

INTRODUCTION:

Monocrotaline (MCT) is a toxic pyrrolizidine alkaloid of plant origin (Bull *et al.*, 1968; Heath, 1969). When administered to rats in high doses, MCT causes acute hepatic necrosis and death (Mattocks, 1986). However, rats given low doses of MCT develop delayed and progressive lung injury characterized by pulmonary inflammation, vascular remodeling, pulmonary hypertension and compensatory right cardioventricular hypertrophy (Turner and Lalich, 1965; Valdivia *et al.*, 1976ab; Kay and Heath, 1969; Mattocks, 1986; Ghodsi and Will, 1981). The pathophysiology is similar to certain chronic vascular diseases of people, and rats treated with MCT are useful animal models for primary pulmonary hypertension and the late phases of the adult respiratory distress syndrome (Voelkel and Reeves, 1979; Snow *et al.*, 1982).

Pyrrolic metabolites formed during hepatic bioactivation via the P450 monooxygenase system are the proximal toxicants responsible for the lesions that occur after exposure to MCT (Mattocks, 1968; Mattocks and White, 1971). Intravenous administration of chemically synthesized monocrotaline pyrrole (MCTP) to rats causes delayed and progressive lung injury, pulmonary hypertension and right cardioventricular hypertrophy that are similar to that caused by the parent alkaloid, MCT (Butler, 1970; Butler *et al.*, 1970; Bruner *et al.*, 1983a).

Fibrin and platelet thrombosis of the pulmonary microvasculature is reported after administration of MCT and MCTP to rats (Turner and Lalich, 1965; Valdivia *et al.*, 1967ab; Chesney *et al.*, 1974a; Lalich *et al.*, 1977; Schraufnagel and Schmid, 1989). Several investigators hypothesized that these thrombi contribute to the lung

injury and pulmonary hypertension that occur after toxicant administration (Lalich *et al.*, 1977; Valdivia *et al.*, 1967ab). Thrombosis may result from an imbalance in procoagulant and fibrinolytic influences but its cause(s) in MCT intoxication are unknown. Results of a previous investigation revealed that rats treated with MCTP do not develop excessive procoagulant activity in the systemic circulation (Schultze *et al.*, 1991b). Fibrinolytic activity has also been examined in rats treated with MCTP (Schultze and Roth, 1992). Rats that received MCTP had decreased fibrinolytic activity in lung tissue within 3 days after toxicant administration. In addition, these rats developed a mild increase in plasminogen activator inhibitor (PAI) activity in blood from 8-14 days after exposure to MCTP, a change that would favor thrombosis.

Endothelial cells are one of the first cell types to develop biochemical and histologic lesions after treatment of rats with MCT or MCTP (Gillis *et al.*, 1978; Hilliker *et al.*, 1983a, 1984a; Molteni *et al.*, 1984, 1985). Morphologic changes associated with exposure to these toxicants include endothelial cell swelling, blebbing and hypertrophy (Reindel *et al.*, 1990). Biochemical evidence of endothelial cell dysfunction in rats after treatment with MCT or MCTP includes changes in activity of angiotensin-converting enzyme, decreased 5HT clearance by the lungs and platelet sequestration in the lungs suggesting loss of the normally nonthrombogenic surface of quiescent endothelium. *In vitro*, a single administration of MCTP to the medium of endothelial cells causes an inhibition in cellular proliferation, an increase in cell detachment and the progressive release of lactate dehydrogenase into the medium In addition, marked cellular hypertrophy of surviving cells and an increase in

synthesis of DNA, RNA and protein occur (Reindel and Roth, 1991; Reindel *et al.*, 1991; Hoorn and Roth, 1992a).

Endothelial cells contribute to the hemostatic process by producing procoagulant and fibrinolytic substances (Rodgers, 1988; Stern *et al.*, 1988; Ge *et al*, 1991; Gertler and Abbott, 1992). Some of these include tissue factor and Factor V, which are important in the formation of fibrin thrombi, and plasminogen activators and plasminogen activator inhibitors, which contribute to fibrinolysis. It is possible that treatment with MCTP upsets the balance of these substances and thereby contributes to thrombosis.

We hypothesized that exposure of endothelial cells to MCTP results directly in altered production of procoagulant and/or fibrinolytic factors that might favor thrombosis within the pulmonary microcirculation. The objectives of this investigation were to describe the changes in procoagulant and fibrinolytic properties of endothelial cells treated with MCTP *in vitro* and to determine if such changes are associated with cellular toxicity and whether they are consistent with occurrence of thrombosis observed *in vivo*.

MATERIALS AND METHODS:

Preparation of endothelial cells. Bovine endothelial cells (BECs) were derived from segments of freshly excised pulmonary artery using the methods of Jaffe *et al.* (1973) and Booyse *et al.* (1975) as modified by Reindel and Roth (1991). Segments of the mainstem pulmonary artery obtained from mature cows were rinsed in Puck's saline prepared from Hank's Balanced Salt Solution (HBSS, Sigma Chemical Co., St. Louis, MO) containing 3% penicillin (300 units/ml), streptomycin (300 μg/ml) and fungizone (0.75 μg/ml) (Antibiotic-Antimycotic, Gibco, Grand Island, NY). The adventitia and external elastic laminae were removed by blunt dissection. Vascular segments of 5-6 cm length were incised longitudinally and placed luminal surface down in 60 mm petri dishes that contained 2 ml of collagenase solution (type IV, 0.1%, Sigma Chemical Co., St. Louis, MO). After 3-5 minutes incubation at room temperature, sheets of endothelial cells were gently brushed from the luminal surface with a rubber policeman and placed into 100 mm petri dishes that contained 6-8 ml Ca^{+2} Mg^{+2}-free HBSS. Small clusters of endothelium were transferred into 6 well plates (Costar, Cambridge, MA) containing Medium-199 (Gibco, Grand Island, NY). BECs were grown in Medium-199 containing 10% fetal calf serum, 0.01 mM thymidine and 1% Antibiotic-Antimycotic. Cultures were maintained by changing media each 48 hours. BECs were passed by enzymatic disassociation with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (0.025% trypsin, 0.27 mM EDTA in Ca^{+2} Mg^{+2}-free HBSS). BECs from passages 4-12 were utilized in these investigations. The identity of BECs was

confirmed by their characteristic cobblestone morphology, ability to accumulate acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Di-I-Ac-LDL, Biomedical Technologies Inc., Stoughton, MA) and positive staining for Factor VIII related antigen.

MCTP synthesis. Monocrotaline pyrrole, synthesized from monocrotaline (Trans World Chemicals, Washington, DC) via an N-oxide intermediate (Mattocks, 1969), was dissolved in the vehicle, N,N-dimethylformamide (DMF) at a concentration of 20 mg/ml and stored in the dark under nitrogen at 0°C.

Treatment regimen. BECs were plated in 6 well culture dishes and allowed to proliferate until monolayers were confluent, typically 3-4 days. On day 0, monolayers received no treatment or were exposed to a final concentration of 5 μg MCTP/ml media or an equal volume of N,N-dimethylformamide (DMF) control vehicle. The dose of MCTP utilized in this investigation was selected for its marked cytostatic effects in the face of only limited cytolysis (Reindel and Roth, 1991). At 4, 24, 48 or 120 hours, endothelial cells and/or culture media were harvested for analysis.

Cellular release of lactate dehydrogenase (LDH). Leakage of LDH into culture medium was used as a marker of cytotoxicity. The activity of LDH in culture medium and cell lysates was analyzed by the method of Bergmeyer and Bernt (1974). Culture medium was removed from cell monolayers and spun in a centrifuge at 600*g* for 10 minutes at room temperature. The supernatant was retained for analysis. Cell lysates were prepared by rinsing monolayers three times with 1 ml HBSS and lysing

cells with 20 μl Triton X 100 (Sigma Chemical Co.) in 2 ml fresh HBSS. LDH activity was expressed as a % of total for each well and was calculated as follows:

$$\% \text{ release} = \left(\frac{\text{LDH in culture medium - medium blank}}{\left(\begin{array}{l}\text{LDH in lysed cells +} \\ \text{LDH in culture medium}\end{array} \right) \text{- medium blank}} \right) \text{X } 100$$

(medium blank = LDH activity in culture medium containing fetal calf serum)

Enumeration of attached cells. Culture medium was removed and BEC monolayers were washed three times with 1 ml HBSS to remove non-adherent cells. Remaining cells were detached from the plates by treatment with 1 ml of trypsin-EDTA solution (Gibco) in Ca^{+2} Mg^{+2}-free HBSS and incubation at 37°C for 30 minutes. Cells were dispersed by vigorous trituration and were enumerated using a hemocytometer.

Protein determinations. BEC monolayers had culture media removed with a pipette and were rinsed three times with 1 ml HBSS. Medium-199 (0.5 ml) without serum was added to each well and the BECs were rapidly frozen at -20°C. Lysates were then prepared by rapid freezing at -20 °C/thawing of BECs three times followed by sonication (Model W-220 Cell Disrupter, Heat Systems-Ultrasonics, Plainview, NY). The protein concentration of BEC monolayers was determined by the method of Lowry *et al.* (1951).

Tissue factor activity. The procoagulant activity of BEC monolayers was analyzed with a single stage clotting assay as described by Schorer and Moldow (1988). Aliquots of BEC lysates (100 μl) were added to 100 μl of Factor XI deficient citrated plasma (Helena Laboratories, Beaumont, TX) and incubated for 1 minute at room temperature. To each sample, 100 μl activated partial thromboplastin time reagent (APTT Reagent, Helena Laboratories, Beaumont, TX) was added and the sample was incubated for 1 minute at 37°C. The sample was recalcified with 100 μl of $CaCl_2$, and the clotting time was measured on a BBL fibrometer coagulation timer (BBL Microbiology Systems, Baxter Healthcare Corporation, McGaw Park, IL). Units of tissue factor activity were arbitrarily defined by conversion from a standard curve derived from dilutions of a commercially available preparation of rabbit brain tissue thromboplastin (Dade Thromboplastin-C, Baxter Healthcare Corporation, McGaw Park, IL). Aliquots of cell lysates from BEC monolayers treated with α-thrombin were used as positive assay controls in measurements of tissue factor activity.

Factor V activity. Culture medium was spun in a centrifuge at 600*g* for 10 minutes, and the supernatant was removed for analysis. It was serially diluted in Factor V deficient plasma (Baxter Healthcare Corp., Dade Division, Miami, FL). A one stage clotting assay for determination of the activity of Factor V was performed (Brown, 1973; Sirridge, 1974). An aliquot of prewarmed tissue thromboplastin (Dade Thromboplastin-C, Baxter Healthcare Corporation, McGaw Park, IL) was added to each sample, the prothrombin time was measured on a BBL

fibrometer coagulation timer and the results were reported in seconds. Units of Factor V activity were arbitrarily defined by conversion from a standard curve developed from dilutions of culture medium, taken from BEC monolayers that received no treatment and diluted in Factor V deficient plasma. Aliquots of culture medium taken from BEC monolayers treated with α-thrombin were used as positive assay controls in measurements of Factor V activity.

Plasminogen activator activity. The plasminogen activator activity in culture medium was determined in microtiter plates (Corning Glass Works, Corning, NY) using a chromogenic substrate assay (COA-SET tPa, Helena Laboratories, Beaumont, TX). Briefly, samples had plasminogen activated to plasmin by the addition of tissue plasminogen activator (tPa). The conversion of plasminogen to plasmin was increased markedly by the addition of tPa stimulator. The amidolytic action of plasmin on the chromogenic substrate S-2251 was measured to determine the final activity of plasminogen activators in the samples. The subsequent generation of p-nitroaniline was proportional to the activity of plasminogen activators in the samples. The reaction was monitored at 405 nm using an EIA microplate reader (BIO-TEK Instruments, Inc., Queue Systems, Parkersburg, WV). An aliquot of previously analyzed, pooled normal rat plasma was run with each batch of samples as a control.

Plasminogen activator inhibitor activity. The activity of plasminogen activator inhibitor in culture medium was assayed with a chromogenic substrate assay (COATEST PAI, Kabi Diagnostica, Stockholm, Sweden). Using microtiter plates, a standard amount of tPa was added to each sample. The excess of plasminogen

activator, in the presence of tPa stimulator, caused conversion of plasminogen to plasmin. The release of p-nitroaniline from the chromogenic substrate, S-2403, was monitored at 405 nm on an EIA microplate reader. The activity of plasminogen activator inhibitor in the sample was inversely proportional to the amount of plasmin formed. To activate latent plasminogen activator inhibitors, aliquots of culture medium were dialyzed against 4 M guanidine-HCl as described by Erickson *et al.* (1986). Aliquots of commercially prepared control plasma with normal and high activity (PAI Control Normal and PAI Control High, Kabi Diagnostica Stockholm, Sweden) were run with each set of tests.

Statistical analysis. All determinations were run in duplicate and averaged values were considered a replication. Data are expressed as means $\pm$ SE. Homogeneity of variance was assessed with the F-max test. A log transformation was performed on any nonhomogeneous data. If variances were homogeneous, data were analyzed using an analysis of variance. Individual means were compared using the least significant difference test. Data with heterogeneous variances were analyzed with the Student's t test for independent means (Steel and Torrie, 1980). The criterion for significance was $p < 0.05$.

RESULTS:

Control cells. For all markers measured, results from BECs treated with DMF vehicle were not different from those of cells that received no treatment (data not shown).

Markers of cytotoxicity. The activity of LDH released into the culture medium of BECs treated with DMF control vehicle was low and relatively constant (Figure 1). In contrast, BECs treated with MCTP had a marked increase in LDH in the culture medium at 48 and 120 hours after treatment. The number of adherent cells in BEC monolayers treated with MCTP decreased in a time dependent manner (Figure 2). Decreased cell numbers were apparent at 24 hours and this progressed through 120 hours. These effects of MCTP are consistent with those reported previously (Reindel and Roth, 1991). The protein concentration of adherent cells in BEC monolayers treated with MCTP or DMF was not significantly different at any time (Figure 3).

Procoagulant activity. Tissue factor is a membrane-bound glycoprotein of stimulated endothelial cells that promotes coagulation via interaction in both the extrinsic and intrinsic pathways (Nemerson, 1988; Stern *et al.*, 1988). We measured tissue factor activity in BECs treated with MCTP and expressed the activity in several ways. When results were expressed as tissue factor activity per cell attached to the culture well, there was a significant increase in the quantity of tissue factor in lysates of BECs treated with MCTP at 48 hours (Figure 4a). The tissue factor activity per MCTP-treated cell increased markedly by 120 hours. However, when results of these

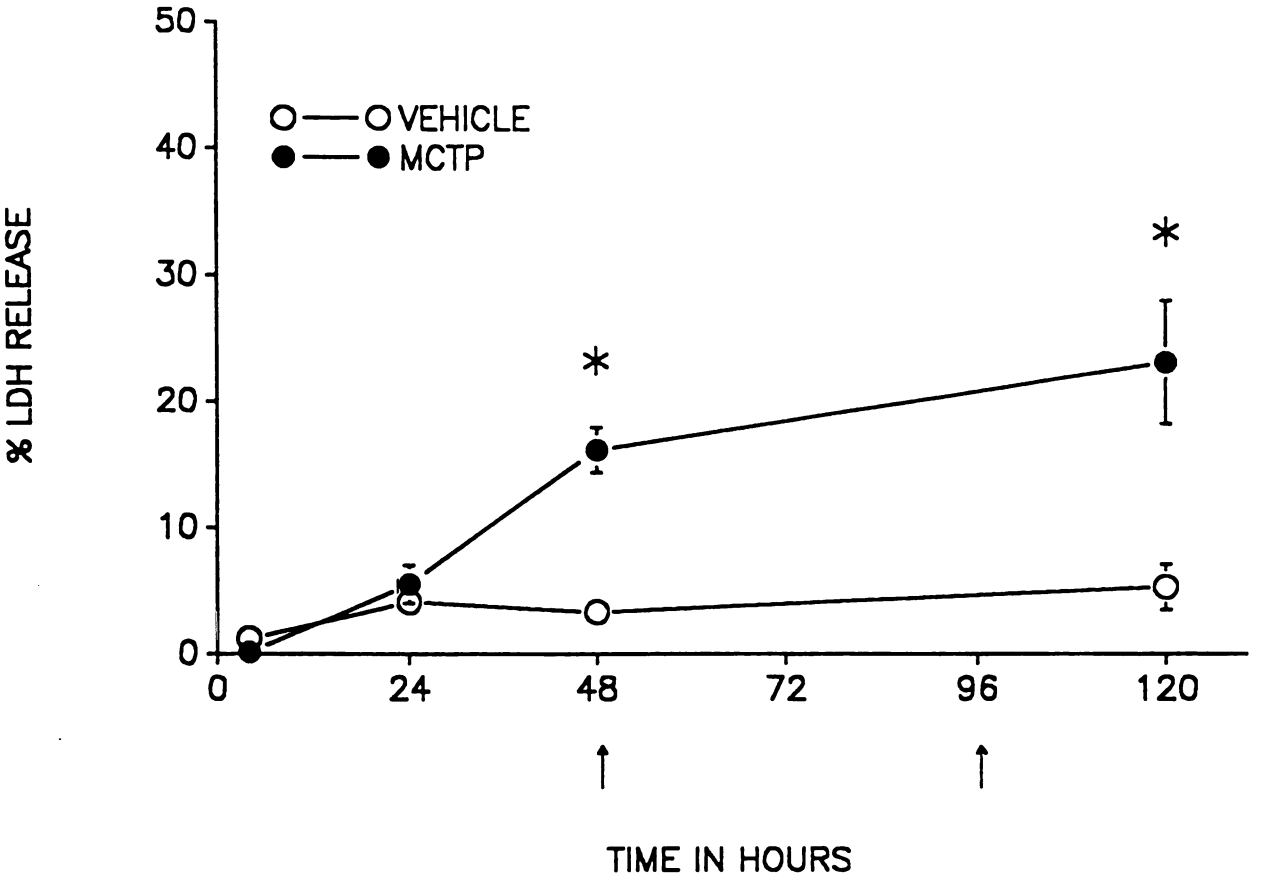


Figure 1: Effect of monocrotaline pyrrole (MCTP) on release of LDH into medium of endothelial cells grown in culture. Bovine endothelial cell monolayers were exposed to medium containing MCTP (5 μg/ml) or DMF vehicle at time 0. Values represent mean $\pm$ SE. Those points without bars had SE less than area covered by symbol. Arrows indicate times at which culture medium was replaced with fresh medium containing no MCTP. *Significantly different from DMF control. N=6.

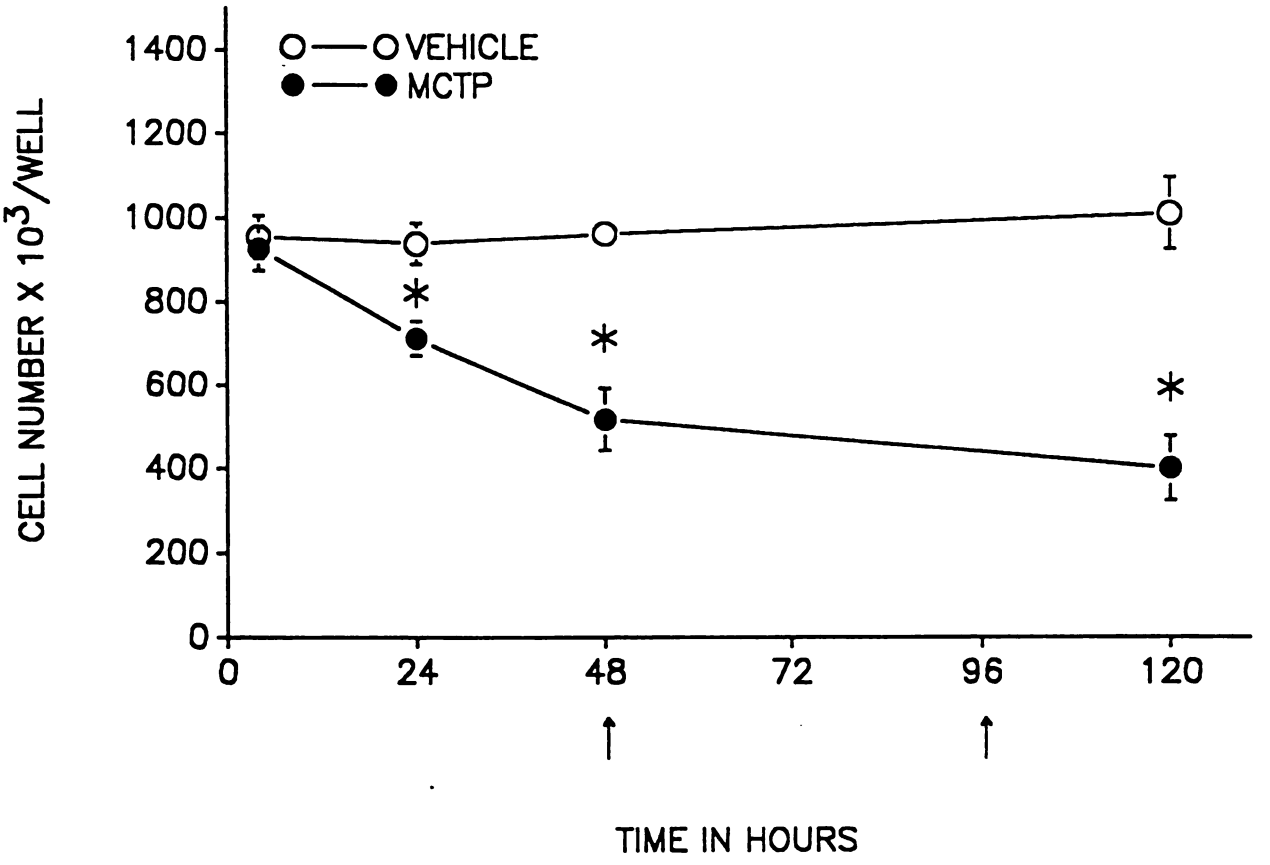


Figure 2: Effect of monocrotaline pyrrole (MCTP) on cellularity of endothelial cells grown in culture. Bovine endothelial cell monolayers were exposed to medium containing MCTP (5 μg/ml) or DMF vehicle at time 0. Those points without bars had SE less than area covered by symbol. Values represent mean $\pm$ SE. Arrows indicate times at which culture medium was replaced with fresh medium containing no MCTP. *Significantly different from DMF control. N=6.

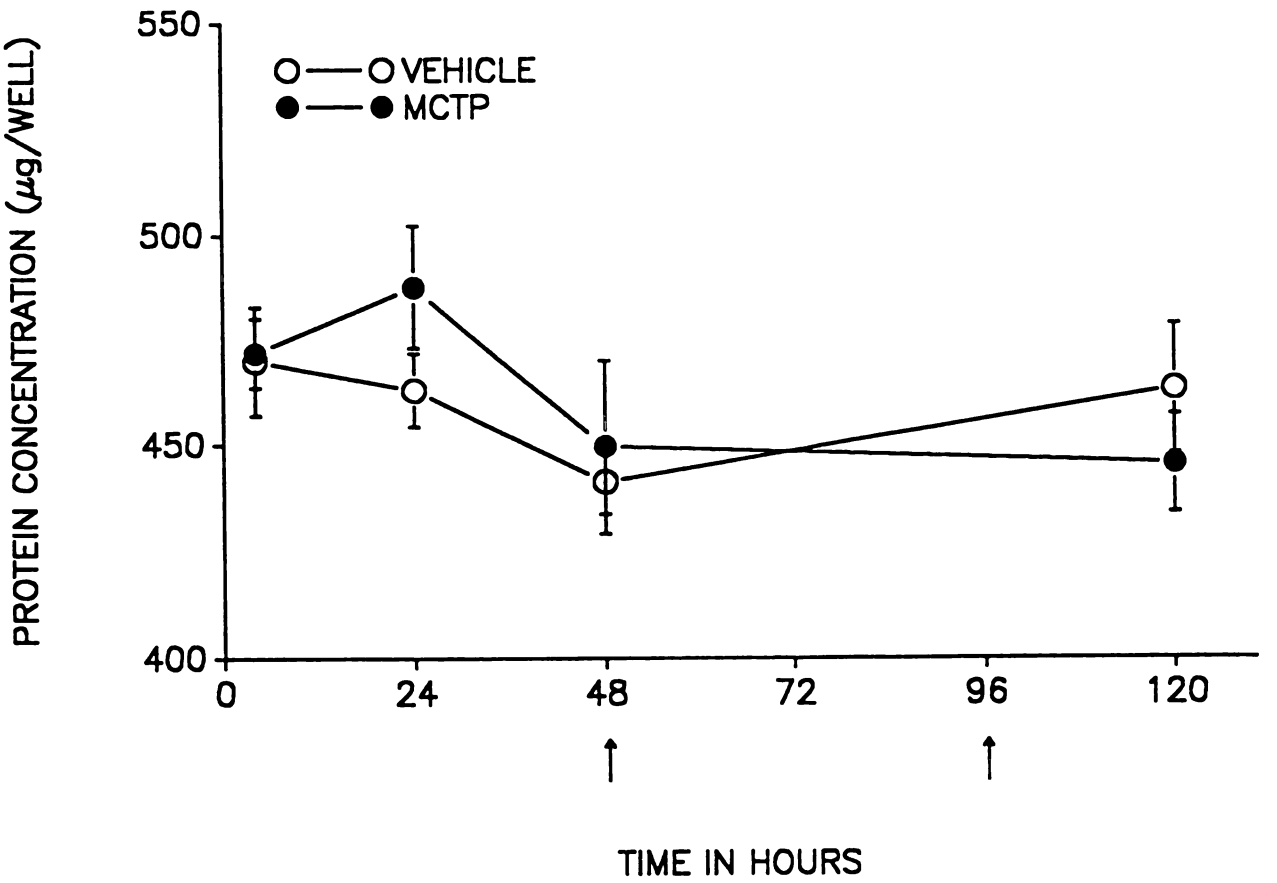
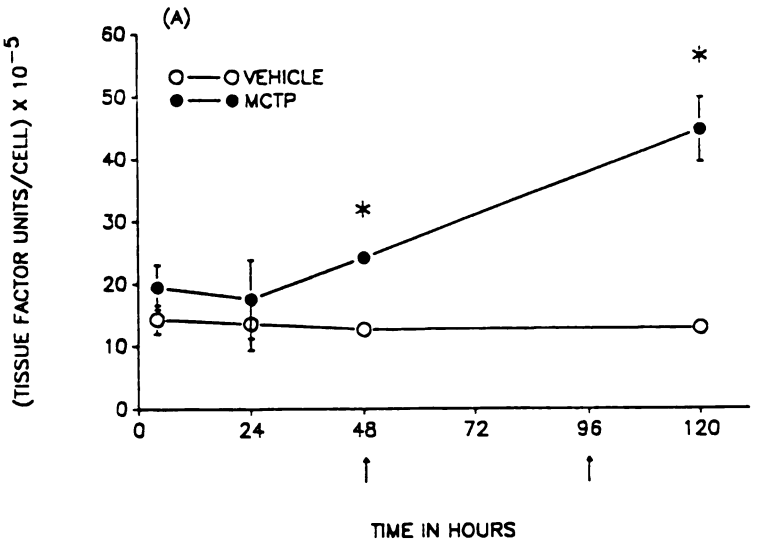
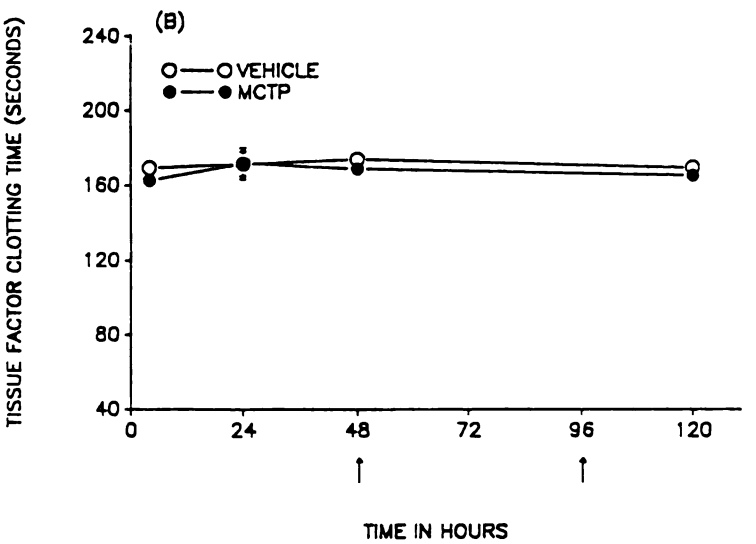
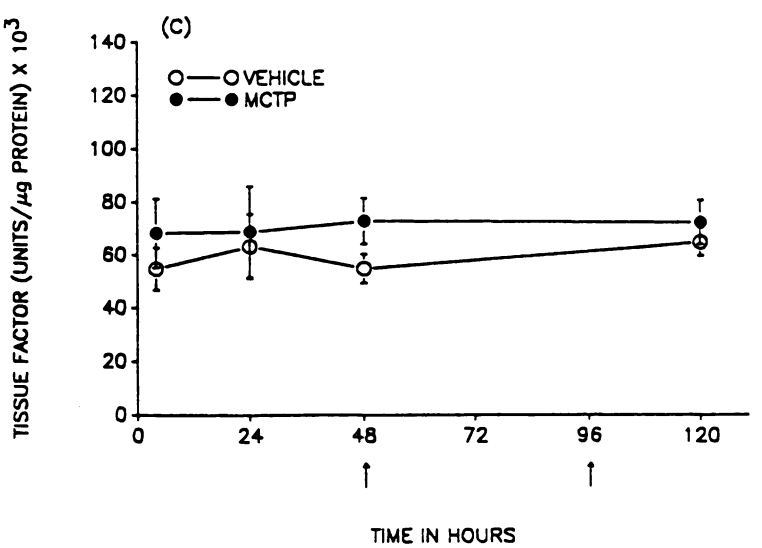


Figure 3: Effect of monocrotaline pyrrole (MCTP) on protein concentration of lysates from endothelial cells grown in culture. Bovine endothelial cell monolayers were exposed to medium containing MCTP (5 μg/ml) or DMF vehicle at time 0. Values represent mean ± SE. Those points without bars had SE less than area covered by symbol. Arrows indicate times at which culture medium was replaced with fresh medium containing no MCTP. *Significantly different from DMF control. N=6.

Figure 4: Effect of monocrotaline pyrrole (MCTP) on tissue factor activity expressed as **(A)** tissue factor activity/cell number from lysates of endothelial cells grown in culture, **(B)** clotting time of lysates from endothelial cells grown in culture or **(C)** tissue factor units/μg protein cellular lysates from endothelial cells grown in culture. Bovine endothelial cell monolayers were exposed to medium containing MCTP (5 μg/ml) or DMF vehicle at time 0. Values represent mean $\pm$ SE. Those points without bars had SE less than area covered by symbol. Arrows indicate times at which culture medium was replaced with fresh medium containing no MCTP. *Significantly different from DMF control. N=6.


(A)
O—O VEHICLE
●—● MCTP
(TISSUE FACTOR UNITS/CELL) X 10^{-5}
60
50
40
30
20
10
0
*
*
0
24
48
72
96
120
TIME IN HOURS


(B)
O—O VEHICLE
●—● MCTP
TISSUE FACTOR CLOTTING TIME (SECONDS)
240
200
160
120
80
40
0
24
48
72
96
120
TIME IN HOURS


(C)
O—O VEHICLE
●—● MCTP
TISSUE FACTOR (UNITS/μg PROTEIN) X 10^{3}
140
120
100
80
60
40
20
0
0
24
48
72
96
120
TIME IN HOURS

experiments were expressed as tissue factor activity from entire lysates or tissue factor activity per μg cellular protein, there were no significant differences between BECs treated with MCTP and those treated with vehicle (Figures 4b and 4c respectively).

Synthesis of Factor V, a cofactor for the common pathway of coagulation, is a second important procoagulant function of endothelial cells (Gertler and Abbott, 1992). There was a slight but statistically significant elevation in the activity of Factor V/cell in culture medium of BECs treated with MCTP and compared to control at 120 hours (Figure 5). When data were expressed as Factor V activity/monolayer, BECs treated with MCTP had less activity at 48 and 120 hours (data not shown).

Fibrinolytic properties. Bovine endothelial cells synthesize and release two activators of plasminogen: tissue plasminogen activator and urokinase-like plasminogen activator (Podor *et al.*, 1988; Saksela and Rifkin, 1988; Gerard and Meidell, 1989). Both activators cause the conversion of the zymogen, plasminogen, to the enzyme plasmin (Erickson *et al.*, 1986; Saksela and Rifkin, 1988; Gerard and Meidell, 1989). The activity of plasminogen activators in culture medium from BECs treated with MCTP was increased at 48 and 120 hours compared to vehicle treated controls (Figure 6). The pattern of response in plasminogen activator activity was not different whether data were expressed on a per cell basis or by activity per cell monolayer (data not shown).

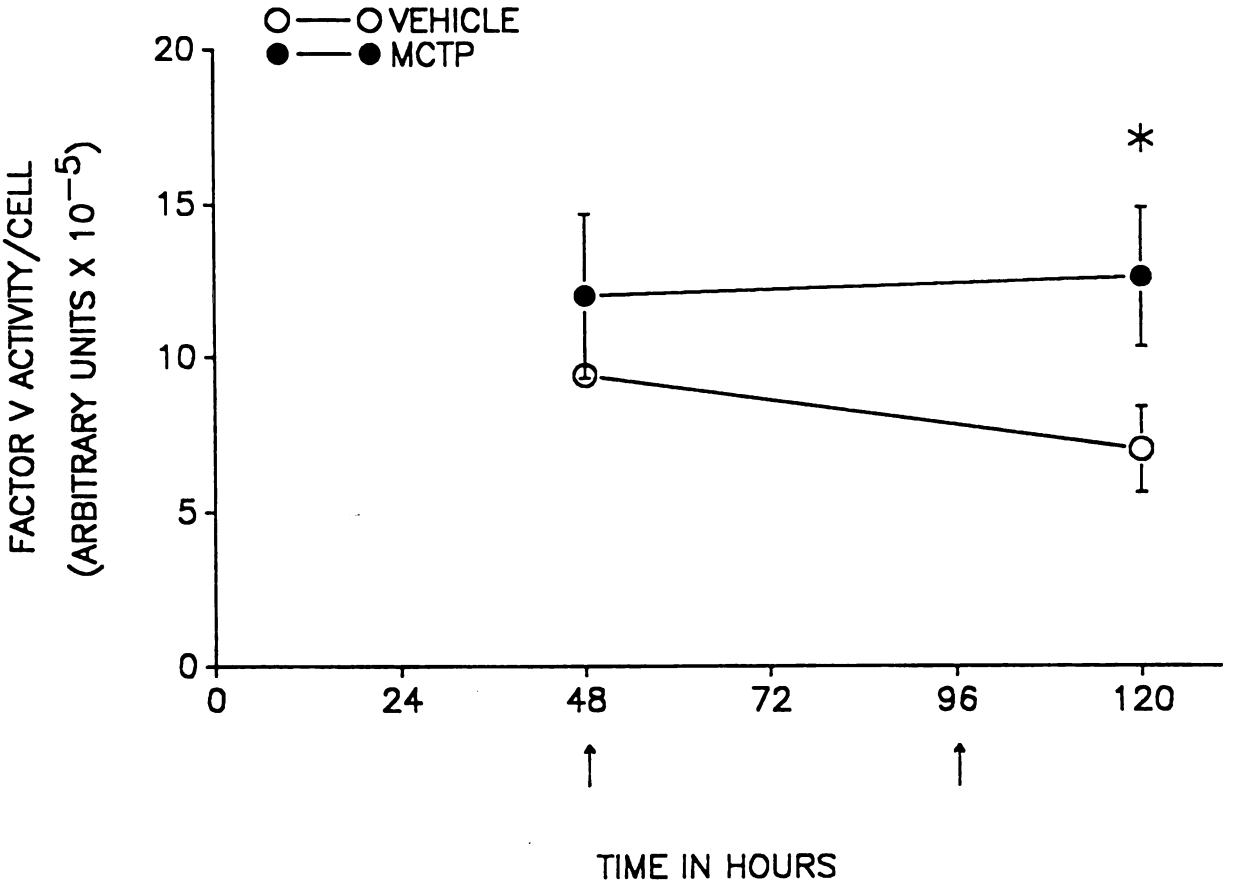


Figure 5: Effect of monocrotaline pyrrole (MCTP) on Factor V activity/cell in medium from endothelial cells grown in culture. Bovine endothelial cell monolayers were exposed to medium containing MCTP (5 μg/ml) or DMF vehicle at time 0. Values represent mean $\pm$ SE. Those points without bars had SE less than area covered by symbol. Arrows indicate times at which culture medium was replaced with fresh medium containing no MCTP. *Significantly different from DMF control. N=6.

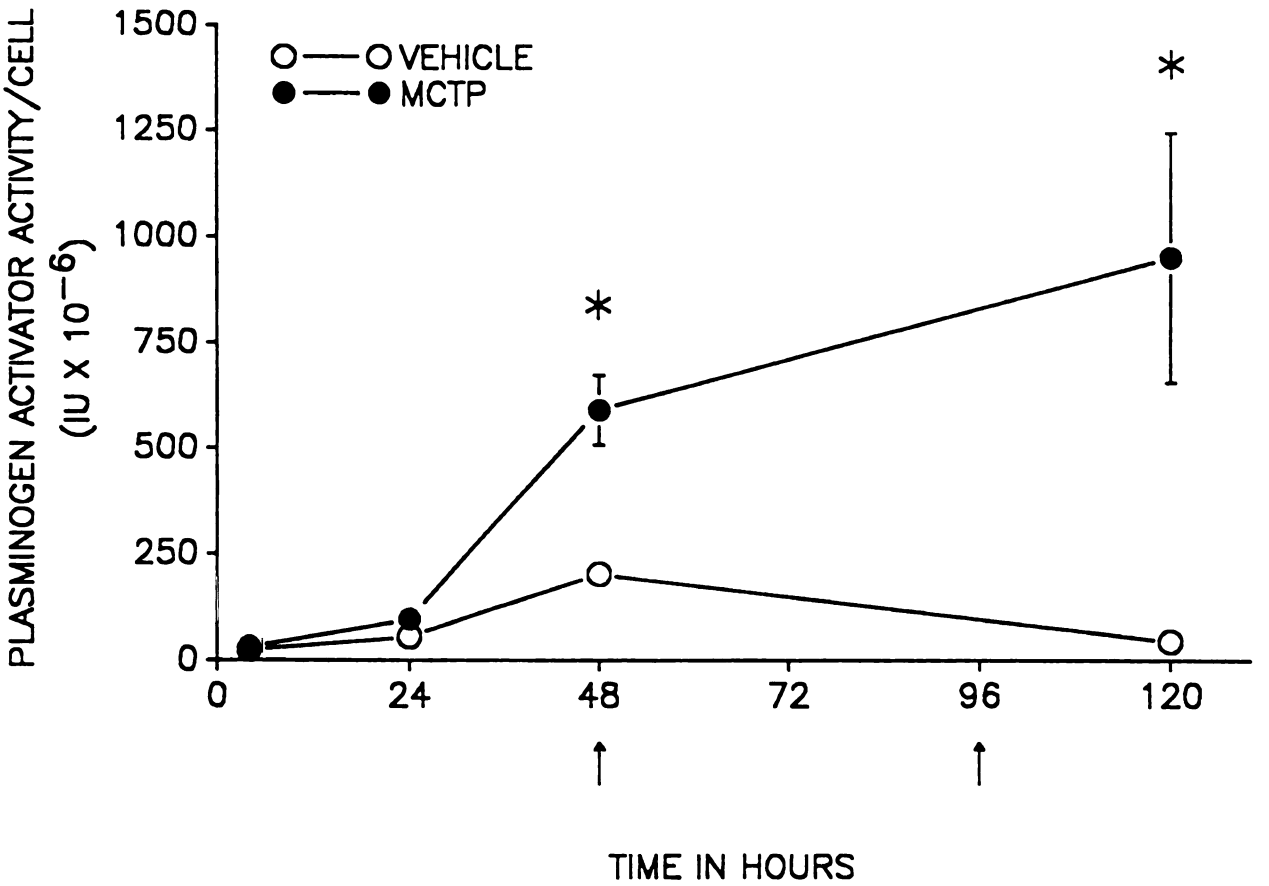


Figure 6: Effect of monocrotaline pyrrole (MCTP) on plasminogen activator activity/cell in medium from endothelial cells grown in culture. Bovine endothelial cell monolayers were exposed to medium containing MCTP (5 μg/ml) or DMF vehicle at time 0. Values represent mean $\pm$ SE. Those points without bars had SE less than area covered by symbol. Arrows indicate times at which culture medium was replaced with fresh medium containing no MCTP. *Significantly different from DMF control. N=6.

Plasminogen activator inhibitor-1 (PAI), an endothelial cell-derived glycoprotein, inhibits both plasminogen activators and thereby can prevent the conversion of plasminogen to plasmin (Loskutoff, 1988). The activity of PAIs in medium of BECs treated with either MCTP or vehicle decreased with time. However, at 48 and 120 hours there was significantly less PAI activity in the medium from BECs treated with MCTP compared to controls (Figure 7). Activation of latent PAIs via dialysis with 4 M guanidine increased the activity in all samples, but at no time did the activity of PAIs in medium from BECs treated with MCTP exceed that of controls (data not shown). There were no differences in the pattern of data when expressed per cell or as activity per monolayer (data not shown).

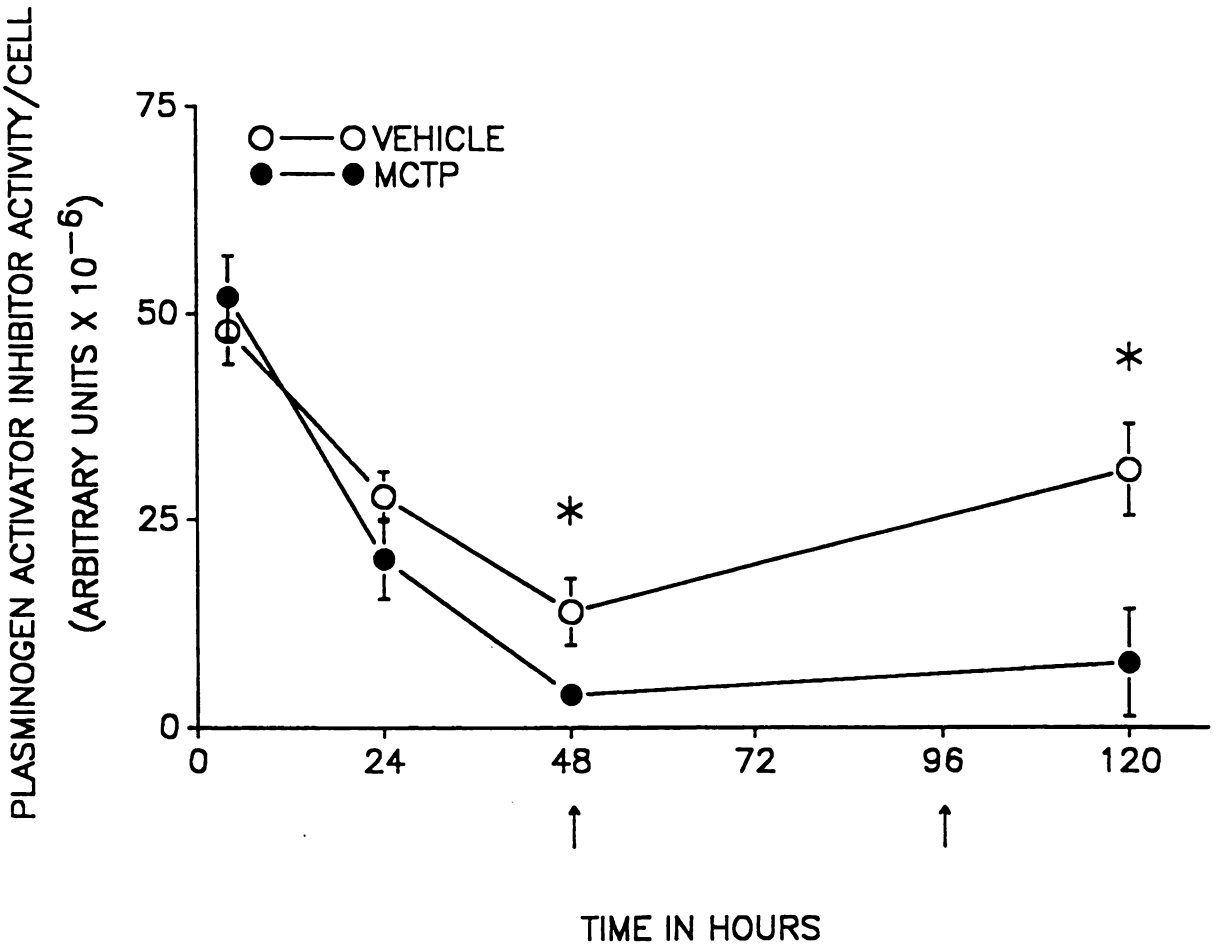


Figure 7: Effect of monocrotaline pyrrole (MCTP) on plasminogen activator inhibitor activity/cell in medium of endothelial cells grown in culture. Bovine endothelial cell monolayers were exposed to medium containing MCTP (5 μg/ml) or DMF vehicle at time 0. Values represent mean $\pm$ SE. Those points without bars had SE less than area covered by symbol. Arrows indicate times at which culture medium was replaced with fresh medium containing no MCTP. *Significantly different from DMF control. N=6.

DISCUSSION:

Recurrent pulmonary thromboembolism has been suggested as a cause of chronic pulmonary vascular disease in people (Harrison *et al.*, 1966; Wyngaarden and Smith, 1985; Tomashefski *et al.*, 1983). Pulmonary microvascular thrombosis occurs in a model of pulmonary vascular disease in which animals are treated with MCT or MCTP. Characteristics of this model include structural and functional changes in pulmonary vascular endothelium (Gillis *et al.*, 1978; Hilliker *et al.*, 1983a, 1984a; Molteni *et al.*, 1984; 1985; Reindel *et al.*, 1990). In this investigation, we sought to determine whether exposure of endothelial cells to MCTP *in vitro* resulted in cellular dysfunction that might favor thrombosis. BECs were used for several reasons. They grow readily in culture and much information is available regarding their procoagulant and fibrinolytic properties. In addition, the response of cultured BECs to MCTP has been characterized (Reindel and Roth, 1991).

The time-dependent decrease in the number of adherent cells in BEC monolayers treated with MCTP was typical for exposure to this toxicant. The delayed and progressive release of LDH from monolayers exposed to MCTP also was consistent with previous studies *in vitro* (Reindel and Roth, 1991) and with the pattern of increased LDH activity in bronchoalveolar lavage fluid from rats treated with MCTP (Schultze *et al.*, 1991a). The protein concentration of BEC monolayers treated with MCTP had not been determined previously. No significant difference in protein concentration occurred despite decreasing cell numbers. This result is similar to that observed in porcine pulmonary artery endothelial cells treated with

MCTP (Hoorn and Roth, 1992a) in which cellular protein concentration of adherent cells increased with time. This finding is also consistent with the results of morphometric analyses demonstrating cellular hypertrophy in BECs treated with MCTP (Reindel and Roth, 1991; Reindel *et al.*, 1991). The cell enlargement allows substantial maintenance of monolayer integrity until many days after exposure to MCTP.

Thrombosis may result from increased procoagulant activity within the blood (Schafer, 1985; Haake and Berkman, 1986; Bauer and Rosenberg, 1987). Tissue factor, also called tissue thromboplastin, is the primary factor associated with endothelial cell procoagulant activity (Nemerson, 1988; Stern *et al.*, 1988). Quiescent endothelial cells produce minimal amounts of this membrane-associated glycoprotein, but when stimulated they can increase tissue factor synthesis within 2-6 hours by 1000 fold (Schorer and Moldow, 1988). Inflammatory mediators such as tumor necrosis factor-α, interleukin 1-β, bacterial endotoxin and phorbol 12-myristate 13-acetate increase tissue factor synthesis markedly (Busso *et al.*, 1991). MCTP exposure caused a pronounced increase in tissue factor activity when the data were expressed as units of tissue factor activity/cell in culture. However, when values were expressed as total tissue factor activity in culture wells or as activity/μg protein in monolayers, there were no differences in tissue factor activity between BECs treated with MCTP and those treated with DMF vehicle. Tissue factor is a glycoprotein bound to plasma membrane of cells. The hypertrophy of BECs exposed to MCTP *in vitro* likely explains the differences in these results, since the surface area per cell increases.

That the total tissue factor activity in cell monolayers did not change with MCTP treatment argues against a significant contribution of tissue factor to increased endothelial procoagulant activity *in vitro*.

Another procoagulant property of endothelial cells is the synthesis and release of coagulation Factor V. The activated form of this coagulation factor, also called accelerator globulin, markedly amplifies the Factor Xa-mediated conversion of prothrombin to thrombin. In addition, Factor V, via its interactions with several other coagulation factors and fibrinolytic proteins, exerts control over aspects of the later stages of coagulation (Colman, 1987). Large increases in the amount of Factor V have been observed after treatment of endothelial cells with endotoxin, homocysteine and interleukin-1 (Gertler and Abbott, 1992). The importance of the subtle but significant increase in the activity of Factor V/cell in the culture medium of BECs treated with MCTP is currently unknown.

Since thrombosis may also be the result of decreased fibrinolytic activity, we evaluated fibrinolytic activity in rats treated with MCTP. In previous investigations of MCTP-induced pneumotoxicity and pulmonary hypertension, we observed a decrease in the net fibrinolytic activity of lung tissue within 3 days after treatment of rats with MCTP as well as an increase in the PAI activity in blood within 8 days after MCTP intoxication (Schultze and Roth, 1992). Accordingly, we hypothesized that the medium from cultured endothelial cells treated with MCTP might have decreased plasminogen activator activity, increased plasminogen activator inhibitor activity or both. Our results do not support this hypothesis, since we observed the opposite

effect, an increase in plasminogen activator activity and a decrease in plasminogen activator inhibitor activity. Since the majority of PAIs in medium of cultured BECs is in the latent phase (Erickson *et al.*, 1986) we measured latent PAI activity. Even after dialysis of samples with guanidine-HCl to activate latent PAIs, we could not detect an increase in the PAI activity in medium from BECs treated with MCTP compared to controls. The results we obtained suggest that BECs responded to MCTP exposure by changes in their fibrinolytic properties that would favor increased fibrinolysis, a protective influence against thrombosis.

A similar response to injury was detected by Bell and Madri (1990) who modulated the angiotensin system of bovine aortic endothelial cells *in vitro*. They observed that treatment with the angiotensin-converting enzyme inhibitor, lisinopril, or the angiotensin II receptor antagonist, sar^1, ile^8-angiotensin II, increased cell migration and increased urokinase-plasminogen activator in bovine aortic endothelial cells. Administration of exogenous angiotensin II prevented the effect of lisinopril on endothelial cell migration and urokinase-plasminogen levels. Bell and Madri suggested that administration of exogenous angiotensin-converting enzyme inhibitors may reduce vessel wall injury by promoting endothelial cell migration over denuded surfaces and decreasing the risk of thrombosis in affected vessels. These data may correspond with the findings of Hoorn and Roth (1992b), who observed a decrease in the angiotensin-converting enzyme activity of bovine pulmonary artery endothelial cells treated with MCTP 2-4 days previously. It is tempting to speculate that MCTP mediated an increase in plasminogen activators either through alterations in

angiotensin-converting enzyme activity or through alterations that occurred coincidentally to changes in the angiotensin-converting enzyme system.

The reasons for the lack of similarity between the decreased fibrinolytic activity in lung tissue observed *in vivo* and the changes in fibrinolytic properties of BECs treated with MCTP could be numerous. For example, BECs in culture might not mimic closely the procoagulant and fibrinolytic properties of the rat or other mammalian species exposed to toxic pyrrolizidine alkaloids. Alternatively, endothelial cells may require the presence of other supportive vascular cells such as smooth myocytes in order to respond as they would *in vivo*. It also seems possible that the pulmonary microvascular thrombosis and decreased fibrinolytic activity of lung tissue observed *in vivo* may not be due to a direct effect of MCTP on endothelial cells but rather may occur secondarily as a result of messengers released from other lung cells. Since many cells produce plasminogen activators and PAIs, it is also feasible that the decrease in fibrinolytic activity observed *in vivo* may not be the result of changes in endothelial cell fibrinolytic properties. Macrophages and neutrophils increase in number in the lungs of rats treated with MCT or MCTP and are altered in their cytomorphologic and biochemical properties, and these cells might contribute to the effects observed *in vivo*.

In summary, we studied the major procoagulant properties of BECs, including tissue factor activity and Factor V activity, after a single exposure to MCTP and evaluated changes in fibrinolytic properties, including plasminogen activators and PAIs. Results of these studies revealed that treatment of cultured BECs with MCTP

does not produce significant changes in procoagulant or fibrinolytic properties that would favor thrombosis.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

The studies described in this dissertation were designed to explore the involvement of the hemostatic system in MCTP-induced lung injury and pulmonary hypertension. The rationale for this investigation was as follows.

The administration of MCT(P) to rats causes pulmonary vascular thrombi that are associated with lung injury and vascular remodeling. The sequelae of these lesions include persistent pulmonary hypertension and compensatory hypertrophy of the right cardiac ventricle. The mechanism of thrombosis and the relationship of the thrombi to pneumotoxicity and pulmonary hypertension in this model have not been elucidated.

Studies described in the second chapter of this dissertation were designed to assess the onset and development of lung lesions in rats treated with a single, iv injection of MCTP. It is at these early times before damage is observed as pronounced changes in markers of pneumotoxicity or development of clinical signs that thrombi have been reported to begin to form in the lungs of rats treated with the toxicant. Examination of changes in markers of pneumotoxicity at early times after administration of MCTP to rats revealed that lung inflammation begins much earlier that has been reported previously. Significant changes included a mild increase in the wet lung-to-body weight ratio, increased protein concentration and activity of LDH activity in the cell-free BALF and an increase in the number of nucleated cells in the BALF. In addition, there was a significant increase in the number of segmented neutrophils in the BALF of rats treated with MCTP and killed at 4 or 120 hours after treatment. The degenerate cytomorphologic characteristics of

these cells suggested a pulmonary milieu that was hostile to health and survival of the neutrophils. Some of these early changes appeared to be reversible. For example, the wet lung-to-body weight ratio temporarily returned toward control values and the number of neutrophils in the BALF decreased. After a brief period, these markers of pulmonary inflammation increased dramatically with time. Other markers, such as the protein concentration and LDH activity in cell-free BALF and the number of nucleated cells in BALF, were moderately elevated early after treatment but later increased dramatically. Overall, pronounced changes in markers of lung injury were delayed for several days after a single iv injection of a low dose of MCTP. The fact that lung injury was measurable at times when thrombi have been reported to occur in the lungs is consistent with potential involvement of these thrombi in the lesions that occur after toxicant administration.

The formation of thrombi implies some imbalance in one or more components of the hemostatic system: vessels, platelets, soluble procoagulant or fibrinolytic proteins. A thorough review of the literature revealed that two components of this system have been the subject of extensive study. Investigations of vessel reactivity revealed exaggerated vasoconstriction in response to various stimuli after treatment of rats with MCT(P). In addition, there is some degree of luminal obstruction due to endothelial cell hypertrophy and/or hyperplasia after treatment of rats with MCT(P). Both the excessive vasoconstriction and the endothelial lesions may slow blood flow, increase contact activation and thereby increase procoagulant activity in the blood, potentially favoring thrombosis.

Several studies have focused on the involvement of platelets in MCT(P)-induced pneumotoxicity and pulmonary hypertension. It is clear that administration of MCT to rats causes thrombocytopenia and that platelets are sequestered in the lungs of rats treated with the putative toxic metabolite, MCTP. These observations suggest that platelets may be important in the pathogenesis of lesions due to MCT(P) administration. Antibody-mediated thrombocytopenia attenuates the pulmonary hypertension but not the lung injury after treatment of rats with MCTP. Although the subject of several investigations, no specific platelet mediator has been identified that is solely responsible for the role of the platelet in MCT(P)-induced pulmonary hypertension.

There have been no previous investigations of the procoagulant activity in blood of rats treated with MCT(P). A variety of screening tests (prothrombin time, modified prothrombin time and activated partial thromboplastin time) and several specific tests (antithrombin III activity, plasminogen concentration, fibrinogen concentration, and platelet count) were selected to provide an overview of the hemostatic system in the blood of rats treated with MCTP and to assess the probability that a hypercoagulable state existed in the peripheral blood. Major alterations were restricted to hyperfibrinogenemia at day 11 post-treatment and increased antithrombin III activity at days 8-11. Although these results indicate some functional alterations occur in the coagulation system of rats treated with MCTP, most changes were modest and transient and therefore unlikely to be the cause of the fibrin and platelet thrombi that form in the lungs.

Since thrombosis may also be the result of decreased fibrinolytic activity, the fibrinolytic system of rats treated with MCTP was examined. Studies of blood demonstrated increased PAI activity from 8-14 days after MCTP administration in the face of unchanged plasminogen concentration, plasminogen activator activity and α-2-antiplasmin activity compared to controls. The increase in PAI activity was modest and its biologic significance open to debate. These results suggest that decreased fibrinolysis may occur in the lung without causing decreased fibrinolytic activity in the systemic blood. Examination of the net fibrinolytic capacity of the lung tissue revealed that decreased fibrinolytic activity begins 3 days after treatment. This onset is consistent with the early onset of lung injury and occurs well in advance of the pulmonary hypertension. This time period is also consistent with previous studies that indicated a trend toward decreased fibrinogen concentration in blood of rats treated with MCTP and results of histologic examinations that showed thrombi deposited in the lungs of rats treated with the toxicant. It is possible that the trend for decreased plasma fibrinogen concentration was due to conversion of fibrinogen to fibrin and deposition of small thrombi in capillaries of the lungs.

To explore possible mechanisms of thrombosis due to endothelial cell dysfunction, BECs were grown in culture, treated with MCTP and examined for changes in major procoagulant properties. There appeared to be a significant increase in the amount of tissue factor per cell. However, when data were expressed as tissue factor/μg of protein in BEC lysates, a normalization believed to be more reflective of endothelial cell coverage within a vessel, no significant change could be

detected. Analysis of Factor V activity/cell in culture medium revealed a mild but significant increase at 5 days after treatment with MCTP. Analysis of plasminogen activator in culture medium showed a marked increase in activity that began 2-5 days after treatment. Analysis of PAI activity revealed a gradual decrease consistent with the increase in activity of plasminogen activators.

Results of these studies in cultured endothelium did not reveal a reason for the decreased fibrinolytic activity observed within the lung tissue *in vivo*. Possible reasons for the contrasting results are numerous. For example, the procoagulant and fibrinolytic properties of rat endothelial cells may not resemble closely those of bovine cells. Alternatively, endothelial cells cultured *in vitro* may not behave like endothelial cells *in vivo*. Thirdly, the microvascular thrombosis *in vivo* may require mediators that affect endothelium but are released from other indigenous cell types affected by MCT(P). Perhaps the decrease in fibrinolytic activity observed in lung tissue *in vivo* is due to changes in cell types other than endothelial cells. Pulmonary macrophages and segmented neutrophils increase in number and develop changes in their cytomorphologic characteristics after treatment of rats with MCTP. Since both cell types produce plasminogen activators and PAIs, it is possible that changes in their cellular fibrinolytic properties cause the fibrinolytic activity of lung tissue to change.

From the information compiled in this dissertation, one can conclude that the most probable cause for the pulmonary thrombosis lies in reduced fibrinolytic activity. This conclusion is supported by the observations of an increase in PAI

activity in blood and a marked decrease in net fibrinolytic capacity of lungs from rats treated with MCTP.

More detailed studies are needed to clarify the involvement of the hemostatic system in this model of human pulmonary hypertension. Many of the tests that are currently available for use in rats for detection of procoagulant activity in systemic blood lack the degree of sensitivity needed to detect microvascular thrombosis within the lung. Adaptation of a sensitive assay for fibrinopeptide A in blood of rats treated with MCTP may be useful in evaluating whether a subtle increase in procoagulant activity occurs. Another alternative would be to compare procoagulant activity in blood from samples taken before and after circulation through the lungs. It is possible that previous studies were limited by a dilution effect of blood. In the event a definitive increase in procoagulant activity was detected, then appropriate anticoagulant therapy with new coumarin derivatives, heparin or the leach extract, hirudin, might be effective. If pulmonary microvascular thrombi are truly important in the genesis of lung injury and pulmonary hypertension, then any method to prevent thrombosis may attenuate, delay, or prevent the sequelae of MCTP treatment.

The decrease in net fibrinolytic capacity of lung tissue observed in rats treated with MCTP is definitely worth further investigation. It would be advantageous to know if the decrease in fibrinolytic activity was due to an increase in the activity of PAIs, a decrease in activity of plasminogen activators or both within the lung. Perhaps examination of lung tissue homogenates using the activity assays for plasminogen activators and PAIs would be helpful. The commercial availability of

plasminogen activators such as tPa and urokinase may provide a useful tool for pharmacologic manipulation of this system. Administration of plasminogen activators in relatively high doses prior to and during the treatment of rats with MCTP may decrease pulmonary microvascular thrombosis and thereby attenuate, delay or prevent the lung injury and pulmonary hypertension. This manipulation would be especially beneficial to our understanding of thrombosis in this model. In addition, it would be beneficial to examine the role of Protein C in the MCTP-treated rat. A decrease in Protein C, a natural inhibitor of the PAI-1, may cause increased activity in PAI in blood and thereby limit fibrinolysis.

The MCT(P)-treated rat is a useful animal model for the study of two human pulmonary vascular diseases, primary pulmonary hypertension and the proliferative stages of the adult respiratory distress syndrome. The mechanism(s) of pulmonary hypertension have not been elucidated, and medical treatment of people affected with these chronic pulmonary vascular diseases has not been very successful. Further investigations of the involvement of the hemostatic system in MCTP-induced pneumotoxicity and pulmonary hypertension may lead to preventive measures or effective treatments for people suffering from chronic pulmonary vascular diseases.

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